Influence of the Centrosome on the Structure of Nucleated Microtubules

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ABSTRACT The capacity of the centrosome to influence the lattice structure of nucleated microtubules was studied in vitro. Brain microtubules self-assembled to give predominantly (98%) 14-protofilament microtubules. However, under exactly the same conditions of assembly they grew off of purified centrosomes from neuroblastoma cells to give mostly (82%) 13-protofilament microtubules. Thus, the nucleation sites on the centrosome constrained the microtubule lattice to yield the number of protofilaments usually found in vivo.

In both interphase and mitotic animal cells most microtubules are anchored at one end in the centrosome, a region comprising the centriole and associated dense material (1, 2). In several cases it has been shown that when microtubule depolymerization is induced by drugs, reassembly occurs at the centrosome (3, 4). Hence, the centrosome is not merely a point of anchorage but also a nucleation site for the microtubules, which arise from the pericentriolar material (5). Little is known at present as to how nucleation occurs at the centrosome or what components are involved. In vitro studies of microtubule assembly, however, suggest that polymerization proceeds readily off of existing microtubules, even from heterologous sources (6, 7), and that when assembly occurs spontaneously it may involve a complex mechanism that uses several unusual protofilament aggregates, which rearrange to give a microtubule seed (8, 9). However, one feature that clearly distinguishes spontaneous polymerization in vitro from assembly in vivo is the regularity of the microtubule structures formed. Microtubules assembled in vitro form a mixture of structures with 13, 14, or 15 protofilaments, which have different lattice arrangements (10–12). Microtubules formed in vivo in most animals are uniformly 13 protofilaments, presumably of the A lattice (11, 13–16). Some invertebrate species consistently form structures of 11, 12, or 15 protofilaments (14, 17, 18). This discrepancy between in vivo and in vitro structures could be due to differences between solvent conditions or co-factors in vivo and in vitro or to the presence of specific nucleation sites in vivo. The persistent effect of nucleation on the lattice structure of microtubules has been demonstrated by Scheele et al. (16). They showed that tubulin, which usually polymerizes to give microtubules of primarily 14 protofilaments, could elongate the A subfibers of flagellar axonemes to give almost exclusively microtubules of 13 protofilaments. If the centrosome were analogous structurally to flagellar axonemes and were therefore composed of microtubule fragments, the consistency of there being 13 protofilaments in vivo could be explained simply by the influence of this microtubule template. However, there is no evidence to suggest that stable microtubule fragments are present in the pericentriolar material of the centrosome, and it seems just as plausible that the centrosome nucleates assembly by promoting polymerization without acting as a structural template.

Recently we purified centrosomes from Chinese hamster ovary and neuroblastoma cells and demonstrated their capacity to nucleate assembly of purified tubulin in vitro and function in vivo to induce asters upon injection into frog eggs (19, 20). These centrosomes contain centrioles and pericentriolar material. In this article we examine the influence of purified centrosomes on the protofilament number of nucleated microtubules and compare this with the protofilament number of microtubules assembled spontaneously under exactly the same conditions. The results suggest that centrosomes constrain the protofilament number; hence, the centrosomal nucleation site provides structural information, which can propagate some distance along the microtubule.

MATERIALS AND METHODS

Microtubule protein from bovine brain was purified by three cycles of polymerization and depolymerization by a modification of the procedure of Shelanski et al. (21, 22) and stored as pellets of frozen microtubules at −70°C. Centrioles were prepared from N115 neuroblastoma cells (gift of M. Nirenberg, National Institutes of Health Bethesda, MD) by the procedure of Mitchison and Kirschner (19) and stored at −70°C.

Polymerization Protocol

Microtubule protein was thawed, homogenized in PB1 (80 mM Na PIPES, 1 mM EGTA, 1 mM MgCl₂, pH 6.8, plus 1 mM GTP), and cleared by

1 Abbreviation used in this paper: PB. 80 mM Na PIPES, 1 mM EGTA, 1 mM MgCl₂, pH 6.8, plus 1 mM GTP.
sedimentation at 100,000 g for 30 min at 4°C. It was then diluted to 1.2 mg/ml in PB at 0°C either with or without centrioles at 5 x 10⁶/ml. The two samples were then incubated at 37°C for 4 min at which point 5 µl of 50% glutaraldehyde (EM grade; Polysciences Inc., Warrington, PA) was added for 3 min.

