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Centrosomal control of microtubule dynamics

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ABSTRACT **In many animal cells, minus ends of microtubules (MTs) are thought to be capped by the centrosome whereas plus ends are free and display dynamic instability. We tested the role of the centrosome by examining MT behavior in cytoplasts from which the centrosome was removed. Cells were injected with Cy3–tubulin to fluorescently label MTs and were enucleated by using a centrifugation procedure. Enucleation resulted in a mixture of cytoplasts containing or lacking the centrosome. Fibroblast (CHO-K1) and epithelial (BSC-1) cells were investigated. In fibroblast cytoplasts containing the centrosome, MTs showed dynamic instability indistinguishable from that in intact cells. In contrast, in cytoplasts lacking the centrosome, MTs treadmilled—shortened at the minus end at about 12 μm/min while growing at the plus end at the same rate. The change in behavior of the plus end from dynamic instability to persistent growth correlated with an elevated level of free tubulin subunits (78% in centrosome-free cytoplasts vs. 44% in intact cells) generated by minus-end depolymerization. In contrast to fibroblast cells, in centrosome-free cytoplasts prepared from epithelial cells, MTs displayed dynamic instability at plus ends and relative stability at minus ends presumably because of specific minus-end stability factors distributed throughout the cytoplasm. We suggest that, in fibroblast cells, a minus-end depolymerization mechanism functions to eliminate errors in MT organization and that dynamic instability of MT plus ends is a result of capping of minus ends by the centrosome.**

The microtubules (MTs) of animal cells are typically organized into a radial array with minus ends anchored at the centrosome and plus ends free and extending to the periphery (1). The plus ends exist in phases of rapid shortening or slower growth (2–4), displaying behavior known as dynamic instability (5). Dynamic instability is presumed to be the major mechanism of MT turnover and allows MTs to explore intracellular space (6).

An alternative mechanism of MT turnover recently has been described for microsurgically produced cytoplasmic fragments of melanophores lacking the centrosome (7). MTs in the fragments did not display dynamic instability but rather persistently grew by addition of subunits to their plus ends and shortened by loss of subunits from their minus ends. MTs were both nucleated by and released from the aggregate of pigment granules, which apparently served as a MT organizing center. Short MTs translocated through the cytoplasm by treadmilling—polymerization and depolymerization at opposite ends. Thus, instead of the conventional plus-end turnover pathway (dynamic instability), melanophore fragments turned over their MTs by minus-end depolymerization balanced by plus-end growth.

The surprising results obtained with cytoplasmic fragments of melanophores prompted two kinds of question. First, was the treadmilling behavior unique to melanophore fragments or was it a more general property of cytoplasmic MTs? Second, what was the mechanism by which the dynamic instability behavior of MTs became switched to treadmilling behavior? A subsequent study of mammalian epithelial cells in culture revealed constitutive release of MTs from the centrosome followed stochastically by minus-end depolymerization (8). However, the frequency of release was low and the resulting minus-end depolymerization did not lead to balanced treadmilling. Treadmilling also was observed, albeit at low frequency in two further studies of MTs in vertebrate animal cells (9, 10). However, the significance of these limited observations of MT treadmilling remained uncertain.

Concerning the mechanism by which dynamic instability behavior could be switched to treadmilling behavior, we proposed in our study of melanophore fragments a simple mechanism based on steady-state considerations (7). Minus-end depolymerization of MTs released from a putative organizing center, the pigment aggregate, would elevate the concentration of tubulin subunits. Elevated tubulin concentration, in turn, would suppress catastrophes at the plus end and facilitate persistent growth. In the steady state, plus-end growth would balance minus-end shortening, resulting in treadmilling. Thus, uncapping of MT minus ends normally attached to the centrosome was predicted to drive the transition in MT behavior.

In this study, we attempted to determine whether treadmilling of MTs was a general property of cytoplasmic fragments of cells. Further, we sought to test the hypothesis of centrosomal control of MT dynamics pattern by comparing the behavior of MTs in cytoplasts containing or lacking the centrosome.

