Interzonal microtubules are dynamic during spindle elongation

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Summary

The pattern and extent of microtubule assembly during spindle elongation has been examined in PtK1 cells by microinjection of biotin-tubulin and immunolocalization of biotin-tubulin-containing microtubules using antibodies to biotin. PtK1 cells were microinjected at 30°C, incubated for various intervals to allow incorporation of biotin-tubulin into microtubules, then lysed, fixed and stained for biotin-tubulin and total tubulin. When mid- to late anaphase cells were examined at short times post-injection, using conventional fluorescence light microscopy, rapid incorporation of biotin-tubulin was detected throughout the interzonal region, between the separating chromosomes, and in the spindle asters. Using confocal fluorescence microscopy, the segments of biotin-labeled microtubules in the interzonal region were found to be continuous with the distal, or plus-ends, of unlabeled microtubules. When telophase cells were examined, a marked decline in the extent of incorporation was apparent.

Quantitative analysis of the total length of labeled polymer in the interzonal region of cells from mid-anaphase through telophase further reveals that the extent of incorporation was maximal during late anaphase, and decreased during telophase. The measured rate of interzonal microtubule growth remained relatively constant during this period. Our results provide direct evidence for plus-end elongation of interzonal microtubules during spindle elongation and further reveal that interzonal microtubules are highly dynamic during late anaphase spindle elongation. The implications of these results for the mechanism of anaphase B are discussed.

Key words: microtubules, spindle elongation, anaphase.

Introduction

Anaphase chromosome motion consists of both chromosome-to-pole motion, anaphase A, and the separation of the poles, anaphase B. In cultured animal cells, pole-pole separation begins after chromosome-to-pole motion is nearly complete and continues for several minutes, depending on cellular morphology. In addition, the rate of pole separation is slower than the rate of chromosome-to-pole motion (Brinkley and Cartwright, 1971).

Structural analysis of the arrangement of spindle microtubules (MTs) throughout mitosis has indicated that those spindle MTs that overlap at the equator lengthen during spindle elongation, or anaphase B (McIntosh et al. 1985). These interzonal MTs are composed of MTs extending from each pole and thus are of opposite polarity, plus-ends distal to the pole from which they originated (Euteneuer and McIntosh, 1980). Recent experiments in which fluorescent tubulin has been microinjected into living cells suggest that these interzonal MTs elongate by addition of subunits to their distal or plus-ends during spindle elongation, although individual MTs could not be resolved in these experiments (Saxton and McIntosh, 1987a). Further analysis of pattern photobleaches in the interzone of late anaphase/telophase cells (a stage that corresponds to our telophase cells) revealed that fluorescence recovery was slow and that the two half-spindles slid apart (Saxton and McIntosh, 1987b). However, the rate of sliding in these cells was very limited (approximately 0.09 μm min⁻¹).

Additional information concerning the mechanism of anaphase B comes from analysis of elongation of the diatom central spindle. In these cells, the central spindle consists of a very regular, interdigitated array of overlapping MTs that slide apart during elongation, reducing the extent of overlap (McIntosh et al. 1979; Candé and McDonald, 1985). These central spindles can be isolated and reactivated in buffers containing ATP and exogenous tubulin (Masuda and Candé, 1987, 1988). When labeled tubulin is present in the reactivation solution, MT elongation at the ends of the region of overlap is observed; as the spindle elongates, the zones of new MT growth approach each other. When central spindle reactivation is prevented by omitting ATP, MT polymerization continues, but the spindles do not elongate (Masuda and Candé, 1988). Thus, ATP-dependent force generation within the overlapping array of MTs contributes to spindle elongation; MT assembly increases both the rate and extent of spindle elongation in vitro (Masuda and Candé, 1988).

In the experiments presented here, the pattern and extent of MT polymerization during spindle elongation in cultured cells has been examined by microinjection of biotin-labeled tubulin (Mitchison et al. 1986; Wadsworth et al. 1989). At various intervals post-injection, the cells are lysed, fixed and prepared for immunocytochemical

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localization of both biotin–tubulin and total tubulin using either conventional or confocal fluorescence microscopy (White et al. 1987). Our experiments demonstrate that MTs remain highly dynamic during late anaphase as evidenced by the rapid incorporation of labeled subunits. Furthermore, we document elongation of individual interzonal MTs during anaphase B. Quantitative measurements reveal time-dependent changes in the amount of total polymer assembled in the interzonal region from anaphase through telophase. The maximal extent of incorporation occurs during late anaphase, coincident with anaphase B pole–pole separation in these cells.

