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Quantitative determination of the proportion of microtubule polymer present during the mitosis-interphase transition

Ye Zhai* and Gary G. Borisy

Laboratory of Molecular Biology, University of Wisconsin-Madison, Madison, WI 53706, USA

*Author for correspondence

SUMMARY

We have developed a new method for determining levels of tubulin polymer, based on quantitative fluorescence detection of x-rhodamine tubulin microinjected into living cells and we have applied this method to analysis of the mitosis-interphase transition. LLC-PK cells in interphase and mitosis were microinjected, then cooled and rewarmed to drive tubulin incorporation. Total tubulin fluorescence in individual, living cells was quantified using a cooled, scientific grade CCD image sensor. Cells were then washed and lysed into a microtubule-stabilizing buffer to extract the soluble pool. Total tubulin polymer fluorescence was determined for the extracted cells in the same way as for living cells. Fluorescence images were corrected by flat-fielding and background subtraction. The ratio of extracted cell fluorescence/living cell fluorescence for individual cells, was taken as the proportion of tubulin as polymer. Cells in M-phase, G_1 and random interphase were analyzed. G_1 cells had almost the same proportion as random interphase cells. Mitotic cells gave a value of 90±5% of G_1 cells at 37°C. Within M-phase, levels of tubulin as polymer in metaphase and early anaphase were not significantly different. In contrast to the general expectation of microtubule depolymerization at anaphase onset, these results indicate that as cells exit mitosis, the overall proportion of tubulin as polymer does not change dramatically even though the mitotic spindle disassembles. We conclude that the mitosis-interphase transition is accompanied by a redistribution of tubulin at an essentially constant polymer level. Therefore, a global shift to depolymerization conditions is not the driving force for anaphase chromosome movement.

Key words: tubulin, microtubule, interphase, mitosis, fluorescence microscopy

INTRODUCTION

Two different classes of model have been put forward to explain the mechanism of chromosome movement during anaphase. One class proposes that chromosome movement may be the result of disassembly of the microtubules to which the chromosomes are attached (Inoue and Sato, 1967; Margolis and Wilson, 1981; Koshland et al., 1988; Coue et al., 1991). The force for motion is derived from the free energy change upon depolymerization (Hill, 1981, 1985). For example, in the experiments of Koshland et al. (1988) and Coue et al. (1991) microtubules were attached to isolated chromosomes or particles and then diluted in buffers lacking both ATP and GTP, and containing a lower concentration of tubulin, to promote microtubule depolymerization. Upon dilution of the tubulin, either the minus ends of the microtubules moved towards the chromosome or the chromosome moved towards the minus ends of microtubules. More recently, studies using microinjection of biotin-tubulin into anaphase cells revealed that elevated concentrations of biotin-tubulin can induce transient elongation of kinetochore microtubules and reversal of chromosome-to-pole motion, suggesting that tubulin polymerization can drive the chromosome towards the plus end (Shelden and Wadsworth, 1992). Together, these experiments suggested that microtubule dynamics can be linked to the production of force and, specifically, that disassembly may be the driving force for anaphase chromosome movement. The second class of models proposes that chromosome movement may be driven by motor proteins (Rieder and Alexander, 1990; Hyman and Mitchison, 1991), such as a dynein or a kinesin-like molecule, located at the kinetochore (Steuer et al., 1990; Pfarr et al., 1990). In either case, microtubules depolymerize primarily at the kinetochore during chromosome movement. If motor molecules are the sole driving force for movement, one might not expect to see a change in tubulin polymer levels. Microtubule disassembly during chromosome movement would be expected to be accompanied by a redistribution of tubulin subunits from the spindle structure to the cytoplasmic network. However, if the disassembly of microtubules is the driving force, then a global shift to depolymerization conditions would be expected to decrease tubulin polymer. Clearly, there is a need to distinguish between these two distinct models, and investigate the possibility of a global shift and the extent of such shifts in the polymerization level in individual cells.

Previous studies have not established clearly whether there is more or less tubulin polymer in the mitotic spindle than in

the interphase network. Existing methods available for measuring abrupt changes in tubulin polymer during the mitosis-interphase transition are rather limited. Previous electron microscopic analyses have established a microtubule count in a given region of a cell, but cannot indicate free monomer levels (McIntosh et al., 1975; Rieder, 1981b; McDonald et al., 1992). Although many biochemical, pharmacological and immunological methods have been developed for the determination of tubulin in cells or tissue extracts (Borisy, 1972; Rubin and Weiss, 1975; Pipeleers et al., 1977; Hiller and Weber, 1978; Ostlund et al., 1979; Beertsen et al., 1982; Thrower et al., 1991), almost all have been designed for analyzing large populations of cells and rely on measuring tubulin by indirect assays, i.e. binding of colchicine or anti-tubulin IgG. Multiple steps in the assay procedures lead to large variation in the results obtained, and none of the methods lends itself to measuring abrupt changes at a defined cell stage such as the metaphase-anaphase transition.

We have developed a new method for determining the amount of tubulin as polymer based on the quantitative fluorescence detection of x-rhodamine tubulin injected into living cells before and after extraction in a microtubule-stabilizing buffer. We use the term 'fluorescence ratio assay' for this new method. This method is direct and fast, since fluorescently labeled microtubules can be directly measured with a cooled, charge-coupled device (CCD) image sensor after microinjection. In addition, it provides the high temporal resolution needed for determining the proportion of tubulin as polymer during the mitosis-interphase transition in individual cells. We show that the proportion of tubulin as polymer in the cell does not change dramatically from mitosis to interphase. We suggest that chromosome movement and onset of anaphase are not driven by a global shift in the cell to depolymerizing conditions.

MATERIALS AND METHODS

Cell culture

Porcine kidney epithelial cells of the line LLC-PK (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's (DME) medium (Sigma Chemical Company, St Louis, MO) containing 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 20 mM Hepes, and antibiotics. Two days prior to an experiment, cells were transferred to an etched locator coverslip (Bellco Biotechnology, Bellco Glass, Inc., Vineland, NJ) mounted over a hole in the bottom of a 35 mm culture dish modified for microinjection (Gorbsky et al., 1987) and grown at 37°C in 10% CO_2. In order to reduce fluorescence background for quantification of fluorescence, the normal DME medium in a culture dish chamber was changed to DME medium without Phenol Red a few hours before an experiment.

Preparation of x-rhodamine tubulin and microinjection

Pure tubulin was prepared from microtubule protein as previously described (Borisy et al., 1975; Vallee and Borisy, 1978), and then conjugated by the addition of *N*-hydroxysuccinimidyl x-rhodamine (Sammak et al., 1987). After 10 minutes at 37°C the mixture was pelleted at 20,000 ***g***, at 37°C for 20 minutes. Labeled tubulin was then further purified by two or three cycles of polymerization at 37°C in 10% DMSO and depolymerization at 0°C without DMSO. Depolymerized x-rhodamine tubulin at 6-8 mg/ml in injection buffer (100 mM PIPES, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM GTP, pH 6.9) with 1% DMSO was divided into 10 μl aliquots and stored in liquid nitrogen until the day of use.