The free microtubules (0.5 ml) were diluted twofold with 0.5 ml of PB containing 2% tannic acid (see reference 21) and incubated at 4°C for 2–3 h. They were then pelleted for 10 min at 30 psi in a Beckman airfuge (Beckman Instruments, Inc., Fullerton, CA). The pellet was then postfixed with 1% OsO₄ in PB for 1 h at 22°C, washed in 30 mM veronal acetate buffer pH 7.4 for 15 min, block stained in 0.5% uranyl acetate in veronal acetate buffer for 30 min at 22°C, and rapidly dehydrated in an acetone series and embedded in Araldite (grade CY-212 [British]; Polysciences Inc.).

Separation of Centrosomes from Free Microtubules

The centrosome regrowth assay and the efficient separation of free from nucleated microtubule has depended on the development of a simple convenient apparatus for sedimenting structures onto glass coverslips through a glycerol-containing cushion. This apparatus is shown in Fig. 2. For electron microscopy 0.9 ml, and for light microscopy 0.1 ml of the fixed reaction mixture was layered onto a 5-ml cushion of 30% glycerol in PB and spun at 4°C for 15 min at 13,000 rpm (27,000 g) in a Beckman JS-13 rotor in a Beckman J-2-21 centrifuge (Beckman Instruments, Inc.), onto a 12-mm round coverslip precoated with polylysine. The first milliliter was aspirated off, the remaining glycerol was then aspirated, and the coverslip was removed with a tool. For light microscopy the coverslip was postfixed in methanol at −20°C for 5 min, and the microtubules were then visualized by antitubulin immunofluorescence as described previously (19). For electron microscopy the coverslips were washed three times in 2% tannic acid in PB for 2–3 h and then osmicated, block stained, and dehydrated as described above. The coverslip was then flat embedded and after polymerization of the Araldite the coverslip was removed by immersion in liquid N₂. For light microscopy of the entire reaction mix (Fig. 3B), which is mostly free microtubules, the fixed sample was diluted 10⁴-fold with PB and spun onto a 4-mm square coverslip precoated with polylysine in the Beckman airfuge EM90 rotor at 30 psi for 15 min (19) and visualized by immunofluorescence as above. For length determination of free and nucleated microtubules, immunofluorescent fields were photographed at 250× and digitized as described (19). 100 microtubules were measured to determine average length.

Electron Microscopy

Isolated centrosomes without microtubules were prepared for electron microscopy as described (23) except that after sedimentation, the pellet was stained with tannic acid and processed as above for free microtubule pellets.

Silver sections were cut from the embedded specimens with a diamond knife and applied to 75 mesh carbon-coated Formvar grids. They were stained in 2% uranyl acetate in 12.5% methanol and 35% ethanol for 20 min, and in 0.4% lead citrate in 0.1 M NaOH for 2 min (24). The grids were then examined in an Philips 400 electron microscope (Philips Electronic Instruments Inc., Santa Clara, CA). Microtubule cross-sections were photographed at a magnification of 90,000 and printed at 2.5 times that magnification. Three independent observers were asked to examine the prints and count the protofilaments. Any discrepancies (<1% of the total) were discarded.

RESULTS

Structure of Isolated Centrosomes

Centrosomes isolated from N115 neuroblastoma cells contained the expected nine triplet microtubules (Fig. 1B). They are surrounded by fibrous electron dense material, most of which adheres closely to the centriole cylinder. In tannic acid stained (Fig. 1, A and B) and in conventionally stained (data not shown) preparations we observed no microtubule stubs or ordered protofilament structures in the fibrous material. We estimate that 20-nm-long microtubules would have been positively identified in both cross section and longitudinal sections.

Conditions for the Study of Both Spontaneous and Nucleated Assembly

The conditions for the observation of spontaneous polymerization and nucleated polymerization are somewhat incompatible. Purified centrosomes will nucleate the assembly of phosphocellulose-purified tubulin at a concentration well below that required for spontaneous assembly (19). However, to study spontaneous assembly the purified tubulin requires seeds or unusual and nonphysiological solvent conditions such as high Mg²⁺, glycerol, or dimethylsulfoxide to promote assembly (25, 26). The use of seeds to study spontaneous assembly is unacceptable since Scheele et al. (16) have already shown that the seeds can influence the structure of the elongated polymer. The alternative material is tubulin containing microtubule-associated proteins, which polymerizes well under physiological conditions. However, when such tubulin is used for nucleated polymerization studies spontaneous po-
Polymerization swamps out nucleated assembly. In sectioned material it would usually be impossible to distinguish the nucleated microtubules from the much larger number of spontaneous polymers. Since the conditions that determine protofilament number have not been well established we did not want to vary to even the slightest degree the concentration, time of incubation, or solution composition in comparing spontaneous to nucleated assembly. This necessitated finding conditions under which both would occur together but the nucleated polymers could be separated from spontaneous polymers and examined separately.