Materials and methods

Preparation of biotin–tubulin

Biotin–tubulin was prepared as described (Mitchison et al. 1986; Wadsworth et al. 1989). Small samples were stored at -70°C in injection buffer (20 mM sodium glutamate, 1 mM EGTA and 0.5 mM MgSO4, pH 7.2). Protein was adjusted to 1 mM GTP and centrifuged to maximum speed in an Eppendorf microcentrifuge before use. For all of these experiments, biotin–tubulin was used at a concentration of 3–4 mg ml⁻¹ in the injection pipette as determined by a modification (Schatzler and Pollock, 1973) of the method of Lowry et al. (1951).

Cell culture and microinjection

PtK2 cells were grown at 37°C in Ham's F-12 medium supplemented with 10% fetal bovine serum, 10 mM Hepes and antibiotics. For use in experiments, cells were plated on glass coverslips and allowed to grow for 36–48 h before use. Coverslips were then mounted in a laboratory-constructed microinjection chamber (Wadsworth et al. 1989), which was mounted on the stage of a Zeiss IM-35 microscope. In this chamber, individual cells could be observed, injected and readily relocated following staining. Temperature was maintained at 30°C using an OptiQuip Red Beam Incubator calibrated with a YSI Telethermister. Microinjection was performed using a Narishige micromanipulator and an Eppendorf model 5242 microinjector. Needles were pulled to a final tip diameter of approximately 0.5 µm on a Sutter Instruments P-80 Brown-Flaming micropipette puller, using Microdot capillaries. Pipettes were back-loaded with approximately 0.5 µl of biotin–tubulin using a Hamilton syringe. Chromosome position in injected cells was recorded photographically on 35 mm film. Cells incubated for less than 30 s post-injection were photographed just after injection and again just after lysis. For some experiments in which cells were incubated longer than 30 s, the living cell was photographed after injection and just before lysis, to record any change in chromosome position.

Immunofluorescence

Following microinjection, cells shown in Figs 1 and 2 (below), and control cells used for quantitation (see Quantitative methods), were lysed for approximately 50 s in lysis buffer containing 80 mM Pipes, 5 mM EGTA, 1 mM MgSO4 and 0.5% Triton X-100, pH 6.8 (Cassimeris et al. 1986). Cells shown in the remaining figures, and the cells quantitated in Fig. 6, were lysed as described above, then rinsed for an additional 50 s in saline at room temperature before fixation. The saline rinse reduced the number of spindle MTs allowing a more precise determination of the extent and pattern of biotin–tubulin incorporation (see Discussion). Cells were fixed for 25 min in 2% paraformaldehyde and 0.1% glutaraldehyde in phosphate-buffered saline (PBS), pH 7.3, and rinsed in PBS containing 0.1% Tween and 0.02% azide (PBS–TWEEN–Az) and for 5 min in 1% sodium borohydride. For some other experiments, cells were placed in fixative before lysis. Antibody incubations were performed in humid chambers at room temperature. Cells were incubated first with rabbit anti-biotin antibodies (Enzo Biochemicals, New York, NY) at a dilution of 1:50 in PBS–TWEEN–Az containing 1% BSA (bovine serum albumin) for 30 min, rinsed in PBS–TWEEN–Az and incubated with fluorescein-labeled goat anti-rabbit and then fluorescein-labeled rabbit anti-goat antibodies. Cells were then incubated in a mouse monoclonal anti-tubulin antibody (generous gift from Dr J. R. McIntosh) for up to 3 h followed by rhodamine-labeled goat anti-mouse antibodies. All fluorescent secondary antibodies were purchased from Organon, Teknika, West Chester PA, and used at a final dilution of 1:50 in PBS–TWEEN–Az containing 1% BSA, for 30 min at room temperature. Stained cells were mounted in 0.1% n-phenyldiamine in glycerol and sealed with nail polish.

