THE PERICENTRIOLAR MATERIAL IN CHINESE HAMSTER OVARY CELLS NUCLEATES MICROTUBULE FORMATION

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ABSTRACT

The structure and function of the centrosomes from Chinese hamster ovary (CHO) cells were investigated by electron microscopy of negatively stained whole-mount preparations of cell lysates. Cells were trypsinized from culture dishes, lysed with Triton X-100, sedimented onto ionized, carbon-coated grids, and negatively stained with phosphotungstate. The centrosomes from both interphase and dividing cells consisted of pairs of centrioles, a fibrous pericentriolar material, and a group of virus-like particles which were characteristic of the CHO cells and which served as markers for the pericentriolar material. Interphase centrosomes anchored up to two dozen microtubules when cells were lysed under conditions which preserved native microtubules. When Colcemid-blocked mitotic cells, initially devoid of microtubules, were allowed to recover for 10 min, microtubules formed at the pericentriolar material, but not at the centrioles. When lysates of Colcemid-blocked cells were incubated in vitro with microtubule protein purified from porcine brain tissue, up to 250 microtubules assembled at the centrosomes, similar to the number of microtubules that would normally form at the centrosome during cell division. A few microtubules could also be assembled in vitro onto the ends of isolated centrioles from which the pericentriolar material had been removed, forming characteristic axoneme-like bundles. In addition, microtubules were assembled onto fragments of densely staining, fibrous material which was tentatively identified as pericentriolar material by its association with the virus-like particles. We conclude that the pericentriolar material of CHO can initiate and anchor microtubules both in vivo and in vitro.

The centrosome consists of a pair of centrioles surrounded by a fibrous substance called the "pericentriolar material." In mammalian cells, the centrosome serves as a microtubule organizing center in two distinct ways. During interphase, it resides near the nucleus and is apparently the origin of a network of cytoplasmic microtubules that are thought to help maintain the cell's shape (35, 26, 8, 24). During cell division, the centrosomes, one at each pole of the cell, give rise to a spindle-shaped array of microtubules that forms the framework of the mitotic apparatus (40, 3, 26). Although the centrosome has been studied for a century, and although it is a prototype for other microtubule-organizing centers, the mechanism by which it nucleates, orients, and anchors microtubules has remained a mystery.

In their studies of cell division (2, 7, 40), the early cytologists first posed the crucial question, which part of the centrosome gives rise to the

spindle fibers—the centrioles or the pericentriolar material? The centrioles were the most conspicuous and persistent feature of the centrosome (2, 7), but it was the pericentriolar material, seen in the light microscope as a clear zone, that was observed to wax and then wane as cell division progressed (40).

Ironically, the advent of electron microscopy deepened, rather than resolved, the problem. On the one hand, both the centrioles and spindle fibers were revealed in thin sections to be bundles of microtubules (3, 14), leading to suggestions that the centrioles might "spin out" or nucleate the microtubules of the spindle (34). On the other hand, it was soon apparent that while a few spindle microtubules might contact the centrioles directly, the great majority of them originated in the densely staining, apparently amorphous material surrounding the centrioles (28). Hence the old dilemma, instead of being dispelled, reappeared in a new guise.

Recently, the ability of the centrosomes to nucleate microtubule assembly was directly tested in vitro by incubating lysed, dividing cells with purified microtubule protein (36, 18, 32). These experiments confirmed that the centrosome can nucleate microtubules in vitro, and in one report it was suggested that the material associated with the centrioles is the nucleating material (36). Again, however, electron microscopy failed to distinguish unequivocally which component of the centrosome nucleated microtubules.

In this paper we describe the structure and function of the centrosome in vitro, isolated from other cell components, as obtained from lysed Chinese hamster ovary (CHO) cells. We show that the pericentriolar material is actively associated with the formation of microtubules in vivo, and that it nucleates microtubule assembly in vitro. We conclude that it is the pericentriolar material, and not the centrioles themselves, that nucleates the microtubules of the mitotic spindle. In these experiments the pericentriolar material was made easier to follow by the presence of numerous virus-like particles, specific to CHO, which served as markers for the pericentriolar material.

MATERIALS AND METHODS

Cell Cultures

CHO cells were grown as nonconfluent monolayers in Ham's F-10 medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with antibiotics, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) buffer, pH 7.2, and 10% fetal calf serum, at 37°C under 5% CO_2.

Interphase cells were harvested from tissue culture dishes by first gently shaking loose and removing mitotic cells and then treating the adherent cells with 0.25% trypsin in Gibco solution A (Grand Island Biological Co.).

Some cell cultures were blocked in S-phase by treatment with 10 mM thymidine for 12 h (41). Other cell cultures were blocked in mitosis by first treating with 10 mM thymidine for 12–15 h and then replacing the medium with fresh medium containing 0.1 μg/ml Colcemid (27, 9). After 5 h in Colcemid, cells blocked in mitosis were shaken from the dishes, pelleted in a tabletop centrifuge, treated with 1 ml of 0.25% trypsin in Gibco solution A for 1 min at 37°C, and processed for electron microscopy. In a typical experiment, cells from one 60-mm diameter tissue culture dish were used, giving a packed cell volume of ~0.01 ml.