We chose three-times-cycled microtubule protein from bovine brain (21) and N115 centrosomes, which have been shown above to be devoid of microtubule fragments (Fig. 1 and reference 9) for the experiment. In pilot studies, we varied protein concentration and time of incubation to find conditions under which microtubules nucleated from centrosomes had a slight kinetic advantage but bulk spontaneous polymerization also occurred. The extent of nucleated and spontaneous assembly was determined by immunofluorescence assays. We fixed regrown centrosomes in solution and observed them by sedimentation through a glycerol-containing cushion onto a coverslip (19) using the apparatus shown in Fig. 2, and spontaneous microtubules by direct sedimentation of a diluted aliquot of the whole mixture onto coverslips in the airfuge (23). We found the best compromise between nucleated and spontaneous assembly to be with a microtubule protein concentration of 1.2 mg/ml and an incubation time of 4 min at 37°C. Fig. 3A shows the regrown centrosomes from such a mixture which, after fixation, were separated from the free microtubules by sedimentation through a glycerol-containing cushion. Fig. 3B shows a 10^5-fold dilution of the total assembly mixture sedimented in the airfuge. We...

**Figure 2** Modified 15-ml Corex centrifuge tube (Corning Glass Works, Corning, NY) used to sediment regrown centrosomes onto coverslips. The coverslip is placed on the plexiglass insert, and after sedimentation and aspiration of the supernatant, it is removed with the tool.

**Figure 3** Immunofluorescent visualization of regrown centrosomes (A) and free microtubules (B) using anti-tubulin antibody. In A the centrosomes were sedimented at 1/10 of the density used for electron microscopy. The separation from free microtubules is good, though some residual ones can be seen. In B the free microtubules were diluted 10^5-fold and sedimented in the airfuge. × 1,500.
estimate that the centrosomes grew 50–200 microtubules of mean length 5.5 μm, and a spontaneous polymer of mean length 6.0 μm was formed.

As can be seen in Fig. 3, A and B, the glycerol cushion is successful in separating the vast majority of the free microtubules from the regrown centrosomes. For the experiments in which protofilament number was determined, the same sedimentation technique was used; the number of centrosomes per coverslip was ~10 times that in Fig. 3A. After sedimentation the coverslip was incubated in tannic acid, postfixed in osmium, washed, block stained, and dehydrated. It was then flat-embedded, and after removal of the coverslip, the block was sectioned parallel to the surface. We examined the first few gray sections.

For examination of spontaneous microtubules, identical parallel incubations without centrosomes were fixed and treated with tannic acid in solution, and then pelleted in the airfuge, postfixed in osmium, washed, block stained, dehydrated, and embedded. We examined random gray sections though the pellet.

Measurement of Protofilament Number

Fig. 4 A shows a low magnification view of centrosomal microtubules at the plane of the centrosome. Fig. 4 B shows a low magnification view in a region where protofilament cross-sections were generally evaluated. The centrosome is below the plane of section, and the radial array of microtubules is sectioned ~1 μm above it. We estimate that most microtubules were examined at 1–2 μm from the center of the centrosome. We calculated this distance by fitting the distribution of microtubule cross-sections to the geometry of a plane that cut a spherical array of lines and then calculating from that the origin of the array. Sections, at the centrosome, are shown at higher magnification in Fig. 5 A and B. It is clear from many such examples that most of the microtubules arise from pericentriolar material present in satellites or adherent directly to the centriole. Microtubules seem in some cases to penetrate the pericentriolar material, which potentially allows for interactions from both the end and the side of microtubules. We saw only a few microtubules that arose by extension of the centriole triplets. To observe the protofilaments it is necessary to cut the microtubule with an almost perfect transverse section.

We performed three separate paired experiments of centrosome regrowth and spontaneous assembly. We scored only

\[ \frac{N_1}{N_2} = \frac{\tan l_1}{\tan l_2} \]

For this one can show that the number of microtubules \( N_1 \) cut at a linear distance \( l_1 \) from the center of the distribution in a section, and the number \( N_2 \) cut at a distance \( l_2 \) from the center of the distribution was related to the depth, \( R \), of the source by: \( \frac{N_1}{N_2} = \frac{\tan l_1}{\tan l_2} \). From this the linear distance from the center encompassing 1/2 the distribution is simply \( R \) and the radius of a circle encompassing 1/4 the distribution is also \( R \).
FIGURE 5 Higher magnification views of microtubule nucleation at the centrosome. The microtubules appear to originate in the amorphous pericentriolar material, most of which adheres tightly to the centriole triplets. Note the excellent structural preservation of the isolated centrosomes after storage at -70°C. × 70,000.

those cross-sections where the protofilaments could be counted around the entire circumference. Photographs were examined at a magnification of 220,000 and the number of protofilaments was counted by three individuals, two of whom were blind to the nature of the experiment. Disagreement occurred in only 3 of 361 cases, and these were discarded.