Analysis of chromosome motion

Recordings of chromosome motion were made using a Dage Newvicon camera (model 67M) and a Gyr 1/2 inch time-lapse tape recorder (model TLC 1400). The rate of chromosome motion was determined from traces of chromosome position made on sheets of clear acetate.

Microscopy

For standard immunofluorescence observations, a Zeiss IM-35 microscope with a 63x1.4 NA lens and filters for rhodamine and fluorescein excitation was used. Cells were photographed using TMAX 400 film, developed in HC 110 dilution B. For confocal microscopy, a BioRad MRC500 laser scanning head mounted on a Nikon Optiphot microscope equipped with a 60x1.4 NA objective lens was used. The confocal microscope was operated with the pinhole set for a section thickness of approximately 0.9 µm. Section thickness was determined by imaging 10 µm diameter glass beads using back-scattered light. A step size for the stage motor that gave discernible difference in each sequential image in a Z series through the bead was used. The diameter was then measured at the widest point, and section thickness estimated by dividing the diameter by the number of images collected. A Z series of images through the specimen was collected for both the rhodamine and fluorescein channels. Confocal images were background subtracted and contrast stretched to increase the signal to noise ratio, a 9x9 crisping convolution matrix was applied to enhance the spatial resolution and a final contrast stretch applied. Two-color images were made in a similar manner, but the contrast and brightness of each channel was increased as required, to balance the resulting image. Pairs of images were then simultaneously projected on the monitor; color slides of the monitor were taken with Kodak color slide film.

Quantitative methods

Quantitative measurements of MT lengths were made on a Masscomp computer. MTs in each optical section through the cell were traced using a mouse. The traces generated from each optical section were assigned different grey-scale values and a color look-up table was used to color code each optical section. MTs could then be followed through successive image planes by adding traces to the monitor. When a complete trace of an MT was generated, its length was measured using the mouse, and the trace was removed from the screen using a mouse-driven ‘eraser’ function. The eraser function ensured that MTs were not traced more than once and facilitated tracing in regions dense in MTs. No attempt was made to adjust the final calculated length of each MT for the number of optical sections through which it extended. Interzonal MTs generally did not extend through more than two optical sections, as this region in the cell remains relatively flat during anaphase B. For cells rinsed in saline prior to fixation, successive trials gave an error of ±2.2%. However, the patterns of incorporation, as well as the average growth rates of individual MTs, were similar in cells prepared by both methods (for example, compare Figs 2 and 5). Half of the cells shown in Fig. 6, and one third of the control cells, microinjected more than once to estimate the accuracy of a quantitative method. The computer measurements were calibrated using an objective micrometer as a test object. Rates of
Microtubule elongation were calculated by dividing the average length of biotin–tubulin incorporation by the injection interval for each experiment.

Results

Microtubule assembly during anaphase spindle elongation

To examine the pattern of MT polymerization during spindle elongation, PtK1 cells were microinjected with biotin-labeled tubulin (biotin–tubulin) (Mitchison et al. 1986; Wadsworth et al. 1989) and double-labeled to visualize both biotin-containing MTs and the total MT staining pattern. For these experiments, cells were incubated at 30°C to reduce the number of labile MTs (Wadsworth et al. 1989), lysed and stained as described in Materials and methods. A cell that was injected in mid-anaphase and fixed 60 s post-injection is shown in Fig. 1. Three criteria were used to select mid-anaphase cells: (1) chromosome-to-pole motion was not yet complete, as evidenced by the presence of kinetochore fibers; (2) little change in the pole-to-pole spindle length had occurred; and (3) the chromosomes maintained a characteristic V shape. In injected mid-anaphase cells, short segments of biotin-labeled MTs were detected in the interzonal region between the separating chromosomes (Fig. 1). Biotin–tubulin fluorescence was also detected in the region between the chromosomes and the pole, but was much less intense than the total tubulin staining pattern of this region of the cell (Wadsworth et al. 1989). Astral MTs also incorporated biotin–tubulin (out of the plane of focus in Fig. 1); many of these astral MTs appeared labeled along their entire length.