Cells were microinjected according to general protocols previously reviewed (Kreis and Birchmeier, 1982). Before microinjection derivatized tubulin was spun for 30 minutes at 20,000 ***g***, 0°C to clarify the solution and prevent pipette clogging. Micropipettes (WPI, New Haven, CT) were pulled on a vertical pipette puller (David Kopf Instruments, Tujunga, CA). The micropipette concentration of tubulin was in the range 6.5-7.8 mg/ml and the volume injected was estimated at 5-10% of the cell volume. Cells were microinjected using a Nikon Diaphot inverted microscope (Nikon Inc. Garden City, NY) equipped with phase-contrast optics. Mitotic cells were injected between early prophase and metaphase. Injected cells were chilled at 0°C for 40 minutes and then rewarmed at 37°C to drive copolymerization of injected and endogenous tubulin. Mitotic cells were allowed to incorporate the labeled tubulin for at least 20 minutes before imaging was begun; interphase cells or G_1 cells derived from injected mitotic cells were allowed to incorporate label for 2 hours before imaging.

Cell lysis and tubulin extraction

Fluorescence was quantified in living cells and in the same cells after lysis and extraction. After imaging the living cells, the culture dish was removed from the stage of the microscope and quickly rinsed twice with phosphate buffered saline (PBS), pH 7.4, at 37°C. To extract the soluble pool, the cells were then lysed for 2 minutes in a microtubule-stabilizing buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9) containing 0.5% Triton X-100 detergent (Pierce, Rockford, IL) and 10 μg/ml taxol (National Cancer Institute, Bethesda, MD). Lysis and extraction conditions were based on the study of Schliwa and Van Blerkom (1981), who analyzed many buffer variations and determined a solution that preserved the cytoskeleton well. Taxol was included to specifically stabilize microtubules during extraction, and to maintain their stability during subsequent processing (Schiff and Horwitz, 1980). After cell lysis and tubulin extraction, the cells were immediately returned to the microscope stage, relocated by means of the etched locator grid and imaged again.

Data collection and image analysis

Fluorescence microscopy was performed on a Zeiss IM35 inverted microscope (Carl Zeiss, Inc., Thornwood, NY), using the Zeiss rhodamine filter cassette (ex, 546/12 nm; em, 590 nm long pass), and an Apo ×40/1.0 NA phase objective. The temperature of the medium in the dish chamber was maintained at 37°C by circulating warm water through a brass block holding the dish chamber and through a coil surrounding the objective during the period of monitoring cells. Temperature was measured at the specimen plane with a calibrated thermistor. A layer of mineral oil (E. R. Squibb and Sons Inc. Princeton, NJ) was overlayered on the dish medium to retard gas exchange and evaporation during observations of living cells.

For initial focusing with minimum bleaching, a SIT camera (Dage-MTI Inc. Precision video, Michigan city, IN) was used to collect three video frames (total exposure 0.1 second), which were then averaged using a Quantex QX 9000 image processor (Quantex Corporation, Sunnyvale, CA). For quantitative analysis, fluorescent and phase-contrast images were captured in digital form using the CCD camera (Series 200, camera head, CH220, Photometrics Ltd., Tucson, AZ). Total tubulin fluorescence in individual cells was determined before and after lysis and extraction. All digital images were saved for later image analysis.

Before image analysis, all fluorescence images were corrected by background subtraction and flat-fielding. The light source and optical system introduce some variation in the illumination pattern at the object plane and therefore also over the field of the CCD array. Flat fielding corrects for this uneven illumination if that illumination is a stable characteristic of each object exposure. Flat fielding was accomplished by image arithmetic as follows: $I_C = [(I_R - I_B) \cdot M]/(I_F - I_B)$, where I_C is the corrected image, I_R is the non-corrected or 'raw' object exposure, I_B is an electronic or dark background frame obtained with the shutter closed, M is the mean pixel value of the object exposure,

and I_F is the so-called flat field frame obtained with no specimen but a homogeneous fluorescent field.

The flat-fielded images were analyzed in the following manner. Images of living or extracted cells were displayed on the video monitor. Using a mouse we delineated a rectangle on the monitor surrounding the entire living or extracted cell. Fluorescence intensity values of the pixels within the rectangle were integrated and computed.

For presentation, images were taken using a Planapo ×100/1.3 NA objective and scaled to 8 bits for display, and photographed by a 4000 line film recorder Matrix PCR (Matrix Instruments Inc., Orangeburg, NY) using T-max 100 film (Eastman Kodak Co. Rochester, NY).

RESULTS

Evaluation of quantitative fluorescence technique

Our method of analysis is based on the quantitative fluorescence detection of x-rhodamine tubulin injected into cells and measured in two different states - living and after extraction in a microtubule stabilizing buffer. Quantification of fluorescence in the living state gives a value proportional to total tubulin content, - polymer plus the subunit pool. After extraction of the soluble subunit pool, the remaining fluorescence gives a value proportional to the tubulin in polymer form. The ratio of fluorescence, extracted/living, then gives the proportion of tubulin in microtubules (see Materials and Methods). Validation of this fluorescence assay method requires evaluation of both optical and cell biological parameters. Fluorescence must be quantitatively determined for tubulin throughout the volume of the cell, and the conditions for microtubule stabilization after extraction must neither augment nor diminish the preexisting level of cellular polymer.

The fluorescence ratio assay requires that an image of a living cell is to be compared with an image of the same cell after extraction, presumably at lower intensity because of the extraction of soluble tubulin. Several sources of error may contribute to random or systematic error in the analysis. These could include light-source instability, photobleaching, a change in the fluorescence yield of x-rhodamine tubulin before and after extraction, and imprecision of focus. We evaluated each of these sources of error.

Since fluorescence intensity is proportional to exciting intensity, i.e. emission of the fluorophore molecules is proportional to the intensity of the incident photons, any variations of fluorescence due to fluctuations in the light-source intensity between the times that the living and extracted cell images were recorded would result in variations in the signal captured by the CCD, which would be unrelated to the proportion of tubulin polymer. Significant fluctuation of source intensity was obtained with a conventional microscope lamp and power supply, leading us to replace the original lamp with a more stable source. The light source chosen was a 100 W Hg arc with a current-regulated power supply (Oriel model 68805, ORIEL Corporation, Stratford, CT 06497).