Cell Lysis

Cells were trypsinized as above and pelleted in a tabletop centrifuge, resuspended for 1 min in one to five drops of distilled water, and, in the initial experiments, lysed by the addition of an equal volume of solution containing Triton X-100. The composition of the lysis medium evolved during the course of the investigation and is indicated for each experiment in the figure legends. The final composition of the lysis solution was 0.25% Triton X-100 in 10 mM piperazine-*N*-*N'*-bis[2-ethane sulfonic acid] (PIPES), pH 6.94, 0.1 mM $MgCl_2$, 1 mM ethylene glycol-bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetate (EGTA) (PME buffer). Lysates were fixed by the addition of an equal volume of 2% glutaraldehyde in 10 mM potassium phosphate, pH 7.0.

Preparation of Tubulin

Microtubule protein was purified from pig brain tissue by two cycles of polymerization and depolymerization as previously described (5). The pellet was resuspended in 100 mM PIPES, pH 6.94, 0.1 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP (polymerization buffer), and centrifuged at 230,000 *g* for 90 min as previously described to remove nucleating structures (6, 1). The resulting supernate was used in all polymerization experiments. Protein concentration was determined by the method of Lowry et al. (17).

Incubation with Tubulin

Cells were lysed as described above, by the addition of an equal volume of a 2× concentrated lysis solution (0.5% Triton X-100, 20 mM PIPES, 0.2 mM $MgCl_2$, and 2 mM EGTA). 3 vol of microtubule protein in polymerization buffer were added to the lysate and the mixture was incubated at 31°C for 10 min. The final concentration of microtubule protein in the mixture ranged from 0.9 to 3.0 mg/ml. Polymerization was

stopped by the addition of an equal volume of 2% glutaraldehyde.

For controls, some lysates were fixed with an equal volume of glutaraldehyde immediately after cell lysis. Other lysates were incubated in polymerization buffer lacking tubulin for 10 min and then fixed.

Electron Microscopy

Several drops of fixed lysates were sedimented at 3,200 *g* for 5 min in a tabletop centrifuge onto ionized, carbon- and Formvar-coated electron microscope grids by a modification of the method of Miller et al. (20, 13), either negatively stained with 2% sodium phosphotungstate, or positively stained with 1% uranyl acetate, and observed in a Philips 300 electron microscope. Some lysates were sedimented onto grids before fixation, treated with 10 μg/ml trypsin for 5 min, fixed with glutaraldehyde for 2 min, and negatively stained with 2% phosphotungstate as described above.

Length measurements were made directly from the electron microscope plates with a Gaertner microcomparator (Gaertner Scientific Corp., Chicago, Ill.). Magnification was calibrated with a 21,600 line/cm diffraction grating. The central portions of negatively stained centrosomes were often deeply embedded in stain, thus making printing of the images difficult. In these instances, plates were taken over a range of exposures to allow the subsequent printing, from separate plates, of various regions differing greatly in optical density.

RESULTS

Our strategy in these experiments was to examine the centrosomes in cell lysates that had been deposited on electron microscope grids and negatively stained. In addition to its ease and rapidity, this method of preparation offered several advantages over traditional thin-section preparations. It allowed the high resolution examination of structures which might not be seen in thin sections. It allowed an assessment of the physical continuity between different cell structures, and allowed their examination free from surrounding material. Finally, it was a natural prelude to in vitro studies on the function of cell components.

One limitation of the method is that in disrupting a cell and applying a complex three-dimensional object onto the surface of an electron microscope grid, one perturbs the structural relationships of the object's components. An additional problem arises from the interpretation of images that are two-dimensional projections of three-dimensional objects. Despite these drawbacks, whole-mount electron microscopy of cell lysates, combined with negative staining, is an approach that has not been extensively explored, and, as will be shown, is of particular value in the analysis of supramolecular structures.

These experiments were done in two stages. In the first stage, the association between centrosomes and native microtubules was examined in both interphase and dividing cells. In the second stage, the ability of the centrosomes to nucleate microtubule assembly was directly tested in vitro by incubating them with exogenous microtubule protein. The results described below comprise the observations on several hundred centrosomes.

Centrosomes in Interphase Cells

Two kinds of populations were used in the studies of interphase cells: first, populations of cells in exponential growth were examined, since most of the cells in these cultures could be expected to be in interphase; second, some cultures were treated with thymidine to product relatively homogeneous populations of cells blocked in the period of DNA synthesis (S-phase).

When interphase CHO cells were lysed with detergent and examined in the electron microscope, the centrosomes were found to consist of three parts: a pair of centrioles, an apparently amorphous pericentriolar material, and numerous small particles embedded in this material (Fig. 1).

The particles had an outer diameter of 67 ± 4 nm and frequently displayed an interior lumen with a diameter of 22 ± 2 nm. Similar particles were reported in thin section studies of CHO by Wheatley (38), who observed them only at the centrosomes and suggested that they might be an unusual form of virus that specifically associated with the pericentriolar material so as to be automatically transmitted to the daughter cells during cell division. This suggestion was supported by several of our observations. When the particles were positively stained with uranyl acetate, they showed a densely staining core (Fig. 3*a*). When examined by stereo microscopy, they appeared roughly spherical. Furthermore, when the pericentriolar matrix was removed by brief treatment with trypsin and the particles were negatively stained, many of the particles showed hexagonal profiles, suggesting that they might be icosahedral (Fig. 3*b*). In most of the preparations, some of the particles appeared with and some without cores. These are all typical staining patterns for many nucleic acid-containing viruses (15, 39).