The pattern of tubulin incorporation in late anaphase cells was also examined. In late anaphase cells chromosome-to-pole motion was nearly complete, with only very short kinetochore fibers remaining, and the spindle was more elongate than the mid-anaphase cells. Time-lapse records of PtK1 cells, at 30°C reveals that pole–pole separation occurred in late anaphase after chromosome-to-pole motion was complete. The rate and duration of pole–pole separation were 0.9±0.2 μm min⁻¹ (n=9) and 4.6±1.6 min (n=9), respectively. The rate and extent of pole separation in un.injected and injected cells was indistinguishable. Pole–pole separation was completed before the events of telophase (chromosome decondensation, nuclear re-formation and the initiation of cytokinesis, see below). Thus, by these criteria, we could inject late anaphase cells in which pole–pole separation was occurring.

A pair of optical sections from a cell injected in late anaphase and lysed 30 s later is shown in Fig. 2A–D). These cells have been imaged using a confocal fluorescence microscope (see Materials and methods) to improve our ability to detect labeled MTs (compare Figs 1 and 2). Using this method, short MT segments containing biotin–tubulin, continuous with the distal (or plus) end of interzonal MTs, are readily detected. In some cases, the entire length of a MT appears to be biotin-labeled (Fig 2C,D).

When late anaphase cells were incubated for longer intervals following injection, the total tubulin and biotin-labeled tubulin staining patterns were nearly indistinguishable using the confocal fluorescence microscope. A pair of optical sections from a cell that was injected during late anaphase and lysed 67 s later is shown in Fig. 2E,H. Comparison of the total tubulin staining (Fig. 2G) with the biotin staining pattern (Fig. 2H) reveals that incorporation of biotin–tubulin into spindle MTs is nearly complete within 67 s. Phase-contrast micrographs of the injected cells (Fig 2A,B and E,F) reveal that little or no pole separation has occurred during the incubation interval; thus both cells shown in Fig. 2 contain labeled segments that are much longer than the extent of pole separation during the incubation interval. This is especially noticeable in Fig. 2E–H, where the majority of interzonal MTs, including those in MT bundles, appear to have fully incorporated biotin-labeled subunits. The rapid incorporation of biotin-labeled tubulin into individual interzonal MTs during anaphase B elongation is therefore not directly coupled to pole separation in these cells.

Microtubule assembly during telophase

MT assembly during midbody formation has also been investigated by injecting cells with biotin–tubulin during telophase. Telophase cells were defined by the following criteria: (1) chromosome-to-pole motion was complete, as judged by the lack of kinetochore fibers; and (2) bundles of MTs were present in the interzone. In addition, in most telophase cells cytokinesis and chromosome decondensation had begun. Telophase cells were examined at various intervals post-injection using either conventional

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Fig. 1. A mid-anaphase PtK1 cell, which was injected with biotin–tubulin and processed for immunocytochemistry using anti-tubulin (A) and anti-biotin (B) antibodies. The cell was injected in mid-anaphase and incubated for 60 s prior to lysis. The total tubulin staining (A) reveals fluorescence of the astral, interzonal and kinetochore fiber MTs. In the biotin-stained image (B) labeling is detected in the interzonal region and in the aster. Bar, 10 μm.
or confocal optics. Fig. 3 illustrates a telophase cell that was lysed 55 s post-injection and examined using the confocal microscope. Short segments of biotin-containing MTs are observed in the center of the interzone/forming midbody; longer labeled segments were also present. However, the incorporation was much less extensive than in late anaphase cells incubated for similar post-injection intervals (compare Fig. 2G, H with Fig. 3). Astral MTs were also labeled, frequently along their entire length (Fig. 3).