We evaluated the stability of the light source by taking a series of one-second exposures with a one-second interval or a 59-second interval between each exposure (Fig. 1). The signal was directed to the CCD using a half-silvered mirror in the dichroic mirror position and a metal-coated neutral density filter (0.1% transmission) on the microscope stage. The output of the light source was reflected back to the CCD by the neutral density filter and any variations in the intensity produced variations in the signal captured by the CCD as measured in ADUs (Analog to Digital Units). The current regulated power supply produced a 0.5% standard deviation when measured over a 50-second period and 0.4% standard deviation when measured over a 50-minute period (Fig. 1). Thus, both short-term and long-term fluctuations were negligible under these conditions.

Photobleaching is a second potential source of error and, unlike source fluctuation, which would generate random variation, photobleaching would systematically underestimate true fluorescence in the extracted cell. The bleaching due to exposure using a ×40 oil 1.0 NA lens was evaluated by quantifying successive exposures. Cells were microinjected with x-rhodamine tubulin and successive one-second exposures were taken of each cell. The total cell fluorescence was determined by integrating over a boxed region with the CCD after background subtraction and flat-fielding (see Figs 3, 6, 8, for examples of images). The level of photobleaching (Fig. 2) was negligible (1% per exposure), indicating that the measurement of fluorescence intensity in living and extracted cells can be compared essentially without the need for correction.

Implicit in our fluorescence ratio experiments is the assumption that the fluorescence yield of x-rhodamine tubulin in the intact cell is identical to that in our stabilization buffer following extraction. Although it is not possible to duplicate cytoplasmic conditions precisely, we did examine whether fluorescence yield varied detectably with different buffers, ionic conditions, and over a pH range of 6.4-7.4. In these experiments, interphase cells were injected with x-rhodamine tubulin, allowed to incorporate label for two hours, and then lysed and extracted in taxol-containing buffer. After a brief wash with microtubule stabilizing buffer, fluorescence images of the cells were taken in each of five different buffers, each containing taxol (our standard stabilization buffer, pH 6.9; 100 mM Pipes containing EGTA and Mg, pH 6.4; 100 mM Hepes, pH 7.4; stabilization buffer without EGTA and Mg, pH 6.9; or PBS, pH 7.4). The fluorescence intensity of the same cell was measured in each of the different buffers by sequential replacement of the solutions. None of the solutions significantly

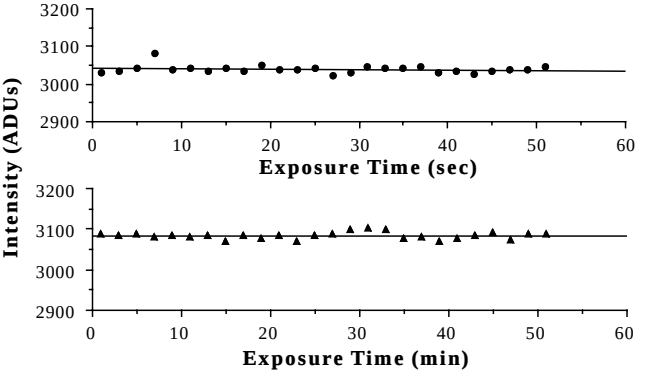

Fig. 1. Stability of light source and rate of bleaching. A series of 1-second exposures were taken with a 1-second wait (upper panel) or a 59-second wait (lower panel) period between each exposure. The light source was a Hg arc with current-regulated power supply (Oriel model 68805). The signal was directed to the CCD with a half-silvered mirror. The results indicate that the signal was steady to within 0.5% on a time scale of 1 second to at least 1 hour.

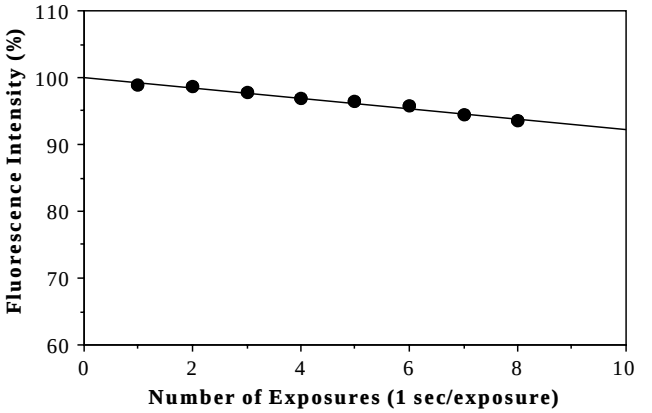

Fig. 2. Cells were injected with x-rhodamine tubulin, successive 1-second exposures were taken, and the total cell fluorescence for each exposure was measured with the CCD. The fluorescence intensity was normalized, with 100% defined as the value of intensity extrapolated to zero exposure. The figure shows the average values for 10 cells. A curve was fitted to the data by a least squares regression and extrapolated to zero exposure. Bleaching of the fluorophore resulted in a decrease in the fluorescence intensity, of about 1% per exposure, under our experimental conditions.

increased or decreased the fluorescence intensity values, indicating that the fluorescence yield of x-rhodamine tubulin is not very sensitive to changes in its aqueous environment. These results are in agreement with previous findings that have shown that rhodamine fluorescence is unaffected by pH over the range 4-8.5 (Murphy et al., 1984).

Since cells have a three-dimensional shape, light from outside the focal plane may influence the fluorescence signal captured by the CCD. The effect of focus on fluorescence measurement was examined by taking phase-contrast and fluorescence images of cells above and below the plane of focus. Fig. 3 shows typical mitotic and G_1 cells, and the fluorescence intensity for each image integrated within a boxed region containing the cell. Although defocussing causes the light to spread out, it can still be largely contained within the defining box. The results demonstrate that under these conditions slight changes in focus do not significantly affect the measurement of fluorescence intensity, even for distances as great as 10 μm above or below focus, in agreement with previous studies (Kajstura and Bereiter-Hahn, 1988).

Perturbation of the normal tubulin polymer state

If the ratio of fluorescence in polymer to total tubulin fluorescence is an accurate indicator of the underlying microtubule polymerization equilibrium, it should behave predictably under known perturbants of microtubule assembly (Keith, 1991). When cells are treated with taxol, the critical concentration for tubulin assembly into polymer is drastically lowered, and intracellular tubulin is driven into polymer (Schiff et al., 1979; Schiff and Horwitz, 1980). The proportion of polymer in the cell should be close to 100% under such a condition. However, when the temperature is lowered, favoring depolymerization of microtubules (Brinkley and Cartwright, 1975) or when cells are treated with nocodazole, which binds to tubulin and inhibits