Although the particles are interesting in themselves, it must be emphasized that we have not found them at the centrosomes of the closely re-

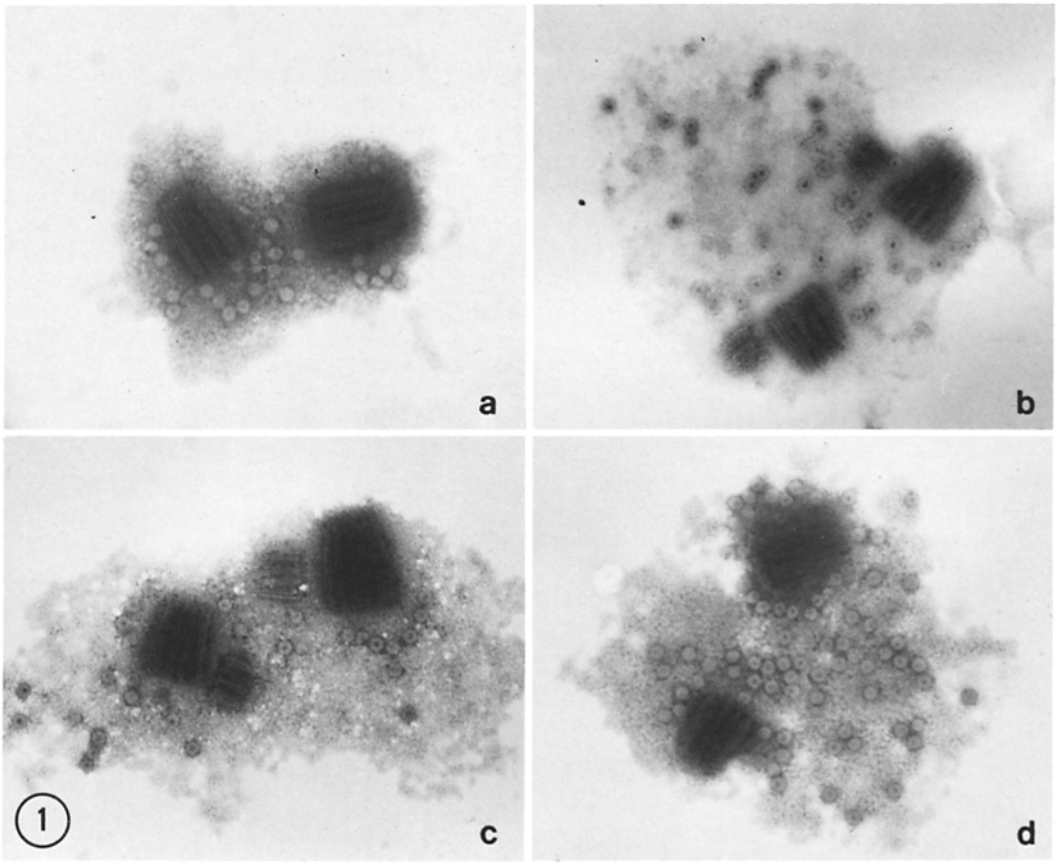

FIGURE 1 Four centrosomes from interphase cells, arranged in a probable temporal sequence. Each centrosome consists of a pair of centrioles, fibrous pericentriolar material, and numerous small particles. In (*a*) the two mature centrioles are perpendicular to each other. In (*b*) and (*c*), each centriole bears a procentriole. In (*d*) the centrosome has probably split, leaving one mature and one nearly mature centriole. From cells lysed in unbuffered 1% Triton X-100. Whole-mount, negatively stained with 2% phosphotungstic acid, pH 6.5. × 25,500.

lated Chinese hamster Don-C line or six other human and mouse lines (HeLa, L929, Nb2a, Nb18, C3H/10T½, and McA10) and therefore they apparently are not a universal component of the centrosome. However, as will be seen in the experiments to be described, these particles were bound specifically and tightly to the pericentriolar material in CHO, so that they served as markers for the pericentriolar material. We have put this fortuitous property to use, since in several of the experiments described below, the amorphous pericentriolar material would otherwise be much more difficult to distinguish in cell lysates.

The four centrosomes from interphase cells shown in Fig. 1 have been arranged in a probable temporal sequence. The stages of the cell cycle that these centrosomes represent can be determined by reference to previous electron microscope studies (33, 9) on the centriole cycle in Chinese hamster cells. These studies have shown that centrioles give rise to procentrioles in S, that the procentrioles elongate as the cell cycle progresses, and that they attain maturity (maximum length) in the next cell generation, usually in G_1. Pairs of parent-daughter centrioles generally separate from each other in prophase to establish the poles of the mitotic spindle. Using these criteria, the centrosome in Fig. 1 *a* is probably from a cell in early interphase: both centrioles are mature, and neither one has yet given rise to a procentriole. In Fig. 1 *b* and *c*, each centriole is attached to a procentriole in the typical perpendicular configuration, representing stages seen later in interphase. The centrosome in Fig. 1 *d* probably repre-

sents one-half of a duplicated centrosome that has separated from its partner and is ready to migrate to one pole of the cell. The daughter centriole has not yet reached its full length, and usually does not do so until early in the next cell generation (33).