When telophase cells were examined following somewhat longer post-injection incubation intervals, nearly complete labeling of astral MTs was observed and increased incorporation into the midbody was detected. In Fig. 4 a cell that was lysed 82 s post-injection is shown; the total-tubulin staining pattern shows intense staining of the forming midbody, and astral MTs extending from each pole. When the anti-biotin staining was observed, nearly all the astral MTs were labeled along their entire length. Thus, within this incubation period, nearly complete turnover of the entire array occurred. Biotin–tubulin was also detected in the forming midbody, but in contrast to the astral MTs, the staining was much less intense than the corresponding total tubulin fluorescence. In this instance, because of the density of midbody MTs, staining of individual MTs was not apparent using conventional fluorescence optics. It is clear, however, that complete turnover of interzonal MTs did not occur during this post-incubation interval, although astral MTs in the same cell appear to have fully incorporated labeled subunits.

Telophase cells, injected and incubated for 1.5- to 2-min intervals, have also been examined using confocal microscopy. Heterogeneous incorporation of labeled tubulin into the bundles of interzonal MTs was observed: some bundles were unlabeled while others were labeled to various extents (data not shown). With progressively longer incubations (3–5 min), nearly all of the bundles of interzonal MTs were labeled. Because of the close
Fig. 3. Confocal micrograph of a telophase cell injected with biotin–tubulin and incubated for 55 s prior to lysis. Anti-tubulin staining is shown in red and anti-biotin staining in yellow/green. A single optical section through the cell is shown. Biotin–tubulin has incorporated into the central region of the mid-body and into the asters (not in focus in this optical section). Bar, 10 μm.

Fig. 5. Confocal micrographs of late anaphase PtKi cells injected with biotin–tubulin. Merged images of anti-tubulin staining (red) and anti-biotin staining (yellow/green) are shown. The cells were incubated for 53 (A) and 58 (B) s prior to lysis. In A a single section through the cell is shown, in B all the optical sections of the cell are shown simultaneously to reveal the total biotin-labeling pattern. Biotin–tubulin incorporation at the ends of interzonal MTs is detected. Bars, 10 μm.
proximity of the MTs, we cannot determine whether all the MTs in a bundle had incorporated label at these longer time points.

**Quantitative analysis of interzonal microtubule assembly**

Our immunofluorescence results indicated that time-dependent changes in the assembly of MTs in the interzone occurred during spindle elongation. To examine these changes, biotin-labeled MTs have been quantitated from complete Z series of images acquired using the confocal microscope (see Materials and methods). Using this technique, fluorescent fibers were detected in individual optical sections of these cells. Many of these fibers were judged to be single MTs on the basis of their similarity to fluorescent fibers in adjacent interphase cells (Osborn et al. 1978); other fibers stained more intensely, and probably represent MTs that were sufficiently close to each other that they could not be detected as two (see Sammak and Borisy, 1988). Biotin-stained segments appeared thicker than the rhodamine staining of total tubulin, due to the use of additional secondary antibodies (see Materials and methods). Discrete biotin-stained segments were clearly detected and were continuous with thinner rhodamine-labeled MTs. Because MT ends are distributed throughout the interzone region during these stages, and because we have analyzed cells after very short times post-injection, most biotin segments occupy a unique location at the end of a growing MT, and therefore are considered individual MTs. Only cells that were lysed within 65 s of injection were used for quantitation; MTs in cells incubated for times longer than this were difficult to measure accurately. Finally, in experiments in which cells were fixed before lysis or fixed and lysed simultaneously (Geuens et al. 1989), individual MTs and segments of biotin-labeled MTs were much more difficult to detect, even in the confocal microscope, due to the superimposition of the more numerous MTs and the higher background staining.

The extent of new MT polymerization from midanaphase through late telophase was quantitated by measuring the length of all biotin-labeled polymer in the interzone/cell; these measurements were normalized with respect to the injection interval. Cells were lysed and rinsed in saline before fixation as described in Materials and methods. Two examples of late anaphase PtK1 cells injected with biotin-tubulin, lysed and examined by confocal microscopy are shown in Fig. 5. Merged images in which anti-tubulin staining appears in red/orange and anti-biotin fluorescence in green/yellow are shown. Short segments of biotin–tubulin-containing MTs, which are continuous with non-biotin-containing MTs, are seen in the interzonal region. As shown in Fig. 6, biotin-labeled polymer in the interzone increases from mid- to late anaphase and decreases sharply as cells progress into telophase. Approximately five times more labeled polymer is present in the interzone in late anaphase cells than telophase cells. When the number of labeled MTs/cell was examined a similar pattern was found, with the maximal number of labeled MTs present in late anaphase cells (data not shown). These quantitative data support our qualitative observations of injected cells and demonstrate that
maximal incorporation into interzonal MTs occurs during late anaphase.