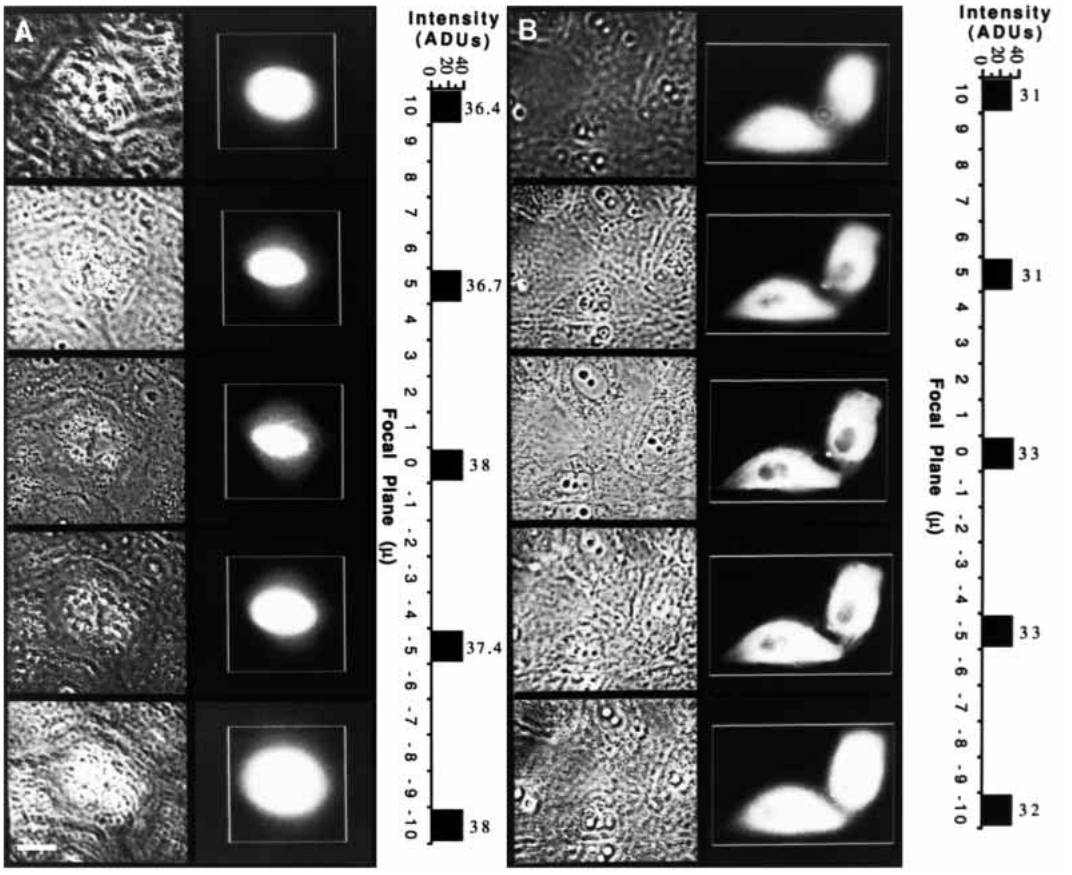

Fig. 3. Effect of focus on fluorescence intensity measurements. Through focus fluorescence images of mitotic (column A), and G_1 cells (column B) were taken at focal planes 5 μm and 10 μm both above and below the in-focus plane (μ=μm). Quantification of the fluorescence intensity from each image in the focal series is shown in bar form at the side of the images. The fluorescence intensity 10 μm above or below focus averaged 96.6% of the level measured at focus. These results indicate that the fluorescence captured by the CCD varies very little with these changes in focus using a ×40, 1.0 NA objective. Bar, 10 μm.

its polymerization (Lee et al., 1980; De Brabander et al., 1986), the proportion of polymer in the cell should be close to 0%.

The boundary conditions of perturbation from the normal tubulin polymer state were examined. Cells injected with x-rhodamine tubulin were treated with either taxol (10 μg/ml) for 12 hours, or nocodazole (40 μg/ml) for 2 hours, or chilled at 0°C for 40 minutes, and then fluorescence images were taken before and after lysis and extraction of cells (Fig. 4A). The fluorescence intensities were measured and quantified as described above. As expected, cells treated with taxol showed high levels of polymer, averaging 89% of total tubulin (Fig. 4B). We also determined the percentage of x-rhodamine tubulin in polymer in the presence of taxol in vitro by a sedimentation assay, and found that over 90% of the tubulin was polymerizable. The remaining 5-10% of tubulin presumably contained assembly-incompetent subunits as the critical concentration for fully active tubulin with taxol is very close to zero (Schiff et al., 1979; Schiff and Horwitz, 1980). In contrast, cells whose microtubules were depolymerized by nocodazole or cold gave lower values, averaging 2% of total tubulin (Fig. 4B). Under perturbing conditions, the shifts in levels in the expected directions support the conclusion that the fluorescence ratio method is a valid indicator of the proportion of tubulin in microtubules.

In our experiments, cells were lysed into a microtubule-stabilizing buffer to extract the soluble tubulin pool. Previous studies using electron microscopy and antibodies against tubulin demonstrated that the microtubule array is well preserved after extraction with the microtubule-stabilizing buffer at 37°C (Schliwa and Van Blerkom, 1981; Schliwa et al., 1981). Using mitotic cells as a sensitive test object because their microtubules are known to be highly dynamic, we found that inclusion of 10 μg/ml taxol in the lysis buffer was sufficient to obtain reproducible fluorescence ratios (57±7%), whereas omission of taxol gave lower and variable values (25%-50%). We performed the same evaluation with interphase cells by lysing them in buffer with and without taxol. Interphase cells were less sensitive than mitotic cells to extraction conditions. Extraction in PHEM in the absence of taxol gave values that were approximately 90% of values in the presence of taxol. We conclude that, by addition of taxol, extraction of soluble tubulin can be performed without significant microtubule depolymerization even in highly dynamic mitotic cells. However, taxol addition might produce a different sort of error. If free cytoplasmic tubulin monomer were driven into polymer during the lysis in the presence of taxol, it would increase the proportion of tubulin polymer and give an erroneous result. This possibility was investigated by pre-incubating mitotic cells with PBS