Table I shows the results of the experiments. In each case the spontaneous microtubules showed a vast preponderance (98%) of 14 protofilament microtubules. Fig. 5B shows examples. In addition, in one experiment a contaminating bundle of spontaneous microtubules, polymerized along with the centrosome nucleated arrays, was identified in a particular corner of a coverslip. Of 15 reasonable cross-sections, 10 were unambiguously composed of, and 5 were likely to be composed of, 14 protofilaments. By contrast most of the microtubules associated with the centrosome (82%) were made up of 13 protofilaments, examples of which are shown in Fig. 5A. There was not significant variation among the three experiments.

DISCUSSION
The results demonstrate that the centrosome influences the structure of the microtubule lattice by producing mostly microtubules with 13 protofilaments under conditions where spontaneous assembly produces almost exclusively microtubules with 14 protofilaments. We cannot say whether the minority of 14-protofilament microtubules associated with

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TABLE I
Number of Protofilaments in Centrosome Nucleated and Spontaneous Microtubules

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the centrosome were truly nucleated or whether they became attached or are trapped adventitiously during assembly or fixation. The latter is possible because a vast majority of the microtubules in the assembly mixture were spontaneous and contained 14 protofilaments.

The consistency of there being 13 protofilaments in vivo could be due to specific solvent conditions, a specific constellation of associated proteins, or templating by the nucleation centers. We have shown here that under solvent conditions that overwhelmingly favor a lattice of 14 protofilaments, the presence of a centrosome will restrict the lattice mostly to 13 protofilaments, which suggests that the centrosome may play a similar role in the cell. These results also suggest that the nucleation site is probably not simply a region that contains promoting materials like microtubule-associated proteins, but a region that species the geometry of subunit assembly. In addition we can say that the local stabilization of the 13-protofilament array must be propagated at least 2 μm, a distance of 250 dimers.

These results are theoretically somewhat surprising since the 14-protofilament lattice must be more stable or kinetically favored under these conditions and is structurally quite different from the 13-protofilament lattice. The 14-protofilament lattice is thought to be composed of a mixture of lattice arrangements with a seam (11), whereas the 13-protofilament lattice is continuous (11, 27). Yet Scheele et al. (16) also observed a persistent 13-protofilament lattice grown from flagellar axonemes. We should point out that those experiments suffered from something that the present experiments do not. To suppress spontaneous polymerization in the flagellar nucleation experiments, the microtubule protein was sedimented hard enough to remove the ring oligomers, which are rich in associated proteins. Thus, the tubulin preparations for the spontaneous and nucleated assembly experiments were not identical. However, in the experiments reported here identical preparations were used, and the spontaneous polymers were separated from the nucleated ones after fixation. We can therefore conclude that despite the structural and presumed energetic differences between the 14- and 13-protofilament lattices, elongation from centrosomes propagates a 13-protofilament lattice stabilized by the nucleation center for a distance of at least 2 μm. In this in vitro situation local chemical properties of the centrosome presumably could not extend that distance, and therefore it must be kinetic propagation during assembly that ensures the presence of 13 protofilaments.

All of these studies bear on the nature of the nucleating material. Numerous electron microscopic studies have failed to reveal any periodic or regular structure in the pericentriolar material, which anchors the microtubules (1, 5). No fragments of a microtubule lattice have been seen in sections of isolated centrosomes (Fig. 1). In addition, no clearly identifiable nucleation structure is present at the ends of the microtubules in vivo (28). However, though amorphous, the pericentriolar material nucleates microtubules with a uniform polarity (19, 29) and favors a specific geometry of the microtubule lattice. In addition we have found that the number of microtubules that can be grown off these centrosomes saturates at high tubulin concentration (19). The limited number of sites, the constant polarity, and the fixed protofilament geometry of the lattice suggest that, though amorphous, the nucleating material may be organized into discrete sites for individual microtubules, at least after the microtubules have been nucleated. Whether discrete sites exist before nucleation, or whether they are organized by the microtubule end itself from a more amorphous but flexible nucleating matrix, remains to be determined.

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