Support for the interpretation of Fig. 1*d* as a separated centrosome at early prophase also comes from images such as those seen in Fig. 2. Here, two separated centrosomes remained attached to the cell nucleus (inset) which did not become dispersed under the lysis conditions employed. Since Chinese hamster cells normally contain just one centrosome throughout most of interphase (33), this micrograph probably represents a stage at the end of interphase and the beginning of prophase. This stage corresponds to the "duplication of mitotic centers" seen by the early cytologists (40). (Note that one pair of centrioles has already given rise to procentrioles, which will mature in the next generation. This premature replication of the centrioles has previoulsy been reported in Chinese hamster cells [33]).

Thus, the general features of the centrosome remain unchanged throughout most of interphase.

The centrosomes seen in Figs. 1 and 2 were from cells that were lysed directly into a cold 1% Triton X-100 solution that did not preserve microtubules. When the cells were lysed at room temperature, however, in the presence of Mg^{2+} and EGTA, the microtubules were well preserved and retained their association with the centrosome (Fig. 4). We have observed up to two dozen microtubules that were anchored in this way. The microtubules at interphase centrosomes typically appeared to arise from a region in the pericentriolar matrix close to the centrioles, but this region was generally too densely stained to determine precisely where the microtubules originated. In all subsequent experiments described in this report, lysis conditions were used which preserved the native microtubules.

Centrosomes in Dividing Cells

When a cell starts to divide, large numbers of microtubules form at the centrosomes. This assembly process is not due to a sudden synthesis of tubulin, since pools of tubulin are known to preexist in cells preparing to divide (30; for reviews see references 22 and 29). Rather, the process might reflect either the proliferation or activation of microtubule nucleating material. Accordingly, centrosomes were obtained from dividing cells to ob-

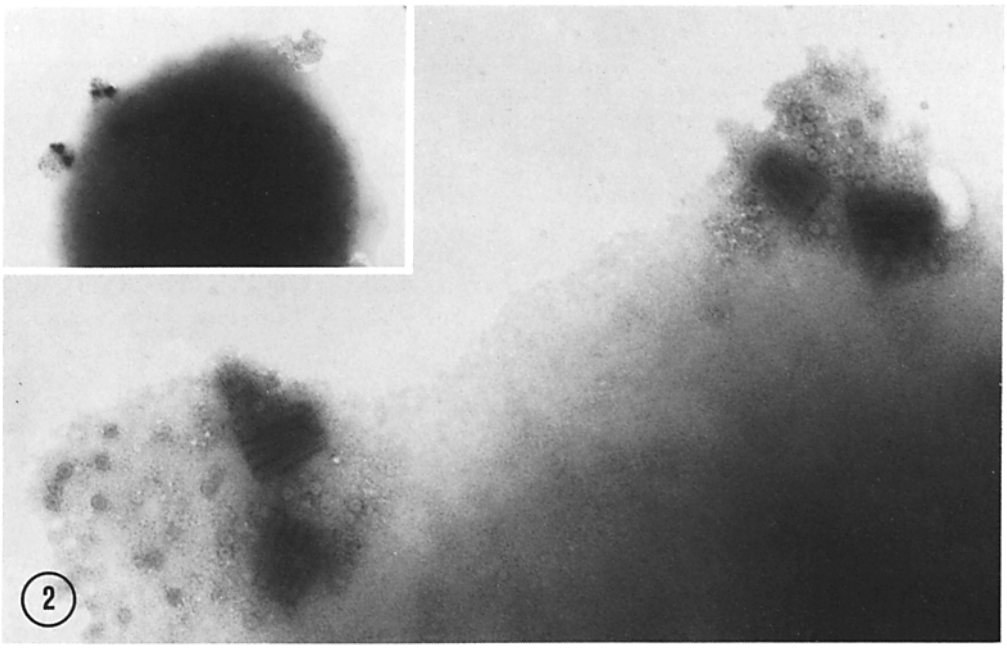

FIGURE 2 Two centrosomes attached to a cell nucleus. This stage probably represents the separation of the centrosomes at the end of interphase. Cytoplasmic microtubules have not been preserved. Cells were lysed in unbuffered 1% Triton X-100. × 24,500; inset, × 2,900.