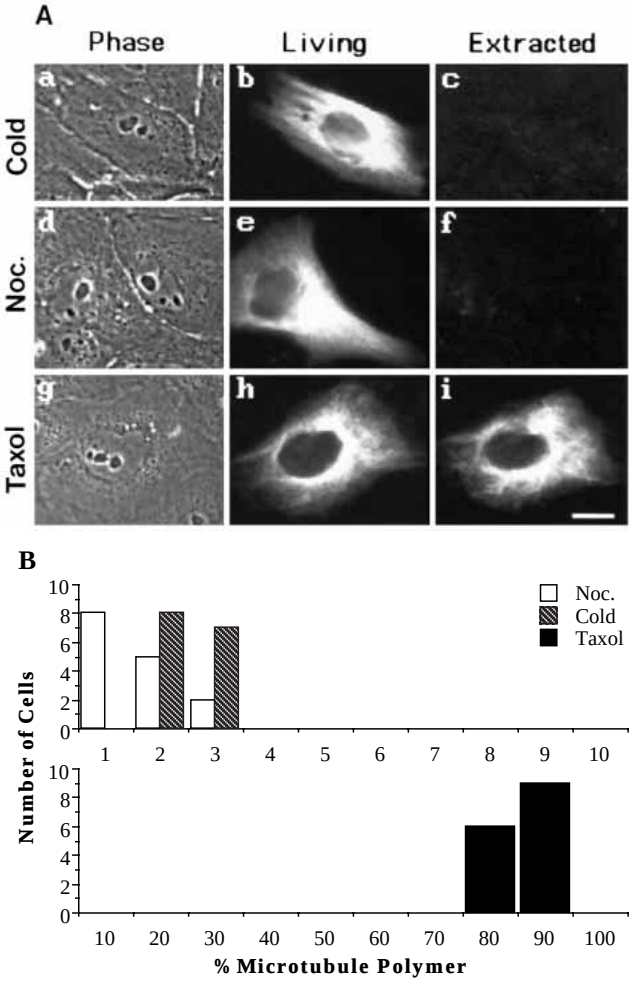

Fig. 4. Perturbation of the normal tubulin polymer state. Cells injected with x-rhodamine tubulin were treated with either taxol or nocodazole, or chilled at 0°C and then rewarmed at 37°C as described in the text. (A) Fluorescence was measured to quantify tubulin polymer after these extreme perturbations from the normal state. (a-c) A cold-treated cell; (d-f) a nocodazole-treated cell; (g-i) a taxol-treated cell. Fluorescence intensities were integrated by a CCD image sensor over a boxed region before and after lysis and extraction. The fluorescence ratio is the intensity of fluorescence in extracted cell divided by the intensity of fluorescence in living cell, multiplied by 100%. (B) Histograms show the fluorescence ratio after nocodazole or cold treatment, and the fluorescence ratio after taxol treatment. A total of 45 cells was analyzed.

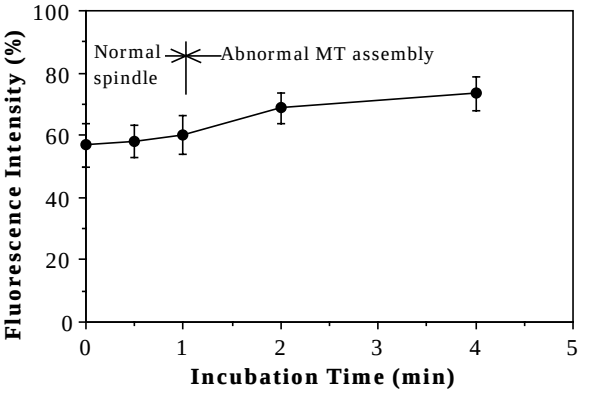

Fig. 5. Effects of preincubation with taxol. x-rhodamine-injected cells were incubated with PBS buffer containing taxol for various times (30 seconds, 1 minute, 2 minutes, 4 minutes). Zero time is defined as no taxol treatment prior to lysis and extraction. After preincubation with taxol, the cells were lysed and extracted in microtubule-stabilizing buffer. Fluorescence images were taken of the cells before and after lysis and extraction. The fluorescence ratio was determined as described in the text. A total of 40 cells were studied, and the results indicated that the time required for taxol to penetrate and exert its effects on intact cells is less than the time needed for cell lysis.

buffer containing taxol (10 μg/ml) for various periods of time (30 seconds, 1 minute, 2 minutes, 4 minutes), and comparing the fluorescence ratios obtained with those for cells that were not pre-incubated or were pre-incubated with PBS not containing taxol. Taxol pre-incubation of 2 minutes or longer induced the assembly of non-spindle free microtubules and an increased level of microtubule polymer (65%-75%), but shorter pre-incubations (<30 seconds) gave normal spindles and results that were not significantly different (Fig. 5) from unwashed cells or cells washed in PBS lacking taxol. The lack of perturbation in cells pre-incubated with taxol for less than 30 seconds indicates that the time required for taxol to penetrate and exert its effects on intact cells is less than the time needed for cell lysis. After lysis, taxol rapidly stabilizes the pre-existing polymer, and the soluble tubulin pool is quickly removed by diffusion and mixing in an essentially infinite volume. Thus, there is not sufficient time for the drug to induce polymerization before the subunit concentration falls to negligible values.

Proportion of tubulin polymer in metaphase, G_1 and interphase cells

LLC-PK cells were microinjected with x-rhodamine tubulin in mitosis and interphase, chilled at 0°C for 40 minutes to depolymerize microtubules and then rewarmed at 37°C to reassemble microtubules from a tubulin pool with a constant mole fraction of x-rhodamine tubulin. This allowed all dynamic microtubules to become as uniformly labeled as possible. Fig. 6a-c shows a typical mitotic cell before (Fig. 6a,b) and after lysis and extrac-

