MICROTUBULE INITIATION AT KINETOCHORES AND CENTROSOMES IN
LYSED MITOTIC CELLS

Inhibition of Site-Specific Nucleation by Tubulin Antibody

DANIEL A. PEPPER and B. R. BRINKLEY. From the Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

ABSTRACT

A lysed cell system was developed to determine whether tubulin antibody can block the nucleation of exogenous tubulin at kinetochores and centrosomes. Mitotic PtK2 cells were pretreated with colcemid to remove all endogenous microtubules and were lysed with Triton X-100 in PIPES-EGTA-Mg** buffer. This procedure left centrosomes, chromosomes, and kinetochores intact as determined by electron microscopy of thin-sectioned cells. Exposure of the lysed cells to phosphocellulose-purified tubulin dimers at 37°C in the presence of 1 mM GTP resulted in site-specific nucleation of microtubules at centrosomes and kinetochores. Treatment of the lysed cell preparations with tubulin antibody before subsequent exposure to the exogenous tubulin resulted in almost complete blockage of microtubule nucleation, especially at kinetochores. Pretreatment of the lysed cell preparations with control antibody or buffer without antibody had no effect on the ability of centrosomes and kinetochores to initiate microtubule assembly. The implications of these results with respect to the molecular composition of centrosomes and kinetochores are discussed.

KEY WORDS Centrosomes, kinetochores, microtubule nucleation, tubulin antibody, electron microscopy

During mitosis, spindle microtubule assembly occurs at specific nucleating sites such as the kinetochore on the chromosomes and the spindle poles (for reviews, see references 1, 15, 17). Such regions, known widely as microtubule organizing centers (MTOCs) (20), have been described extensively but relatively little is known of their molecular composition or the way in which they initiate the assembly of tubulin into microtubules. Several cytochemical studies have indicated that kinetochores, centrioles (basal bodies), and related MTOCs contain RNA which may be involved in the nucleation of microtubule assembly (2, 13, 19, 30). More recently, Pepper and Brinkley (18) presented evidence that kinetochores, centrioles, and pericentriolar material contain tubulin which could be detected by immunoperoxidase staining with tubulin antibodies. Moreover, it was proposed that tubulin was an intrinsic protein of the MTOC and was essential for the initiation of microtubule polymerization at these sites.

To further evaluate the existence of tubulin in MTOCs and to test the notion that intrinsic tubulin of kinetochores is essential for microtubule polymerization, we investigated the assembly of purified 6S bovine brain tubulin at kinetochores and centrioles of lysed mitotic cells before and after exposure to tubulin antibodies.

MATERIALS AND METHODS

Tubulin Purification and Characterization

Microtubule protein was prepared from bovine brain by three cycles of polymerization-depolymerization according to the method of Borisy et al. (5). The microtubule pellet obtained was resuspended in cold buffer A.
containing 50 mM piperazine-N,N'-bis[2-ethane sulfonic acid] (PIPES), 1 mM ethylene glycol-bis-[β-aminooethyl ether]-N,N'-tetraacetate (EGTA), 0.1 mM MgCl₂, and 0.1 mM guanosine triphosphate (GTP), pH 6.9 at room temperature. Dimeric (6S) tubulin free of associated proteins was isolated from this preparation by phosphocellulose column chromatography according to methods described previously (25, 29). Immediately after elution from the column, the tubulin-containing fractions were pooled and brought up to 80 mM PIPES and 0.5 mM MgCl₂ for use in all subsequent experiments. Microtubule-associated proteins (MAPs) were eluted from the phosphocellulose column with a continuous gradient of 0-1.0 M KCl in buffer A. MAP-containing fractions were pooled and dialyzed overnight in buffer B (80 mM PIPES, 1 mM EGTA, and 0.5 mM MgCl₂, pH 6.9) containing 0.1 mM added GTP. Protein concentrations were determined using the Schacterle and Pollack (24) modification of the method of Lowry et al. (14).

The ability of phosphocellulose-purified tubulin to assemble in the absence and presence of MAPs was monitored by absorbance changes at 320 nm with a Gilford Model 240 recording spectrophotometer (Gilford Instruments Laboratories, Inc., Oberlin, Ohio) equipped with a water-jacketed cuvette chamber. Assays were performed by mixing sample components at 4°C in buffer B containing 1 mM GTP and recording turbidity changes upon warming the sample to 37°C in the cuvette.

Cell Lysis and Microtubule Nucleation

Rat kangaroo cells (strain PtK₂) were grown as monolayers in 60-mm Falcon plastic tissue culture dishes (Falcon Labware, Inc., Div. Becton Dickinson & Co., Oxnard, Calif.) containing Ham's F-10 medium supplemented with 10% fetal calf serum and 2 mM glutamine. The cells were maintained in a humidified atmosphere of 95% air and 5% CO₂. I h before each experiment, the growth medium was replaced with the same medium containing 0.15 μg/ml colcemid to disrupt spindle microtubules. All subsequent treatments were carried out at room temperature unless otherwise noted.