MATERIALS AND METHODS

Cell Cultures and Microinjection. CHO-K1 cells, human primary fibroblasts (strain 356), and BSC-1 cells were cultured in F-10 medium supplemented with 10% fetal bovine serum and antibiotics on coverslips with photoetched locator grids. Cells were injected with Cy3-tagged tubulin at a needle concentration of 10 mg/ml for digital fluorescence imaging as described (11) and incubated for at least 1 hr before enucleation to allow incorporation of labeled tubulin into MTs.

Preparation of Cytoplasts Containing or Lacking the Centrosome. Cytoplasts were prepared by a modification of a described method (12). Briefly, cells were treated with nocodazole (1 μg/ml) and cytochalasin D (1.25 μg/ml) for 90 min. Coverslips were placed upside down into centrifuge tubes and centrifuged at 10,000 $\times g$ for 20 min. Enucleation resulted in about equal numbers of cytoplasts containing or lacking the centrosome. Coverslips were washed with fresh medium to remove drugs and incubated for 2–3 hr for complete recovery of MTs in cytoplasts.

Image Acquisition and Analysis. Digital fluorescence images of MTs were acquired as a time-lapse series with a cooled

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Abbreviation: MT, microtubule.
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charge-coupled device as described (11). Each series contained 100 images taken at 3-s intervals. Distances and rates were measured with the use of METAMORPH (Universal Imaging, Media, PA) and analyzed in SIGMAPLOT (Jandel, San Rafael, CA) as described (7). The proportion of MT polymer was determined by using a ratiometric fluorescence procedure as described (13). To quantitate patterns of MT behavior in centrosome-free cytoplasts, time-lapse series of images were analyzed, and stable MTs and MTs displaying dynamic instability and treadmilling were counted at the cell periphery.

Immunostaining. Double immunostaining for α- and γ-tubulin was done as described (8). Briefly, cells were fixed with glutaraldehyde and permeabilized with Triton X-100 (11). Primary antibodies were monoclonal anti γ-tubulin (Sigma) and polyclonal antibody against tyrosinated tubulin (a gift from Chloe Bulinski, Columbia University, New York). Incubation with 4′,6-diamidino-2-phenylindole (DAPI; 0.1 mg/ml) was included to reveal nuclei.

RESULTS

The MT array in fibroblast cells is essentially radial and focused on the centrosome, which typically is juxtaposed to the nuclear surface (Fig. 1 *Upper Left*). Cytoplasts prepared from CHO–K1 cells or human primary fibroblasts by removal of the nucleus through centrifugation after cytochalasin/nocodazole treatment either contained the centrosome and displayed a dense radial MT array (Fig. 1 *Upper Left*) or lacked the centrosome and displayed a loose and sparse network of randomly arranged MTs, in agreement with previous observations (12). Centrosome-lacking cytoplasts also showed a higher background of tubulin staining consistent with a lower level of MT polymer (Fig. 1 *Upper Right*).

The pattern of MT dynamics was evaluated by injecting intact cells with Cy3-tubulin, preparing cytoplasts from them, and acquiring digital-fluorescence time-lapse sequences of images. Living fibroblast cytoplasts containing or lacking centrosomes were readily distinguishable by the high density and radial arrangement of MTs invariably associated with the centrosome. Playback of image sequences permitted MTs to be categorized according to their behavior during the 5-min period of observation. In CHO cytoplasts containing the centrosome, most MTs (68%) displayed dynamic instability behavior with alternating phases of growth and shortening at their distal (plus) ends (Fig. 2 *Top*). Some free MTs (15%) also were detected, which shortened rapidly from their proximal (minus) ends (Fig. 2 *Top*, arrowheads). In addition, some MTs (17%) did not show measurable dynamics within the 5-min period of observation. In contrast, in cytoplasts lacking the centrosome, dynamic instability was never observed. Instead, although some MTs (33%) did not show measurable dynamics, most (67%) appeared to translocate through the cytoplasm (Fig. 2 *Middle*) as previously reported for treadmilling MTs in fragments of fish melanophores (7). MTs in centrosome-free cytoplasts prepared from 356 primary human fibroblasts displayed similar behavior with an even larger fraction of MTs (83%) translocating through the cytoplasm.