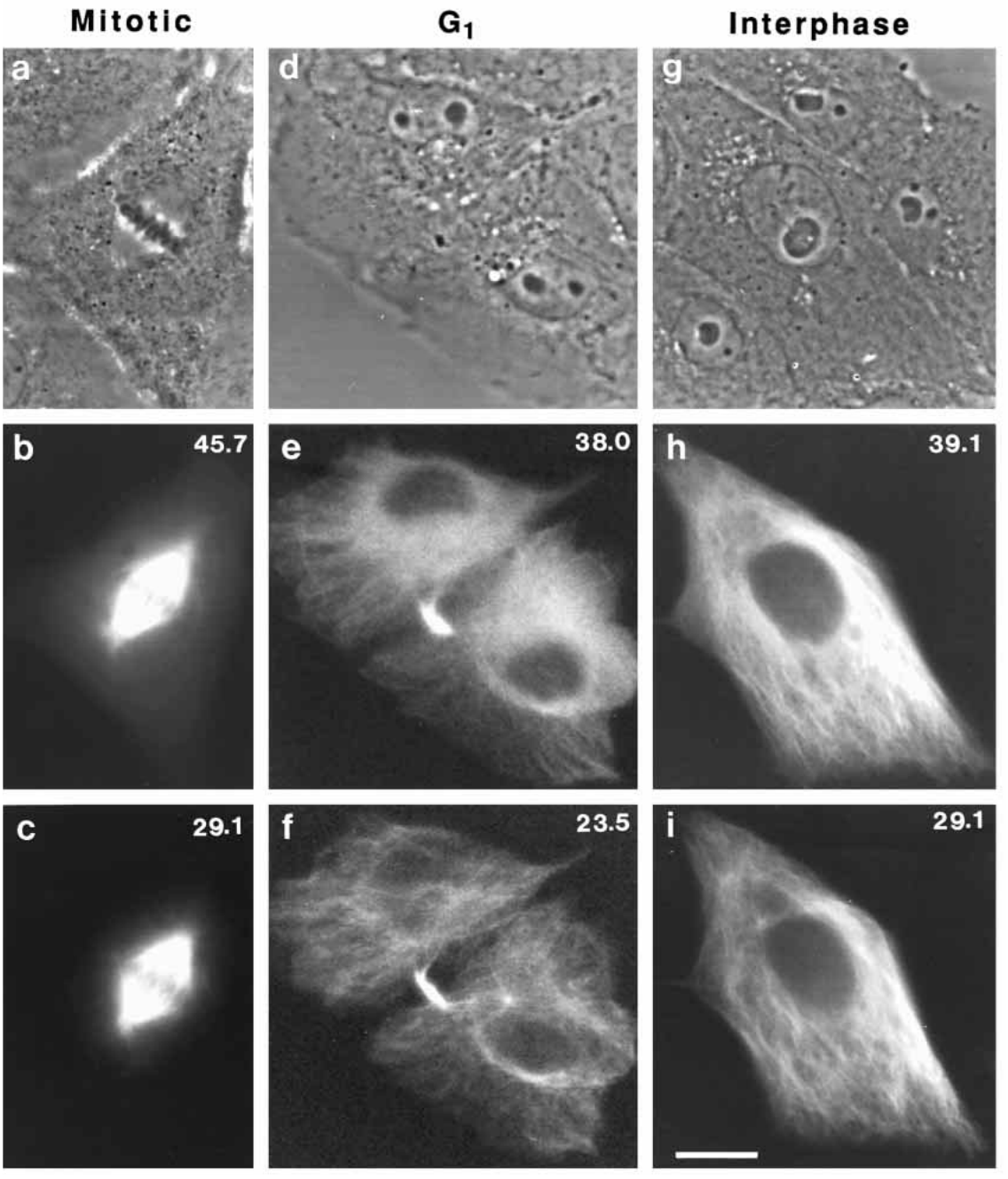

Fig. 6. Quantification of tubulin polymer in mitotic, G_1 and interphase cells. The mitotic cell was microinjected with x-rhodamine tubulin and allowed to incorporate into spindle microtubules for 20 minutes. G_1 cells were microinjected in mitosis and then allowed to progress to G_1 phase. Two hours after injection fluorescence images were taken of the G_1 and random interphase cells before and after lysis and extraction. Phase-contrast images (a,d,g) and fluorescence images (b,e,h) were taken of living cells. After lysis and extraction in a microtubule-stabilizing buffer, a second fluorescence image was taken of each cell (c,f,i). Average fluorescence intensities (arbitrary units) are shown in the top right-hand corners; the proportion of tubulin as polymer is 64% of the total tubulin in this mitotic cell, 62% in the G_1 and 74% in the random interphase cell. Bar, 10 μm.

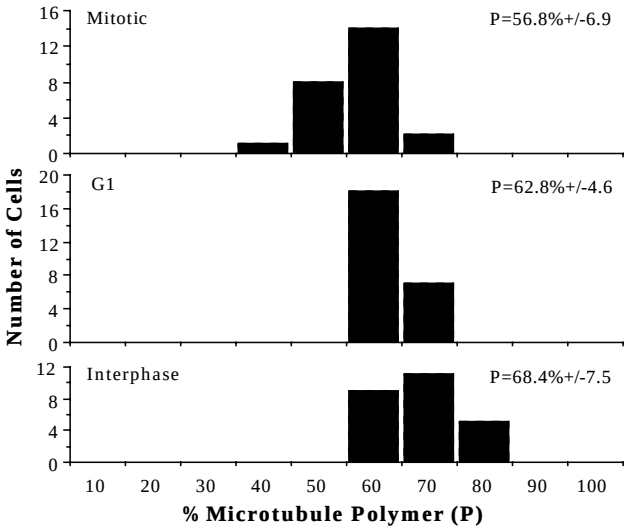

Fig. 7. Histogram of polymer level at different stages of the cell cycle at 37°C. The percentage of tubulin as polymer, measured in 25 mitotic cells (A), 25 G_1 cells (B) and 25 interphase cells (C) as described in the text, are displayed in the histograms. The average proportion of tubulin as polymer in cells, P, is given in the panels.

tion (Fig. 6c). Total fluorescence intensity of tubulin in the cell was determined by integrating the intensity values for all the pixels within the same sized rectangle surrounding the entire cell before and after lysis and extraction (see Materials and Methods). To determine the proportion of tubulin as polymer in G_1 cells, we microinjected mitotic cells and then allowed the cells to progress to form two daughter G_1 cells. The proportion of tubulin polymer in the two daughter cells was determined as one cell. Typical G_1 cells are shown in Fig. 6d-f. The ratio of tubulin polymer in random interphase cells was also determined (Fig. 6g-i). The fluorescence image of living cells showed fibrillar fluorescence over a diffuse background. Lysis and extraction decreased the diffuse background to a negligible level (Fig. 6c,f and i). We interpret the diffuse background as resulting from the soluble tubulin that is removed by the lysis and extraction procedure. Controls in which cells were not stabilized with taxol but rather were fixed and extracted simultaneously gave a higher background, presumably because of the soluble non-extracted tubulin (data not shown). We found that the average proportion of tubulin polymer was 56.8±6.9% for mitotic cells, 62.8±4.6% for G_1 cells and 68.4±7.5% for random interphase cells (Fig. 7).

The differences in tubulin polymer were evaluated by an analysis of variance model using a statistical analysis package (SAS Institute Inc. SAS Circle Box 8000, Cary, NC 27512). The differences in percentage of tubulin as polymer in mitotic, G_1 and interphase cells were significantly different, with a P value less than 0.01. Although the differences in these populations were statistically significant, with G_1 cells having 10% more tubulin polymer than mitotic cells, and random interphase cells 10% more polymer than G_1 cells, the more significant result from the point of view of this study was that the levels of polymer throughout the cell cycle changed so little. Further, the change observed was a small gradual increase rather than a decrease as the cells exited mitosis.