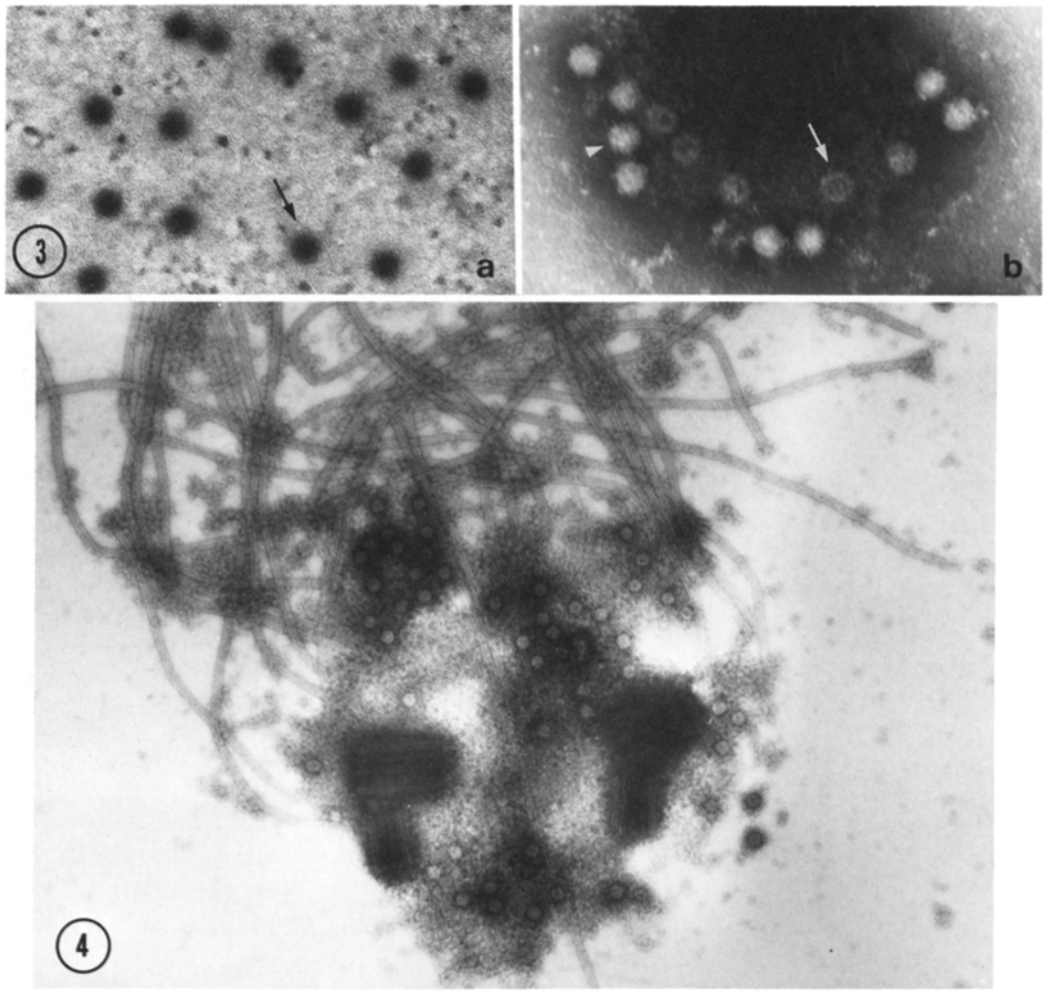

FIGURE 3 Particles at the centrosomes of interphase cells. (*a*) When positively stained with 1% uranyl acetate, the particles appear as dense cores surrounded by a lighter shell (arrow). (*B*) The pericentriolar material has been removed by treatment with 10 μg/ml trypsin for 5 min. Some of the particles have hexagonal outlines (arrowhead). Some of the particles appear to have lost their cores (arrow). × 76,000.

FIGURE 4 A typical centrosome from a cell blocked in S-phase by treatment with thymidine and lysed with 0.25% Triton X-100 in PME buffer. The centrosome consists of two pairs of centrioles, pericentriolar material, and numerous particles. About 20 microtubules remain anchored at the centrosome. × 24,800.

serve what changes, if any, might occur during division.

Our approach was to first block cells in division with the drug Colcemid, which prevents microtubule formation, and then to release cells from the Colcemic block and observe where at the centrosome the microtubules reformed.

COLCEMID-BLOCKED CELLS: Fig. 5 shows two typical centrosomes from Colcemid-blocked mitotic cells. There are now two distinct differences between these and the interphase centrosomes. First, the number of particles embedded in the pericentriolar matrix has substantially increased (from an average of 52 ± 17 to an average of 106 ± 24). Second, the pericentriolar material lies predominantly at one end of the mature centriole, the end that bears the daughter. As determined from electron micrographs of spindles *in situ* (e.g. 21), this is the end of the centriole that apparently faces the spindle during cell division (also R. R. Gould and G. G. Borisy, unpublished observations). Although there was occasionally