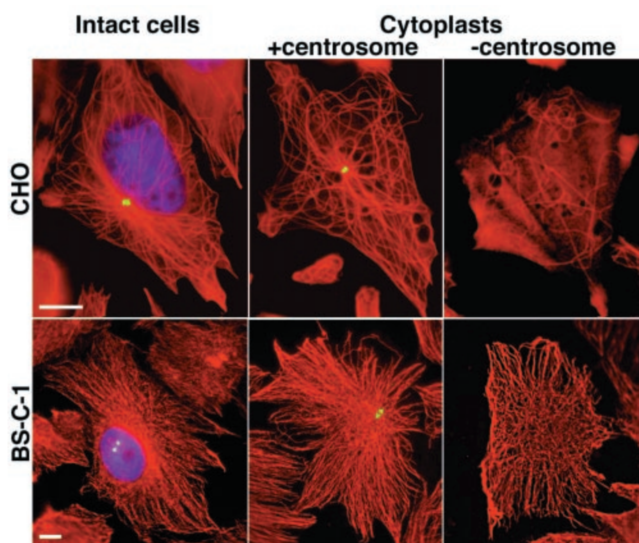

FIG. 1. Distribution of MTs in intact cells and cytoplasts. Intact cells (*Left*) and cytoplasts containing (*Center*) or lacking (*Right*) the centrosome were fixed and triple-stained with α-tubulin antibody for MTs (red), γ-tubulin antibody for the centrosome (green, but superposition makes the spot appear yellow) and 4′,6-diamidino-2-phenylindole (DAPI) for the nucleus (blue). (*Upper*) CHO cells; (*Lower*) BSC-1 cells. (Bar = 10 μm.)

The apparent translocation of MTs through the cytoplasm could be explained by transport of MTs by motor molecules or by treadmilling—polymerization at one end and depolymerization at the other—both mechanisms having recently been demonstrated *in vivo* (7, 8, 9). To discriminate between these mechanisms, we injected cells with a dilute solution ($\leq$1 mg/ml) of fluorescently labeled tubulin. Tubulin at low concentration incorporates nonuniformly into MTs, producing “speckles” along their length (9). In the case of transport, speckles will move with the MTs, and in the case of treadmilling, speckles will remain stationary. Analysis of the speckles behavior demonstrated that they remained stationary, whereas the MTs apparently translocated (Fig. 2 *Bottom*). This result indicates that the MT lattice was stationary and, therefore, that the MTs treadmilled. The possibility that transport may occur along with treadmilling under some specific cytoplasmic conditions is not excluded.

Dynamics were quantified through life-history analysis of individual MTs and instantaneous rate analysis. In CHO cytoplasts containing the centrosome, most MTs showed dynamic instability with excursions at their distal (plus) ends that were small and variable (Fig. 3*A Left*). For this population, growing phases were more frequent (64%) and slower (14 $\pm$ 10 μm/min), whereas shrinking phases were less frequent (36%) and faster (24 $\pm$ 20 μm/min) (Fig. 3*B Left*). A subpopulation of MTs ($\approx$15%) showed rapid shortening (22 $\pm$ 19 μm/min) from their proximal, presumably minus, ends. Similar dynamic-instability behavior and shortening of free MTs occurred in intact fibroblast cells (not shown), although the fraction showing minus-end shortening was smaller (5%). In cytoplasts lacking the centrosome, MTs persistently grew or shortened, and transitions between these states occurred rarely if at all (Fig. 3*A Right*). Instantaneous rates of growth or shortening showed an exponential distribution (Fig. 3*B Right*). However, on average, the rate of growth (11 $\pm$ 8 μm/min) about equaled the rate of shortening (13 $\pm$ 9 μm/min). MTs with speckles lengthened and shortened at similar rates (14 $\pm$ 6 and 12 $\pm$ 5 μm/min, respectively). Thus, the rates did not depend on the concentration of injected tubulin. We conclude that the major condition of treadmilling—similarity of rates of growth and shortening—was satisfied in centrosome-free cytoplasts.