After pretreatment with colcemid, the medium was removed and cultures were rinsed for 30 s with buffer B containing 0.15 μg/ml colcemid. The cells were then gently lysed for 90 s in buffer B containing 0.15 μg/ml colcemid and 0.05% Triton X-100, followed by 3 washes with buffer B, 1 min each. Some lysed cell monolayers were fixed at this point by the addition of 2% glutaraldehyde (in buffer B) to the culture dishes, while others were used in the subsequent experiments described as follows.

After lysis, cell monolayers were incubated for 10-15 min at 37°C in 1.8 mg/ml of purified tubulin in buffer B containing 1 mM GTP. Other lysed cell preparations were first incubated in monospecific rabbit tubulin antibody (prepared according to the method of Fuller et al. [10]) for 15 min at 75-100 μg/ml antibody in buffer B. Controls were incubated in rabbit anti-goat IgG (100 μg/ml in buffer B) or in buffer alone for 15 min. Excess antibody was then washed from the cells with three changes of buffer B, and all preparations were then incubated with tubulin as described above. Incubations in tubulin were then stopped by the addition of 2% glutaraldehyde in buffer B.

Electron Microscopy

The cell preparations were allowed to fix in glutaraldehyde for 10 min and were then washed with buffer B, postfixed with osmium tetroxide, then dehydrated and embedded in Epon directly in the culture dishes using the method described previously (6) for cell monolayers. Selected mitotic cells were punched out of the Epon discs and glued to the tips of blank Epon pegs. Specimens were then serially sectioned with a diamond knife as described previously (18). Sections were stained with alcoholic uranyl acetate and lead citrate, and examined with a Siemens Elmiskop 102 electron microscope at 80 KV.

RESULTS

The polymerization characteristics of the tubulin preparation used in this study are illustrated in Fig. 1 (see figure legend for details). The partially purified microtubule protein used as the initial source material was assembled in vitro at a concentration of 2.0 mg/ml under the present experimental conditions (Fig. 1 a). Tubulin dimers isolated from this source by phosphocellulose column chromatography showed virtually no capacity to

![Figure 1](https://example.com/figure1.png)

**Figure 1** Time course of assembly of microtubule protein fractions at 37°C. Assay conditions are described in Materials and Methods. (a) 2.0 mg/ml whole microtubule protein; (b) 1.8 mg/ml phosphocellulose-purified 6S tubulin; (c) 0.2 mg/ml MAP fraction from phosphocellulose chromatography of whole microtubule protein; (d) 1.8 mg/ml 6S tubulin and 0.2 mg/ml MAP fraction mixed in the same sample.
initiate self-assembly at a protein concentration of 2.0 mg/ml (and as high as 2.5 mg/ml in some assays) under these conditions (Fig. 1b). When the assay mixture was fixed with glutaraldehyde, placed on grids, and stained with phosphotungstic acid, microtubules were not observed in the electron microscope. Similarly, the MAP fraction from the phosphocellulose column showed no capacity for self-assembly (Fig. 1c). However, when the MAP fraction was added back to the purified tubulin dimers (at a tubulin:MAP ratio of 9:1, wt/wt) the ability of the tubulin to assemble into microtubules was restored (Fig. 1d). Therefore the phosphocellulose-purified tubulin alone was incapable of spontaneous polymerization, but could be induced to assemble under the appropriate experimental conditions. The tubulin thus purified retained this potential activity for up to 2 mo after freezing in liquid nitrogen and storage at −80°C, and was used in all subsequent nucleation experiments.

The typical appearance of a mitotic PtK2 cell pretreated with colcemid and lysed with Triton X-100 is shown in Fig. 2. Several chromosomes can be seen surrounding a centriole pair, and the cytoplasmic matrix is extensively extracted leaving
a few mitochondrial remnants, membranous vesicles, and discrete bundles of thin filaments (fig. 2a). The centriole pairs displayed their usual structural appearance (Fig. 2b) in the electron microscope. Serial thin sections of the centrosomal region of these cells displayed clusters of pericentriolar virus-like particles (VLPs) similar to those previously described (11, 18, 27, 31) (see also Fig. 3b). The chromosomes exhibit structurally distinct kinetochores (Fig. 2b and c) typical of colcemid-arrested mitotic mammalian cells (8, 22). There was a conspicuous absence of microtubules in these lysed cell preparations (Fig. 2a), nor could any microtubule remnants be found associated with the centrosomal area (Fig. 2b) or the kinetochore (Fig. 2b and c). Buffer conditions were important and it was found that PIPES concentrations below 80 mM in the system buffer (buffer B) resulted in severe decondensation of the chromosomes. Concentrations above 80 mM induced an abnormal hypercondensation of the chromatin and often caused the chromosomes to coalesce into a single mass. Concentrations of Mg⁺⁺ above 0.5 mM also caused an abnormal hypercondensation of the chromatin.