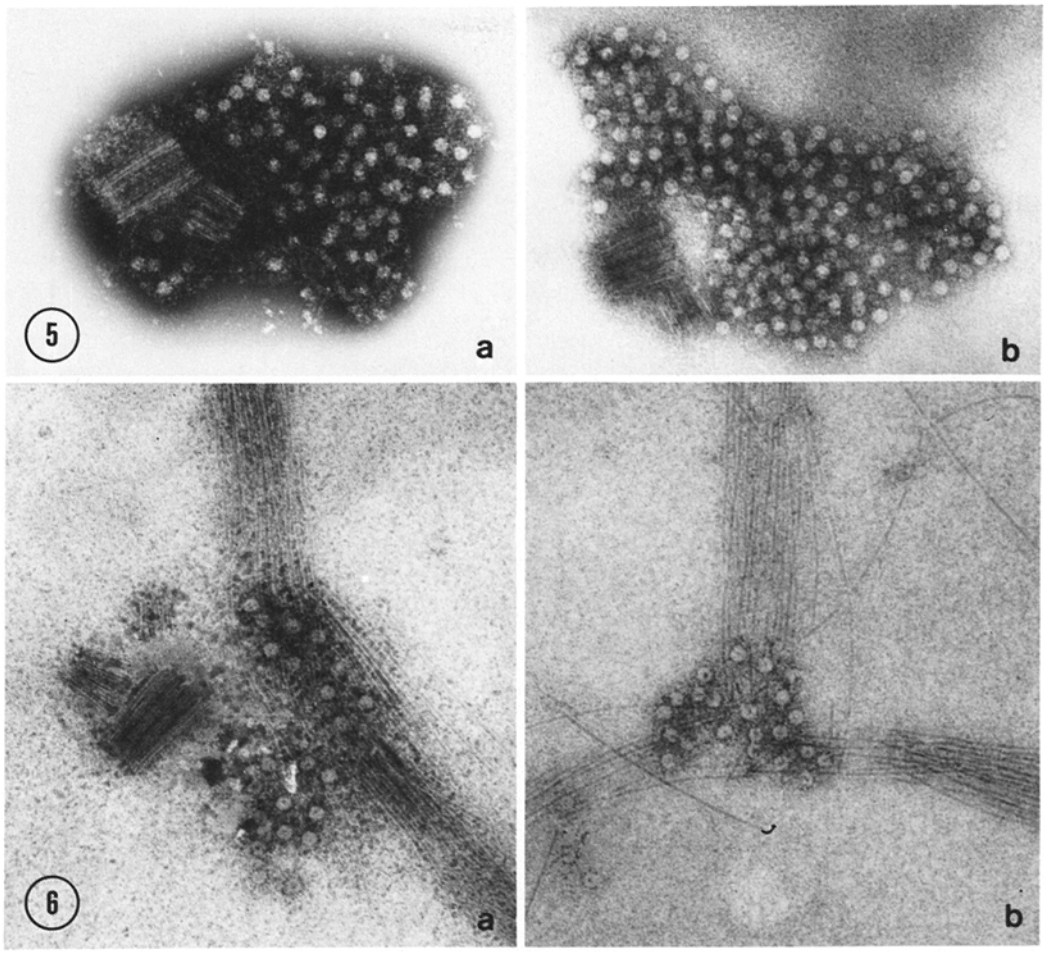

FIGURE 5 Two centrosomes from cells arrested in division with the drug Colcemid, showing the distribution of the pericentriolar material and the increased number of virus-like particles. In (*a*) about 80 particles are present, and in (*b*) about 200 particles are present. The pericentriolar material is positioned predominantly at one end of the centriole, the end which bears the procentriole. From cells lysed with 0.25% Triton X-100 in PME buffer. × 28,800.

FIGURE 6 Centrosomes from dividing cells after 10 min of recovery from Colcemid. Bundles of microtubules emanate from the pericentriolar material. In (*a*), only one or two microtubules contact the centriole, while the remainder arise from the adjacent pericentriolar material. In (*b*) the pericentriolar material with embedded virus-like particles is displaced from the centriole (out of field of view), showing that the microtubules arise directly from this material. Stain has filled the lumens of the microtubules but has been excluded from their exterior by surrounding nucleoplasm. Cells were lysed in 0.25% Triton X-100 in PME buffer. × 30,800.

some disruption of the centrosomal complex during lysis and preparation for electron microscopy, the majority of the centrosomes observed showed this characteristic asymmetric distribution of the pericentriolar material.

RELEASE FROM COLCEMID: In order to determine which part of the centrosome gives rise to microtubules during cell division, dividing cells were released from Colcemid block by resuspending them in fresh medium, allowing spindle microtubules to reform.

Fig. 6 shows a typical centrosome from a cell lysed after 10 min of recovery from Colcemid. It again consists of a pair of centrioles and adjacent pericentriolar material delineated by virus-like particles, but now bundles of microtubules have

formed at the pericentriolar material. The unusual appearance of the microtubules in this preparation apparently resulted from the underlying nucleoplasm, which had excluded stain from the exterior of the microtubules but not from their lumen. Fig. 6*a* shows that only one or two microtubules made contact directly with the centriole; the remainder apparently arose from the adjacent pericentriolar material. In general there was a roughly inverse correlation between the number of microtubules that polymerized at the pericentriolar material and the number of virus-like particles that remained there. In a few instances, no particles at all could be detected. One possible explanation is that the formation of microtubules at the centrosome sufficiently weakened the binding of the particles to allow them to dissociate during cell lysis.

Fig. 6*b* shows a similar portion of densely staining pericentriolar material studded with virus-like particles. This material had been displaced from the centioles (just out of the field of view), probably during sample preparation, and again served as the focal point for bundles of microtubules.

These results suggest that it is the pericentriolar material, and not the centrioles themselves, that nucleates the microtubules in dividing cells.

The Centrosome as a Microtubule Nucleating Center In Vitro

To test the microtubule nucleating capability of the centrosomes in vitro, centrosomes from lysed cells were incubated with exogenous tubulin. The protein used in these experiments was prepared so as to be incapable of self-assembly but fully capable of nucleated assembly (1). Therefore any microtubules that formed during the incubation period would have to have been assembled at preexisting nucleating sites.

INTACT CENTROSOMES AS NUCLEATING CENTERS: To determine whether the intact centrosome would nucleate microtubule assembly in vitro, we lysed Colcemid-blocked mitotic cells and incubated them with microtubule protein. We used Colcemid-treated mitotic cells because they would be expected to contain the most active nucleating centers, and because no microtubules were present before the incubation. The residual Colcemid left in the cells was diluted after lysis and did not interfere with subsequent microtubule assembly.

Centrosomes from Colcemid-treated mitotic cells fixed immediately after cell lysis have already been seen in Fig. 5. No microtubules were present. After 10-min incubation with tubulin, microtubules had polymerized in profusion at the centrosome (Fig. 7*a*). With high beam illumination in the electron microscope, the densely staining focal region of the microtubules was revealed to contain a centriole pair, shown in the same orientation and higher magnification in the inset. Numerous microtubules end in the matrix surrounding the centrioles. No virus-like particles are observable.