We hypothesized previously (7) that treadmilling in centrosome-free cytoplasm is possible because depolymerization of MTs at uncapped minus ends increases the steady-state tubulin concentration and this increase suppresses the transition from growth to shortening at MT plus ends. To test this hypothesis, we determined the proportion of polymerized tubulin by using a single-cell, ratiometric fluorescence assay (13). Total tubulin fluorescence in living CHO cells and in cytoplasts containing or lacking the centrosome first was measured by integrating the fluorescence over the entire cell. Cells and cytoplasts then were extracted into a MT-stabilizing buffer to remove the soluble pool. Correlative light and electron microscopy has

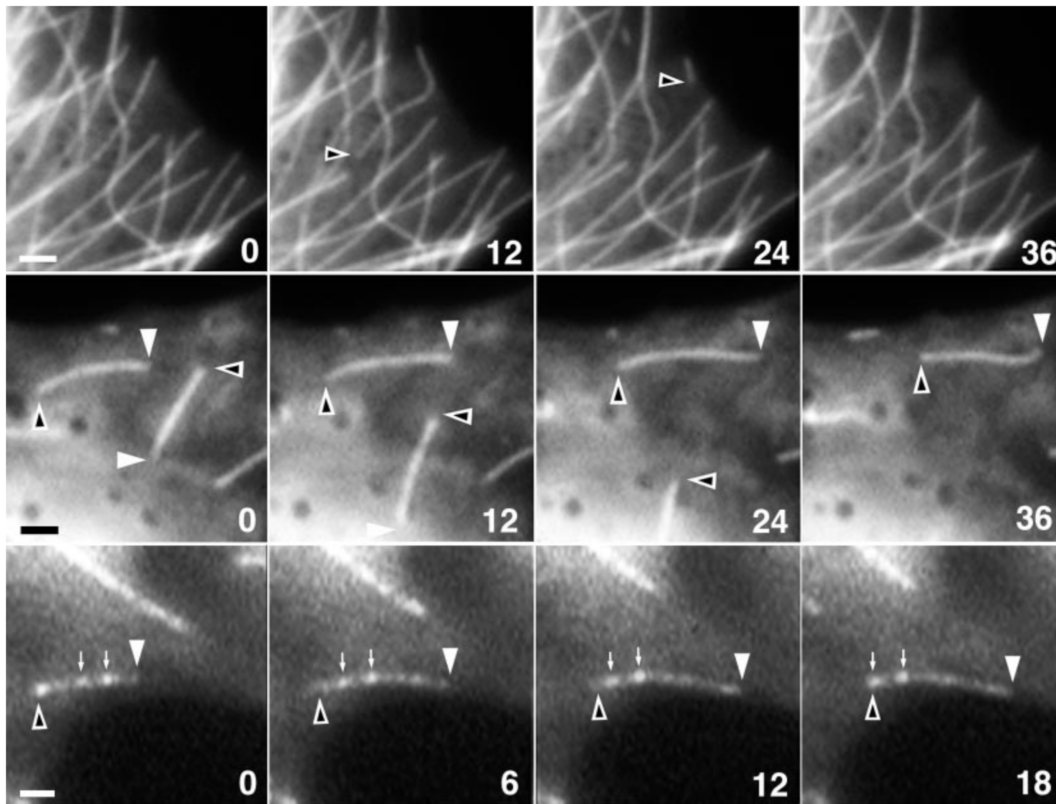

FIG. 2. Fluorescence imaging of MTs in living cytoplasts. In centrosome containing cytoplasts (*Top*), MTs showed dynamic instability at their plus ends whereas free MTs rapidly shortened from their minus ends. In centrosome-free cytoplasts (*Middle*), MTs persistently grew at one end and shortened at the other end. Injection of tubulin at low concentration resulted in nonuniform incorporation of subunits along MTs and produced "speckles" along their length. Speckles remained stationary, indicating that the mechanism of translocation was treadmilling (*Bottom*). Black arrowheads point to shortening and white arrowheads to growing ends. White arrows point to speckles. Numbers indicate time in seconds. (Bars = 1 μm.) Video sequences of MT dynamics in cytoplasts can be seen and downloaded from our web site (http://borisy.bocklabs.wisc.edu).