A cell preparation similarly lysed as described
above and then incubated with purified exogenous tubulin is shown in Fig. 3. Large numbers of smooth-walled microtubules can be seen emanating from the centrosomal area and extending outward for several microns (Fig. 3a). In some instances, microtubules were seen connecting the centrosome with closely adjacent kinetochores. Microtubules nucleated from the centrosomal area usually do not appear to arise directly from the centriole itself, but instead extend from the peri-centriolar mass at many different angles (Fig. 3b). Clusters of VLPs can be seen around the centriolar area and its associated microtubules (Fig. 3b). Several microtubules are found extending from the kinetochores (Fig. 3c) and in most instances, when traced through serial sections, the kinetochore microtubules appeared not to be associated with the centriolar area or any other definable structure.

In all sections, microtubules were found to be associated exclusively with centrosomes or kinetochores, and few if any microtubules were free in the cytoplasm as determined by following microtubule profiles through serially sectioned specimens. Microtubules were absent in cells incubated in buffer B without tubulin.

When cells were lysed, preincubated with tubulin antibody, and then washed to remove unbound antibody, subsequent exposure to exogenous tubulin largely failed to give rise to microtubules (Fig. 4). As compared with Fig. 3, Fig. 4 shows an almost total absence of microtubular structures. An occasional microtubule can be found extending from the centrosomal area outward for a short distance through the chromosomal mass (Fig. 4a and b), although the overall image is similar to that of Fig. 2 and is in sharp contrast to that of Fig. 3. Kinetochore-associated microtubules were conspicuously absent in preparations pretreated in tubulin antibody before incubation in tubulin (Fig. 4b). In control cells which were lysed, preincubated with rabbit anti-goat IgG or buffer B alone, and then exposed to exogenous tubulin (Figs. 5a and b), the centrosomes and kinetochores exhibited many assembled microtubules similar to those in Fig. 3.

DISCUSSION

In vitro initiation of microtubule assembly from intracellular nucleating sites began several years ago when whole microtubule protein was used as a source of exogenous tubulin (16, 28, 30). Subsequent work focused on the use of initiation-incompetent tubulin as a source of polymerizable tubulin subunits for use in lysed mitotic cell preparations (11, 12, 26). The latter studies were the first to address the question of whether spontaneously assembled microtubules became attached to potential nucleating sites or if true site-specific nucleation was actually achieved. We have developed a similar system for studying the site-specific nucleation of microtubules at centrosomes and kinetochores in lysed mitotic cells. Our investigations have utilized purified exogenous tubulin
dimers which show no capacity for self-assembly at concentrations well above those at which the material is used in nucleation experiments with lysed cells.

The mitotic cell preparations used in this study exhibit structurally intact centrosomes (containing centrioles, amorphous pericentriolar material, and pericentriolar VLPs) as well as kinetochores. Apparently, remnant endogenous cellular microtubules were removed by preincubation and lysis of cells in colcemid-containing media. Furthermore, the centrosomes and kinetochores exhibit the capacity to nucleate microtubule assembly using initiation-incompetent tubulin. The characteristics of this nucleation system are in good agreement with previous studies using whole-mounted material (11, 12).

We have also shown that exposure of centrosomes and kinetochores to tubulin antibody inhibits the ability of these structures to serve as microtubule nucleation sites. This inhibition was not due to the binding of antibody to soluble tubulin, since excess (unbound) antibody was washed from the lysed cell preparations before their exposure to exogenous tubulin. In addition, the blockage of nucleation was not due to nonspecific adsorption of antibody protein to initiation sites, since pretreatment of lysed cells with an unrelated rabbit IgG had little effect on the ability of the centrosomes and kinetochores to nucleate microtubule assembly.

The distribution of tubulin in centrosomal and kinetochore structures was described previously (18). We suggested that this tubulin is an intrinsic component of these structures because its preferential association with such specific sites is inconsistent with a process of random adsorption. It was also recognized that this tubulin might indeed serve as a necessary structural/functional component for centrosomes and kinetochores to serve as microtubule initiation sites. If the tubulin present in specific nucleation sites is actually functional in promoting microtubule assembly, then the inhibition of site-specific initiation at centrosomes and kinetochores by tubulin antibody would be expected. This hypothesis is supported by the results of the present study.

Other macromolecules, however, must certainly be involved in maintaining the structure and functional capability of these microtubule nucleation centers. Models have been proposed for the molecular structure of centrioles (7) and kinetochores (7, 23). Some efforts have been made in recent years in attempt to define the molecular components of centrioles and/or kinetochores (2, 3, 4, 9, 13, 18, 19, 21). Evidence has accumulated that nucleic acids (particularly RNA) may serve a structural function in these organelles.

The elucidation of the molecular components and functional control of microtubule nucleation sites would be of great value in understanding the process of mitosis. This is particularly evident regarding the assembly and regulation of the mitotic apparatus. Therefore studies concerning the
composition of kinetochores and centrosomes seem a logical prerequisite for understanding the functional mechanism of these organelles.

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