Fig. 7*b* shows a centrosome that gave rise to over 250 microtubules. The inset again shows the centriole pair at the densely staining center of the centrosome. As will be shown below, the centrioles themselves have probably directly nucleated a few microtubules, but they cannot account for all the microtubules seen in these preparations. The number of microtubules routinely polymerized onto the centrosomes ranged from about 100 to 250. No free microtubules were found on these grids, indicating that only nucleated assembly, but no self-assembly, occurred during incubation.

We conclude that the centrosome is capable of nucleating the assembly of microtubules in vitro in numbers corresponding to the number of microtubules that would ordinarly form there during cell division (19).

CENTRIOLES AS NUCLEATING CENTERS: A few microtubules in dividing cells have been reported to be continuous with centriole microtubules (16, 36). Furthermore, basal bodies from the alga, *Chlamydomonas,* have been shown to initiate microtubule formation in vitro (31). It was thus anticipated that centrioles from CHO would also serve as initiation sites in vitro.

To distinguish the role of the centrioles as a nucleating center from that of the pericentriolar material, it was necessary to examine the nucleating capacity of centrioles in the absence of pericentriolar material. Accordingly, a procedure was developed to remove the pericentriolar material from the centrosomes. Interphase cells were lysed with 0.25% Triton X-100 in PME buffer but omitting the EGTA, and incubated for 10 min at 31°C. Under these conditions, the pericentriolar material was no longer present at the centrosomes. having apparently dissociated from them or broken down during the incubation.

The cell lysates, containing centrioles, were then incubated with microtubule protein for 10 min, and examined by electron microscopy. Microtubules assembled onto both ends of the centrioles (Fig. 8*a*) and procentrioles (Fig. 8*b*), and in every case more microtubules assembled onto