Because the lysis conditions used in these experiments remove labile MTs, interzonal MTs were also quantitated for cells lysed more gently (control cells, see Materials and methods). In these cells, the total length of biotin-labeled polymer was greater than in cells lysed and rinsed in saline for mid-, late anaphase and telophase cells. However, both the pattern of biotin–tubulin incorporation into interzonal MTs occurs during late anaphase.

Discussion

The results of these experiments provide the first direct visualization of plus-end elongation of individual interzonal MTs in PtK1 cells during spindle elongation. Biotin-labeled segments were located at the distal or plus-ends (Euteneuer and McIntosh, 1980) of unlabeled MTs in the interzonal region. This plus-end incorporation was observed from mid-anaphase through telophase, at short times after injection. With longer post-injection incubations the growth of individual MTs became increasingly difficult to detect and the interzonal region became uniformly labeled, at the resolution of the light microscope.

The rapid incorporation of biotin–tubulin into interzonal MTs could occur by de novo nucleation of new interzonal MTs, by treadmilling or poleward flux along existing MTs, by dynamic instability, or by some combination of these methods. Examination of Fig. 2C–D shows that at short time points post-injection, many unlabeled interzonal MTs are present in the interzone. At slightly later time points nearly all spindle MTs have incorporated biotin–tubulin (Fig. 2G–H). De novo nucleation of interzonal MTs cannot account for the conversion of existing unlabeled MTs to labeled MTs as seen in Fig. 2.

Treadmilling and/or poleward flux along existing MTs could account for the conversion of unlabeled MTs to labeled MTs shown in Fig. 2. However, no evidence of a uniform wave of incorporation, as might be produced by synchronous treadmilling of spindle MTs, was detected. Examination of the pattern of incorporation at short time points post-injection reveals unlabeled, partially labeled, and fully labeled MTs in the interzonal region and reveals that the junctions between labeled and unlabeled MT segments are dispersed throughout the interzone. In addition, our measurements show that interzonal MTs elongate at an average rate of about 3 μm min⁻¹ and clearly demonstrate that some MTs can incorporate labeled subunits at rates up to 10 μm min⁻¹ (see Fig. 2D). These values are in contrast to the much slower rates observed for poleward flux in vitro (0.6 μm min⁻¹; Mitchison, 1989) and the rates of treadmilling observed in vitro for microtubule-associated protein (MAP)–rich and MAP-depleted MTs (0.01 and 0.9 μm min⁻¹, respectively; Farrel et al. 1987). Therefore, MT behavior in the interzone during anaphase B elongation is significantly different from previous observations of MTs undergoing subunit flux or treadmilling.

The rapid incorporation of biotin–tubulin detected were measured at the earliest time points examined; values reported here correspond to rates measured following an average post-injection incubation interval of 43 s. These values are most likely underestimates of the actual rate of MT elongation because the average behavior of the population of microtubules is measured. For example, the initiation of elongation could occur at any time during the interval between injection and lysis. Thus, some microtubules would elongate for the entire interval while others would elongate for only a portion of that interval or would begin rapid shortening, both of which would decrease the average measured elongation rate (Mitchison and Kirschner, 1984). Finally, the average polymerization rate measured here is greater than the rate of pole–pole separation in these cells (3.9 μm min⁻¹ versus 0.9 μm min⁻¹, respectively).
throughout the interzonal region could occur by MT dynamic instability behavior (Mitchison and Kirschner, 1984). In this model most MTs continually elongate, while a subset rapidly or catastrophically disassembles. MTs can be rescued from the rapid shortening phase, or if disassembly is complete, can be renucleated from the centrosome (Mitchison and Kirschner, 1984). Thus, a population of MTs will contain both growing and shortening MTs. Dynamic instability behavior has been directly observed in living interphase (Cassimeris et al. 1988; Sammak and Borisy, 1988; Schultz and Kirschner, 1988), and mitotic prometaphase newt lung cells (Hayden et al. 1990). Measurement of dynamic instability in vivo reveal that MTs are capable of rapid assembly and disassembly (average rates of 7.2±0.3 and 17.3±0.7 jummin^-1, respectively, Cassimeris et al. 1988). Therefore, dynamic instability of MTs provides the best explanation for the rapid and heterogeneous incorporation of labeled tubulin and the conversion of unlabeled to fully labeled MTs seen in our experiments.