Proportion of tubulin polymer in anaphase

The analysis described in the preceding section showed that

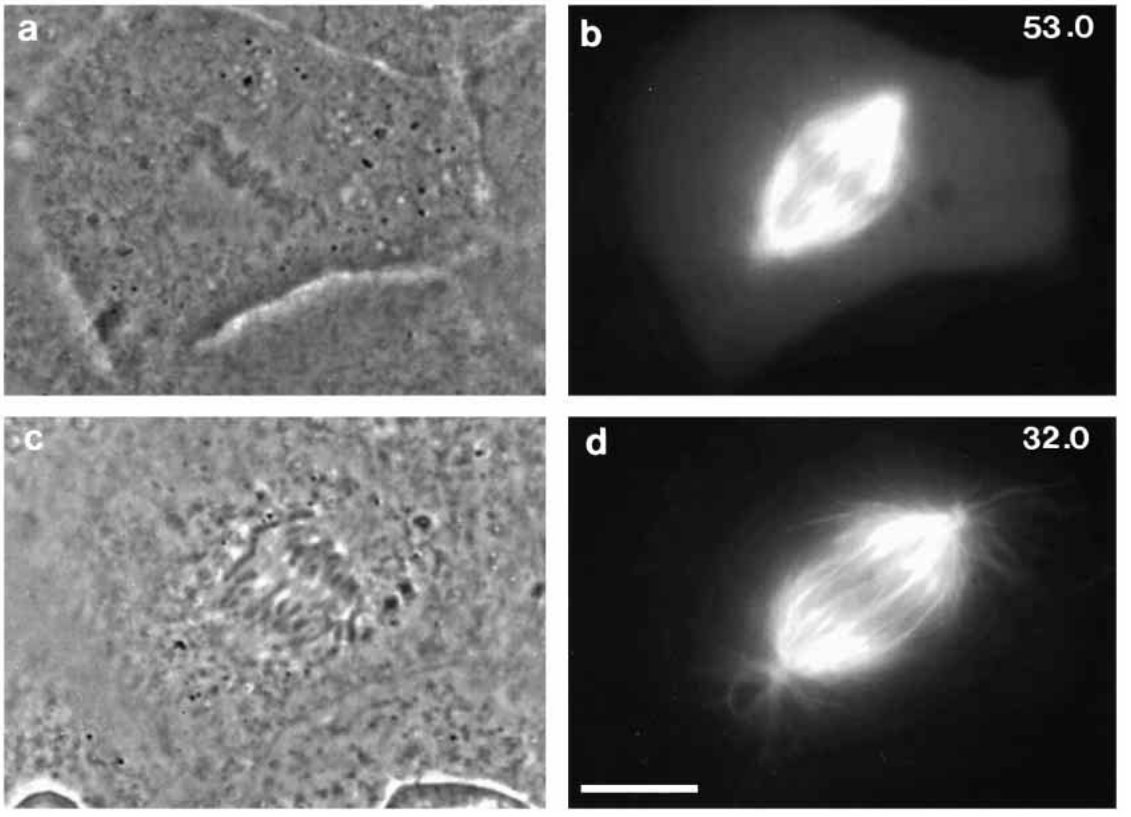

Fig. 8. Quantification of tubulin polymer at the metaphase-anaphase transition. The live metaphase cell (a,b) went into anaphase (c,d) just prior to lysis, so an early anaphase spindle is seen in the image taken after lysis. Average fluorescence intensity is shown in the top right-hand corners. The proportion of tubulin as polymer is 60% of the total tubulin in the cell. Bar, 10 μm.

the level of tubulin polymer in G_1 cells was similar to that in mitotic cells. However, an abrupt and transient decrease in polymer level between metaphase and G_1 might have gone undetected. To examine the possibility that anaphase chromosome movement is induced by a global shift to conditions favoring microtubule depolymerization, we investigated whether the level of tubulin polymer changes abruptly from metaphase to anaphase. Cells at metaphase were microinjected with x-rhodamine tubulin, chilled and then rewarmed before imaging as described above. Fig. 8 shows a typical example of such a cell. A fluorescence image was taken at metaphase (Fig. 8a, b) or anaphase for the living cell. The cell was then allowed to progress into anaphase just prior to lysis and extraction (Fig. 8c, d), so an early anaphase spindle was present in the image taken after extraction. The fluorescence intensities for living and extracted cells were measured as described above.

The proportion of tubulin in polymer obtained from live metaphase or anaphase cells and lysed anaphase cells was the same within experimental error as that obtained from live metaphase and lysed metaphase cells. The average proportion of tubulin in polymer from 10 such cells was 59±5%. Thus, no decrease in polymer level was detected at the metaphase-anaphase transition. At the onset of anaphase, kinetochore microtubules depolymerize but astral microtubules elongate and the midbody develops. Our results indicate that although different microtubule populations within the spindle are undergoing depolymerization and polymerization, the overall proportion of tubulin in polymer remains almost constant.

Effect of lower temperature on tubulin polymer ratio

Although the cultured cells we used grow optimally at 37°C, they will continue to enter and complete mitosis at temperatures as low as 23°C. As assayed by electron microscopy and immunofluorescence, the density of microtubules within the spindle is greatly reduced at lower temperature (23°C) (Brinkley and Cartwright, 1975; Rieder, 1981b; Cassimeris et al., 1990; Wise et al., 1991). The decrease in the number microtubules is most likely due to the loss of non-kinetochore microtubules (Wise et al., 1991). However, precise measure of polymer loss cannot be obtained by these methods. Therefore, we used the fluorescence ratio method to determine the proportion of tubulin as polymer in mitotic cells at 23°C.

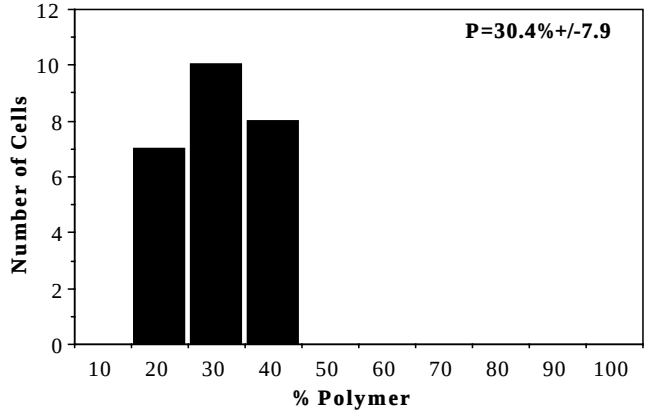

Fig. 9. Effect of temperature on tubulin polymer ratio in mitotic cells at 23°C. A total of 25 LLC-PK mitotic cells were microinjected with x-rhodamine tubulin and the tubulin polymer ratio was determined at room temperature (23°C). The average proportion of tubulin as polymer, 30.4%, was 53% of the value obtained at 37°C.

Cells were injected with x-rhodamine tubulin, cooled on ice and then rewarmed as described above. Before imaging, the cells were placed on a microscope stage where they were allowed to equilibrate to room temperature for 5-10 minutes. After imaging, the cells were lysed and extracted at room temperature. A total of 25 mitotic cells were analyzed and we found that at the lower temperature, mitotic cells gave an average of 30.4±7.9% polymer (Fig. 9), which is about 53% of the total tubulin in polymer at 37°C. The mitotic cells under these conditions still divided, even though almost 50% of the original tubulin polymer of the spindle was gone. This agrees with previous studies in which cells can form a normal spindle and can complete mitosis at the lower temperature (Rieder, 1981a).