demonstrated that these lysis and extraction procedures result in negligible change in the amount or distribution of MTs (14). Images taken before and immediately after extraction confirmed that MTs remained intact in the extracted cytoplasts (Fig. 4*A*). The ratio of fluorescence before and after extraction yielded the proportion of tubulin as MT polymer and showed that the amount of MTs was significantly lower in cytoplasts lacking the centrosome (21 ± 6%; n = 19) as compared with cytoplasts containing the centrosome (44 ± 9%; n = 13) (Fig. 4*B*). Intact cells had similar but somewhat higher levels (56 ± 14%; n = 12) than centrosome-containing cytoplasts, suggesting the possibility that factors beyond the centrosome may contribute to the overall level of MTs but that these factors are less significant than removal of the centrosome. The possibility that cytoplasts containing or lacking the centrosome differed in the proportion of polymerization-competent tubulin was tested by treatment with the drug Taxol, which shifts the equilibrium toward polymer formation. Taxol treatment induced formation of additional MTs, increasing the proportion to >90% in both centrosome-free and centrosome containing cytoplasts and indicating that they were comparable in terms of pools of active tubulin. We conclude that removal of the centrosome per se leads to increase in the level of tubulin monomer at the expense of polymer.

An additional test of the role of the centrosome was to examine MT dynamics in cytoplasts prepared from epithelial cells because detailed light and electron microscopy indicates that many MTs in this type of cell are not anchored in the centrosome (15–17). For such MTs to persist, their stability must depend on factors other than the centrosome. A prediction of noncentrosomal stability factors is that, unlike for fibroblasts, removal of the centrosome from epithelial cells should not significantly affect MT organization or dynamics. Cytoplasts were prepared from the epithelial cell type, BSC-1, and the MT pattern was assessed. Unlike fibroblast cells, cytoplasts prepared from the BSC-1 cells displayed a dense MT array and low background of tubulin staining independent of centrosome presence (Fig. 1 *Bottom*). Cytoplasts of BSC-1 cells also displayed dynamic instability at MT plus ends and stability at MT minus ends independent of centrosome presence (data not shown). Quantitation of MT behavior showed that in centrosome-free cytoplasts prepared from epithelial cells, 99% of the MTs displayed dynamic instability and only 1% depolymerized from the minus end at any given time. As reported previously for intact epithelial cells (13), minus-end stability in epithelial cytoplasts was stochastic, and apparently stable minus ends could become labile and shorten rapidly. Thus, the behavior of MTs in centrosome-free cytoplasts correlated with the degree of centrosome dominance of MT organization in the parental cell. The results suggest that epithelial cells and cytoplasts contain minus-end stability factors distributed throughout the cytoplasm, in contrast to fibroblasts, which apparently are concentrated at the centrosome.

DISCUSSION

Our results demonstrate that MT treadmilling *in vivo* is not a peculiarity of cytoplasmic fragments of melanophores. Rather, it appears to be a widespread, although not universal, property of MTs in centrosome-free cytoplasts of mammalian cells. Centrosome-free cytoplasts prepared from fibroblasts show almost complete and balanced MT treadmilling behavior,