Biotin-tubulin incorporation was also detected in the asters of injected cells. Both elongation of existing astral MTs and nucleation of new MTs from the centrosome was observed. The extent of astral MT labeling was not quantitated, due to the difficulty in measuring the highly three-dimensional astral MT array. However, qualitative comparison of the extent of incorporation into astral and interzonal MT arrays demonstrates that astral MTs were more dynamic than interzonal MTs at this time. Finally, little label was detected in the region of the kinetochore fibers of late anaphase cells, consistent with previous observations that kinetochore fiber MTs was not observed (Fig. 7), we conclude that the loss of MTs is valid only if the loss of MTs during the saline rinse is random and relatively constant from mid-anaphase through telophase. To examine this, we have quantitated the extent of incorporation of biotin–tubulin into MTs in cells lysed and rinsed in saline and cells lysed but not saline rinsed (see Materials and methods). In both cases, similar patterns of tubulin incorporation (compare Figs 2D and 5), rates of MT elongation and MT length distributions were observed. If the loss of MTs in the more highly extracted cells was due to a gradual shortening of all the MTs during lysis, then one would expect a marked shift in the MT length distribution and growth rate compared with that of more gently lysed cells. Since this was not observed (Fig. 7), we conclude that the loss of MTs was random during lysis and suggest that once disassembly was initiated, the entire MT was lost. Lysis might also select for more stable MTs. However, biotin–tubulin incorporation into spindle MTs at long times post-injection was complete or nearly complete in both highly extracted and more gently lysed late anaphase cells (data not shown). Therefore, no sub-class of stable MTs refractory to tubulin incorporation was seen in late anaphase by either method. However, the number of interzonal MTs lost during lysis may be influenced by the stage of mitosis.

The decrease in incorporation we observed as cells enter telophase may be due to the increase in MT bundling. Interactions between MTs, mediated by MT-associated proteins (MAPs), may suppress MT dynamic behavior and thereby decrease the extent of incorporation of labeled subunits. The observation that MAPs decrease MT dynamics in vitro (N. Pryer, personal communication) supports this possibility. An alternative explanation is that the injected labeled tubulin is somehow excluded from MT bundles. While this possibility cannot be formally ruled out, it should be noted that the injected labeled tubulin and secondary antibodies were not excluded from the central region of the midbody of telophase cells in our experiments (Figs 3, 4), as has been previously observed for cells at later stages of mid-body formation. In addition, other experiments, using fluoroescent tubulin and photobleaching methods (Saxton and McIntosh, 1987) in living cells, provide evidence that interzonal MT turnover is reduced when MTs are bundled. In these experiments, cells were injected with labeled tubulin prior to anaphase and allowed to equilibrate; thus all the MTs became uniformly labeled before MT bundling had occurred (Saxton and McIntosh, 1987). These photobleaching results and the data presented here strongly suggest that MT dynamics are greatly reduced within the interzone of telophase cells.

It is unlikely that the time-dependent changes in incorporation that we have observed are due to a perturbation caused by the injection of exogenous tubulin (Schulze and Kirschner, 1986). If incorporation of biotin–tubulin was due to a perturbation, then all cells would be expected to incorporate similar amounts of biotin–tubulin into all classes of MTs. Instead we observe stage-specific changes in the extent of incorporation into the interzone and find that different classes of MTs in the same cell show different extents of incorporation (Figs 3 and 4; Saxton and McIntosh, 1987). Finally, the rate of anaphase B motion is not altered by injection, further suggesting that we have not perturbed the cells but rather are detecting the endogenous MT dynamics.