DISCUSSION

Tubulin polymer determination

We have developed a new fluorescence ratio method for determining the proportion of tubulin polymer in individual cells. The motivation for developing the method was to determine whether net microtubule depolymerization occurred at the metaphase-anaphase transition, but the approach is general and could be applied to any cell type and, in principle, to any monomer ↔ polymer system for which a direct fluorescent probe is available. The method is intrinsically more direct and therefore potentially more linear than indirect methods such as immunoassays, which require multiple steps. As for ratiometric ion imaging (Gynkinewicz et al., 1985), the use of a fluorescence ratio approach obviates the need to independently determine excitation intensity, fluorescence quantum yield, optical constants, detector efficiency, quantity of tubulin injected and specific activity of fluorescence labelling of tubulin.

The method does require the light source to produce a steady output for the measurements of fluorescence intensities at two different times and bleaching of the fluorophore to be minimized. Our results have demonstrated that these conditions are met in our experimental system.

A more serious consideration is whether determination of fluorescence ratio at one focal plane of the cell can provide quantitative information for the entire cell volume. Cells are much thicker than a single focal plane and they round up and increase their thickness during mitosis. The image includes both in-focus and out-of-focus components, and the question may be raised of whether the out-of-focus component is entirely captured by the objective lens. Our results indicate that slight changes in focus did not significantly affect the measurement of fluorescence intensity with a ×40 oil lens when the values were integrated over an appropriately sized box. This agrees with previous studies that indicated that the deviation in fluorescence intensity was below 5% for ±12 μm out of focus in the case of ×40 oil lens (NA 1.0) (Kajstura and Bereiter-Hahn, 1988). Therefore, we conclude that, under our experimental conditions, the fluorescence intensity measured includes photons emitted both from the plane of focus and from planes not in focus, and reflects tubulin content throughout the entire volume of the cell, provided it is ≤20 μm thick.

The extracted/living fluorescence ratio was 89% for the cells treated with taxol and 2% for the cells treated with nocodazole or chilled at 0°C. The fluorescence ratio for the cells treated with taxol was expected to be close to 100%. The experimental result of 89% was similar to what we found when our x-rhodamine tubulin preparation was treated with taxol in vitro, where 5-10% of the x-rhodamine tubulin did not polymerize and was assumed to be inactive (data not shown). Since our results indicated that the fluorescence yield of x-rhodamine tubulin in the same cells in different buffers, ionic conditions, and over a pH range of 6.4-7.4 did not significantly change, it seems reasonable to conclude that the quantum yield for fluorescence of extracted cells is similar to the quantum yield in the living state. Supporting this conclusion is the similarity in amount of tubulin polymerized by taxol in vitro and in vivo (~90%) as assayed by completely different methods: sedimentation in vitro and fluorescence in vivo.

If injected x-rhodamine tubulin adhered to cellular components during the lysis, the apparent level of polymer could be artifactually increased. The result of nocodazole or cold treatment showed that there was very little non-specifically adhering x-rhodamine tubulin. On the basis of known microtubule behavior, these results confirm, as expected, that we can use the ratio of fluorescence after and before extraction as a measure of the proportion of tubulin in polymer in individual cells. Our experiments also demonstrated that the proportion of microtubules in the LLC-PK cell line stable to cold or nocodazole treatment was very low.

Tubulin polymer mass does not change significantly at the mitosis-interphase transition

The major biological question posed by this study was whether net microtubule depolymerization occurs at the mitosis-interphase transition. If anaphase chromosome movement is driven by a global shift in the cell towards depolymerizing conditions, a decrease in tubulin polymer level would be expected. Moreover, recent studies using microinjection of biotin-tubulin into anaphase cells demonstrated that increasing the tubulin concentration could result in tubulin addition at the kinetochore in anaphase, suggesting that tubulin concentration may regulate kinetochore movement during mitosis (Shelden and Wadsworth, 1992). However, our results and a recent study (Jordan et al., 1992) have shown that the fraction of tubulin as polymer was remarkably similar between cells in mitosis and interphase, despite the radical change in microtubule organization during this transition. Instead of a decrease, net tubulin polymer, if anything, increased slightly as the cells entered interphase. More detailed analysis specifically at the metaphase-anaphase transition confirmed the constancy of microtubule levels. No transitory net depolymerization occurred at anaphase onset that might have been missed by comparing mitotic and G_1 cells. These results are inconsistent with the hypothesis that microtubule depolymerization, induced by a global shift to depolymerizing conditions, may be the driving force for anaphase chromosome movement (Inoue and Sato, 1967; Margolis and Wilson, 1981; Koshland et al., 1988; Coue et al., 1991). However, our results do not eliminate a role for local controls, possibly at the level of the individual microtubules, on the assembly and disassembly kinetics. The possibility of local changes in microtubule dynamics mediated by a regulated interaction with the kinetochores is clearly an area warranting further study.

Our results are consistent with qualitative studies that have found that at the onset of anaphase, kinetochore microtubules depolymerize but astral microtubules elongate and the midbody develops. However, our ability to quantify tubulin levels has shown that depolymerization occurring at the kinetochore is balanced by polymerization occurring elsewhere in the spindle and the cell. Thus, the overall proportion of tubulin in polymer remains essentially constant. This conclusion indicates that the mechanism of anaphase chromosome movement in living cells is likely to be more complicated than the simple depolymerization process suggested by the in vitro experiments. The movement of chromosomes not only involves microtubule disassembly, but also the action of motor molecules located at kinetochores (Steuer et al., 1990; Pfarr et al., 1990). If motor proteins provide the driving force for anaphase chromosome movement, then the tubulin polymer level would be expected to be determined by the reaction constants characterizing microtubule dynamics and, a priori, could increase, decrease or remain constant.

Tubulin polymer level could be reduced by lowering the temperature as indicated previously by quantitative studies (Rieder, 1981b; Cassimeris et al., 1990; Wise et al., 1991). At 23°C, mitotic cells had a polymer content of 30.4%, which was only 54% of the value at 37°C. Although lowering the temperature results in a reduction of tubulin polymer and slower mitotic progression, a bipolar spindle is formed. The spindle structure is slightly smaller, but is stable and chromosome segregation proceeds normally. Thus the polymer level normally obtaining at 37°C appears to be in excess over that required to achieve mitosis. Further, the fact that anaphase was neither induced nor prevented by modulating polymer levels by almost a factor of 2 is consistent with the conclusion that shifts in overall polymer levels, per se, do not drive chromosome motion.

In conclusion, the dramatic shift in microtubule organization from mitosis to interphase is not accompanied by a similarly dramatic change in tubulin polymer. Although the level of tubulin as polymer is modulated somewhat, the major event accounting for the reorganization of microtubules at the mitosis-interphase transition is a redistribution of tubulin subunits from the spindle structure to the cytoplasmic network. The redistribution, occurring at essentially constant tubulin polymer level, is presumably a result of intrinsic microtubule dynamics and changes in the pattern of microtubule organizing structures.

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