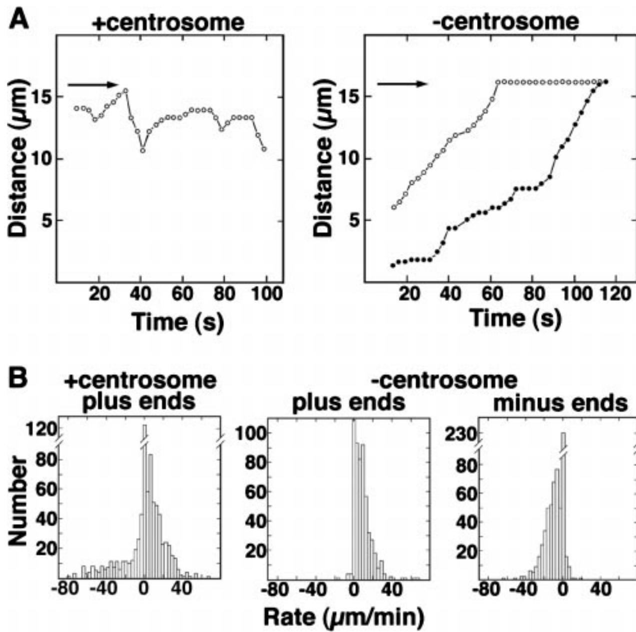

FIG. 3. Quantitation of MT dynamics in cytoplasts. (*A*) Life histories of MTs in cytoplasts containing (*Left*) and lacking (*Right*) the centrosome. Arrows indicate position of cell margin. In centrosome-containing cytoplasts, MT distal ends displayed alternating phases of growth and shortening. In centrosome-free cytoplasts, distal ends (○) persistently grew until they reached the cell margin whereas trailing ends (●) persistently shortened, culminating in MT disappearance. (*B*) Frequency histograms of rates of MT growth and shortening at distal (plus) ends of MTs in centrosome-containing cytoplasts (*Left*) and at distal (plus) and proximal (minus) ends of MTs in centrosome-free cytoplasts (*Center* and *Right*). Change in length was quantified for 41 MT plus ends in 7 centrosome-containing cytoplasts, and for 43 minus ends and 41 plus ends in 10 centrosome-free cytoplasts.

whereas cytoplasts prepared from epithelial cells do not. Rather, epithelial-cell cytoplasts show dynamic instability. However, even epithelial cells and cytoplasts display stochastic minus-end depolymerization, indicating the universality of this pathway. Whether treadmilling appears in centrosome-free cytoplasts seems to depend on cell type-specific factors influencing the stability of the minus end. Thus, minus-end depolymerization may be considered to be a constitutive pathway contributing to MT turnover.

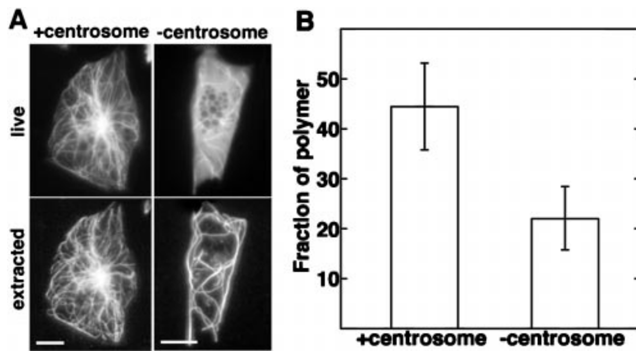

FIG. 4. Proportion of polymerized tubulin in cytoplasts. (*A*) Fluorescence images of MTs in cytoplasts with (*Left*) and without (*Right*) the centrosome before (*Upper*) and after (*Lower*) extraction into a MT-stabilizing buffer. MTs remained stable after lysis, but soluble tubulin was extracted. (Bars = 10 μm.) (*B*) Proportion of polymerized tubulin was determined ratiometrically on individual cytoplasts as integrated fluorescence after extraction divided by integrated fluorescence in the living state (13). Measurements were performed after flat-fielding and background subtraction. Results presented are for 13 centrosome-containing and 19 centrosome-free cytoplasts.

In fibroblast cells, the dynamic instability pattern of MT dynamics [growth and shortening at distal (plus) ends] can be switched to the treadmilling pattern (growth and shortening at opposite ends) by removal of the centrosome. The switch in the pattern of MT dynamics can be explained in terms of steady-state considerations (18) based on the availability of minus ends and the hypothesis shown in Fig. 5. In intact fibroblasts or centrosome-containing cytoplasts, the centrosome functions to cap MT minus ends, thus causing the exchange kinetics of tubulin to be determined by properties of the MT plus end.