In order to quantitate MTs in these cells, it was necessary to rinse cells briefly in saline following lysis in a detergent-containing solution. Our quantitative analysis is valid only if the loss of MTs during the saline rinse is random and relatively constant from mid-anaphase through telophase. To examine this, we have quantitated the extent of incorporation of biotin–tubulin into MTs in cells lysed and rinsed in saline and cells lysed but not saline rinsed (see Materials and methods). In both cases, similar patterns of tubulin incorporation (compare Figs 2D and 5), rates of MT elongation and MT length distributions were observed. If the loss of MTs in the more highly extracted cells was due to a gradual shortening of all the MTs during lysis, then one would expect a marked shift in the MT length distribution and growth rate compared with that of more gently lysed cells. Since this was not observed (Fig. 7), we conclude that the loss of MTs was random during lysis and suggest that once disassembly was initiated, the entire MT was lost. Lysis might also select for more stable MTs. However, biotin–tubulin incorporation into spindle MTs at long times post-injection was complete or nearly complete in both highly extracted and more gently lysed late anaphase cells (data not shown). Therefore, no sub-class of stable MTs refractory to tubulin incorporation was seen in late anaphase by either method. However, the number of interzonal MTs lost during lysis may be influenced by the stage of mitosis. As anaphase progresses, MTs become more bundled, and more resistant to depolymerization during lysis. Interestingly, our quantitative results reveal a decrease in the number of MTs present in the interzone at different stages of the cell cycle.

Our results have several implications for the mechanism of anaphase B. Observations on the diatom central spindle and telophase PtK2 spindles have led to a model for anaphase B in which interdigitated MTs from opposite half-spindles slide apart, presumably due to mechano-chemical motors acting within the interzone (Masuda and...
Recent observations indicate that a dynamic population of astral MTs can exert 'polar ejection forces', which may contribute to chromosome orientation during prometaphase and exclude material from the spindle pole (Bajer, 1980; Bajer et al. 1986; Salmon, 1989). It is possible that dynamic arrays of astral MTs from opposing poles push each other apart during spindle elongation. This model predicts that inhibition of MT dynamics will prevent spindle elongation and suggests that some MT--MT interactions in the interzone may govern, rather than produce, pole separation (Aist and Berns, 1981; Klinebusch and Borisy, 1982). Experiments in our laboratory are currently being conducted to test this hypothesis.

Previous photobleaching experiments on telophase cells have revealed antiparallel sliding of interzonal MTs. However, because telophase cells were examined, most of pole--pole separation had already occurred in these cells and the contribution of sliding at earlier stages remains to be measured. Our data indicate that MTs were most dynamic, as measured by the ability to incorporate labeled tubulin, during late anaphase, when rapid pole--pole separation occurs. As interzonal MTs became more bundled in telophase, MTs became less dynamic, as evidenced by the decrease in incorporation of labeled tubulin and the slow rate of fluorescence redistribution after photobleaching (FRAP) (Saxon and McIntosh, 1987). The decrease in dynamics occurred coordinately with a tenfold change in the average rate of pole--pole separation in these cells (0.9 μm min⁻¹ for late anaphase cells (this report) and 0.09 μm min⁻¹ for telophase cells (Saxon and McIntosh, 1987)). Thus, if sliding interactions produce pole--pole separation in late anaphase, either the interactions must be of short duration, or a small subset of stable interzonal MTs, which were not detected in our experiments, are responsible for force generation. It is possible that a cell must rapidly and continually nucleate MTs from each pole to create a few antiparallel MT interactions that produce sliding, much as many MTs are nucleated at the spindle pole to ensure kinetochore fiber formation.

Interestingly, the decrease in rate of pole--pole separation seen as MT bundling occurs during telophase suggests that pole separation and MT bundling are antagonistic processes and may therefore involve different MAPs.

In summary, these experiments reveal plus-end elongation of individual interzonal MTs, and nucleation and growth of astral MTs, during spindle elongation in PtK₂ cells. Our results reveal that interzonal MTs are highly dynamic during spindle elongation. Quantitative measurements of the extent of incorporation demonstrate time-dependent changes in the incorporation of labeled tubulin during spindle elongation, with maximal incorporation of tubulin detected during anaphase B spindle elongation.

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