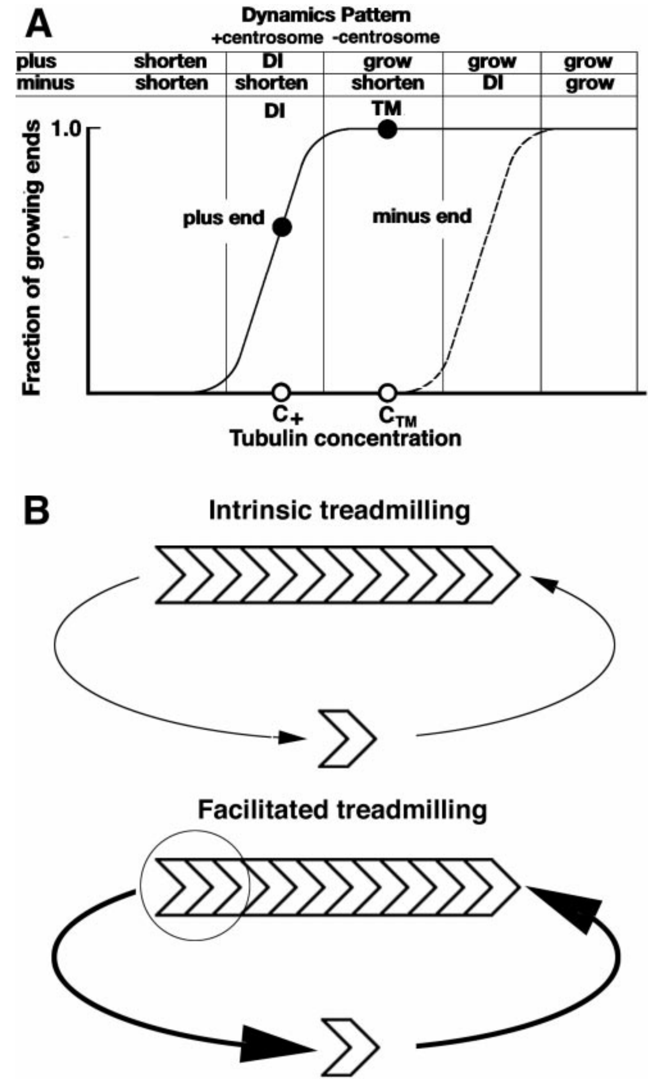

FIG. 5. Hypothesis for MT treadmilling. (*A*) MT dynamics pattern. The probability that a MT end is growing is plotted versus tubulin subunit concentration. When minus ends are capped, the steady-state subunit concentration is set by the critical concentration for the MT plus end, c_+. Below this value, both plus and minus ends of MTs shorten. At c_+, plus ends (●) show dynamic instability—they both grow and shorten—whereas any free minus ends (○) only shorten. If minus ends are uncapped (−centrosome), tubulin subunits released from shortening minus ends will increase the tubulin pool until plus ends persistently grow and a concentration is reached, c_{TM}, at which MTs display balanced treadmilling (TM). Above this value, growth could theoretically occur at minus ends (dashed line), but the range at which this occurs *in vivo* has not been determined. (*B*) Intrinsic vs. facilitated treadmilling. MT treadmilling is rate limited by dissociation of tubulin subunits from the minus end. Treadmilling *in vitro* is slow because this rate is low. In living cells, minus-end shortening is 2–3 orders of magnitude faster. Protein factors may bind to the minus end, facilitating loss of subunits. This would increase treadmilling speed.

The plus ends drive tubulin-subunit concentration to a low steady-state value, and the resulting behavior of the plus ends is dynamic instability (19). Minus ends appearing in this environment would shorten rapidly because the critical concentration for the minus end *in vivo* is higher than for the plus end. In fibroblast cytoplasts lacking the centrosome, all MT minus ends are uncapped and continuously depolymerize, and as a result, the monomer concentration increases to a point where dynamic instability at the plus end is suppressed. At the elevated tubulin monomer levels, the transition probability to the growing state becomes sufficiently high that the plus end persistently grows. Steady state is reached when growth at the plus end balances shortening at the minus end—the condition required for treadmilling. Thus, a minus-end pathway of depolymerization drives the change in pattern of MT dynamics.

Although removal of the centrosome *per se* is the simplest explanation of treadmilling, we cannot exclude other possibilities from contributing to the change in MT behavior. Cytoplasts containing or lacking the centrosome may differ in ways other than simply the presence or absence of the centrosome. Cytoplasmic structures, endomembrane systems, or molecules preferentially associated with the centrosome could have been differentially lost from cytoplasts along with the centrosome. For example, a kinesin family member with a central motor domain has been shown to promote dynamic instability of the plus end by increasing its probability of shortening (catastrophe) (20). Were catastrophe-promoting factors concentrated at the centrosome, their loss with the centrosome would be predicted to decrease instability and lead to higher probability of growth at the plus end. It should be noted, however, that the procedure for enucleation involves treatment with cytochalasin D and nocodazole before centrifugation. Consequently, endomembrane systems such as the Golgi apparatus, normally located near the centrosome, become dispersed throughout the cytoplasm (21). Furthermore, minus-end motors and their cargoes, which tend to concentrate at the centrosome, also become dispersed in the absence of MTs (22). Consequently, any putative differential loss of a cytoplasmic factor during the removal of centrosomes must be MT-independent. An alternative class of explanation is based on the change in organization of MTs. Loss of radial organization resulting from loss of the centrosome may result in loss of spatial sequestration of MT motors and components of the endomembrane system. Such spatial disruption could indirectly lead to changes in MT behavior. However, such changes would nevertheless be a result of removal of the centrosome.

The speed of treadmilling, 12 μm/min, is remarkable in that this value exceeds previously reported values of treadmilling *in vitro* (23–25) by 2–3 orders of magnitude. This disparity in rate suggests the possibility of *in vivo* factors missing in purified systems. Previous experiments (26) indicated that growth rate of MTs in an *in vitro* but complex system, namely egg extracts, was not linearly dependent on tubulin concentration. Our data showed similarity of rates of growth in centrosome-free and centrosome-containing cytoplasts despite an $\approx$twofold difference in tubulin monomer concentration. Both of the sets of results suggest the possibility of rate-limiting factors other than tubulin subunit concentration. Polymerization may be accelerated by plus-end factors, which enhance addition of tubulin subunits to the growing ends of MTs. Alternatively, minus-end factors may increase the rate of loss of subunits from minus ends of treadmilling MTs. For actin filaments (where it has been shown that subunit loss from the pointed end is rate-limiting) a protein factor, ADF/cofilin, which accelerates pointed-end dissociation (27, 28) speeds up treadmilling. By analogy, we suggest that a minus-end dissociation factor may exist for tubulin *in vivo* (Fig. 5*B*). The minus-end factor could be a molecular motor as suggested by studies linking motors with induced depolymerization (29). The rapidity of treadmilling *in vivo* also reinforces the possibility that motors acting as coupling factors (30) may harness the potential of treadmilling to accomplish cellular work. Although MT motors are considered to play the leading role in chromosome movement in mitosis (31) and slow axonal transport in neurons (32), MT dynamics may also play an important role in these processes.

The minus-end pathway can explain how fibroblast cells maintain their pattern of MT organization. Although the centrosome, through its nucleation function, initially establishes the MT array (1), the array is subject to degradation through release of MTs (8), breakage (9, 10), and spontaneous formation in the cytoplasm (10, 33). Were these processes to continue unabated, the cytoplasm would eventually fill up with noncentrosomal MTs, leading to loss of cellular organization. As previously suggested as an implication of treadmilling (18, 24), minus-end shortening may function to eliminate free, uncapped MTs. With minus-end shortening as the default pathway, the centrosome, by capping the minus end, not only determines the pattern of MT dynamics at the plus end but insures that only centrosomal MTs survive, thus maintaining cellular organization.

In contrast to fibroblast cells, minus ends of MTs in epithelial cells did not depend on the centrosome for their stability. This result suggests the existence of stabilizing factors distributed through the cytoplasm. Although the identity of such a factor remains to be established, a likely candidate is the γ-tubulin complex (34, 35). Lack of immunostaining with γ-tubulin antibody of free MT ends in epithelial cells in our study may reflect limitations of the method and does not rule out a possible stabilizing role of the γ-tubulin complex. Our previous study of MT dynamics in PtK cells (8) indicated that MTs released from the centrosome generally are capped at their minus end but that the capping is stochastic and that the MTs may lose the cap and then rapidly shorten from the minus end. Thus, the minus ends of MTs may be associated with either stabilizing or destabilizing factors. The regulation of association of minus-end factors and the cell-type specification of their cellular distribution remain to be determined.

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