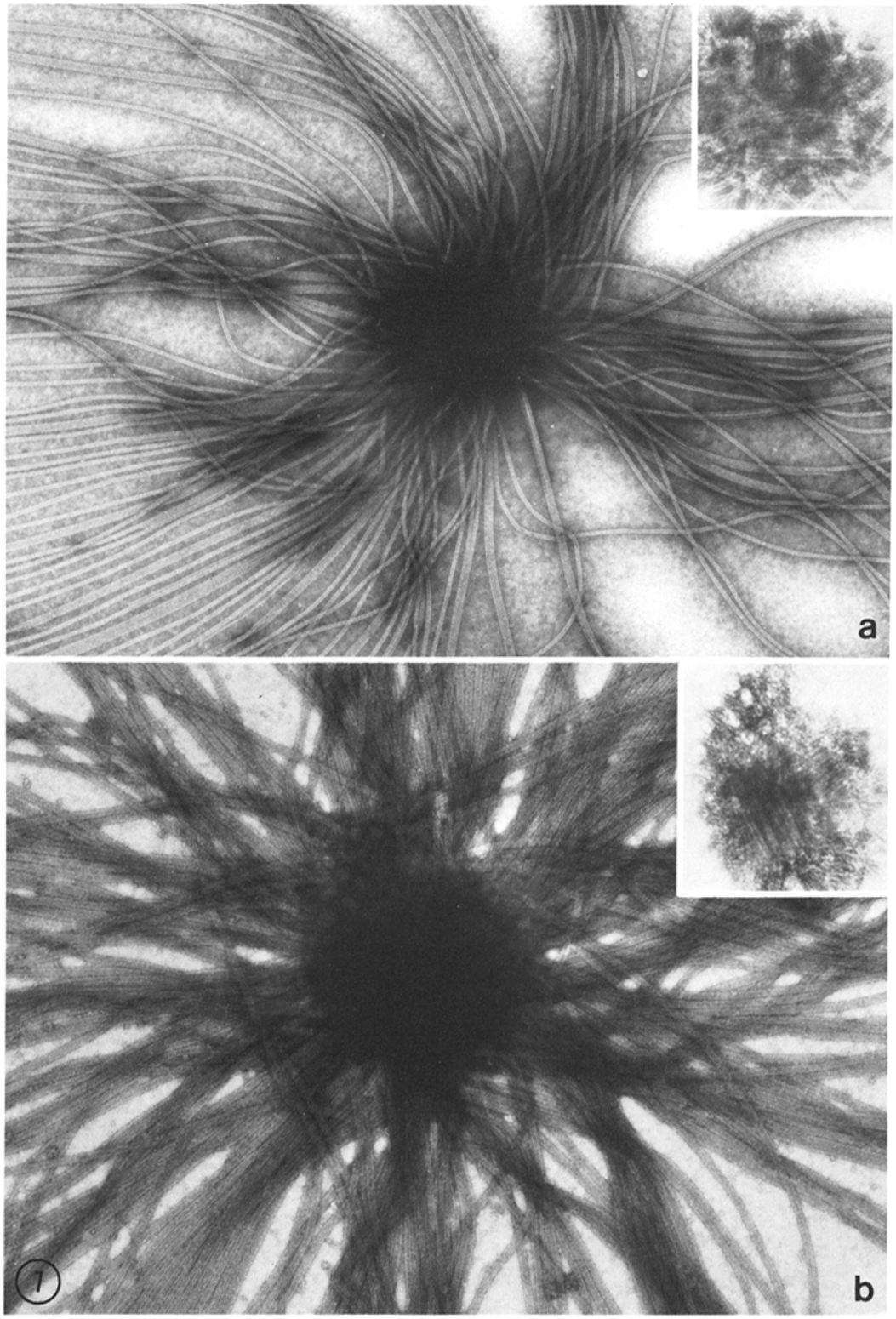

FIGURE 7 Two centrosomes from Colcemid-blocked dividing cells after incubation with tubulin in vitro, showing the range in the number of microtubules polymerized at the centrosomes. In (*a*) 125 microtubules emanate from the densely staining center. The stain conceals a centriole pair, shown at higher magnification and the same orientation in the inset. No virus-like particles are present. In (*b*) about 265 microtubules radiate from a densly staining centrosome, shown at higher beam illumination in the inset. A few particles are present. Cells were lysed in 0.25% Triton X-100 in PME buffer. (*a*) × 20,000; inset, × 37,500. (*b*) × 35,000; inset, × 35,000.

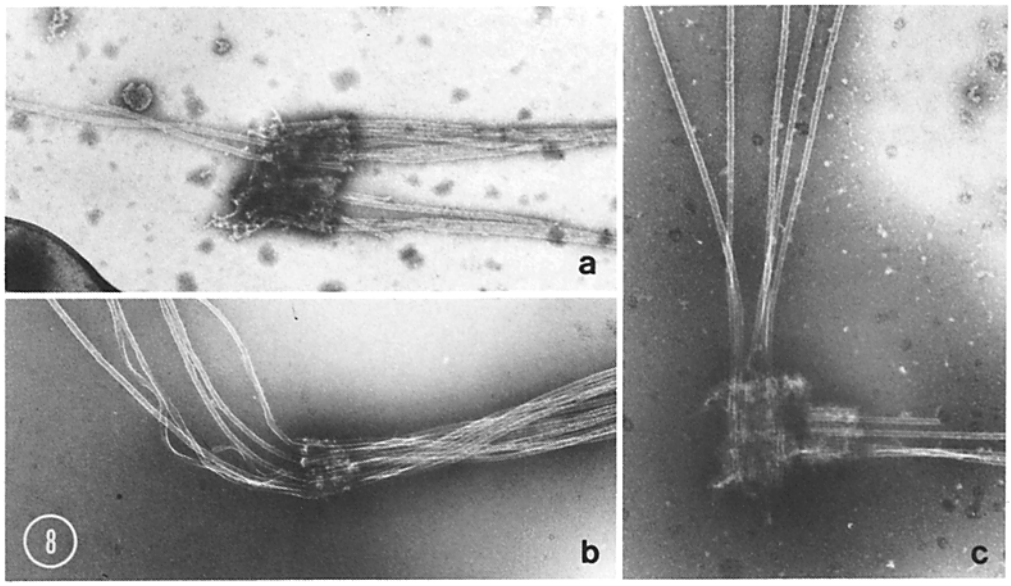

FIGURE 8 Interphase centrioles after incubation with tubulin in polymerization buffer. Tubulin has polymerized onto a centriole pair (*a*), a procentriole (*b*), and a centriole-procentriole pair (*c*). In each case growth onto the distal end is favored. (*a–c*) × 30,100.

one end than the other. Centrioles have an inherent polarity which is unambiguously reflected by the position of the daughter centriole. The end of the centriole that bears the daughter is considered the "generative" or proximal end, as is the end of the daughter which abuts the parent centriole. These ends are often distinguished in thin sections by the presence of a "cartwheel" (12).

Fig. 8*a* and *c* show that it is the distal end onto which microtubules assemble preferentially. A similar conclusion has recently been reported by McGill and Brinkley (18). Thus, both centrioles and procentrioles served as nucleating sites for microtubule assembly in vitro and showed the biased polar growth previously reported for microtubules (23, 10), axonemes (1, 4) and basal bodies (31).

Thus while the centrioles alone can nucleate a few microtubules in characteristic flagellar axoneme-like bundles, they do not give rise to the much larger, divergent array of microtubules as do intact centrosomes containing the pericentriolar material. This strongly suggests that the pericentriolar material nucleated the majority of microtubules seen when intact centrosomes were incubated with tubulin.

ISOLATED PERICENTRIOLAR MATERIAL AS A NUCLEATING CENTER: In many lysates of Colcemid-blocked mitotic cells, patches of pericentriolar material were apparently spontaneously detached from the centrosomes with a low frequency, affording an opportunity to determine whether this material could initiate microtubule assembly in vitro. Isolated fragments of pericentriolar material would ordinarily be difficult to identify because, aside from their generally fibrous appearance, they would be relatively nondescript. In CHO, however, the patches were inferred to be pericentriolar material from the observation that they bound groups of the virus-like particles.

Fig. 9*a* and *b* show two patches of material from lysed cells that have not yet been incubated with tubulin. After 10 min of incubation with tubulin, the isolated patches of pericentriolar material were found with the same frequency in the lysates as before, but this time with numerous microtubules attached. Fig. 9*c* and *d* show two typical patches in which the microtubules originate in the amorphous material. Again, the virus-like particles serve as markers.

We conclude from the above observations that the pericentriolar material can nucleate and anchor microtubules in vitro.

DISCUSSION

These experiments allow several important conclusions to be drawn about the structure and function of the centrosome from the typical mammalian cell line, CHO.

First, the centrosome has been shown to be an

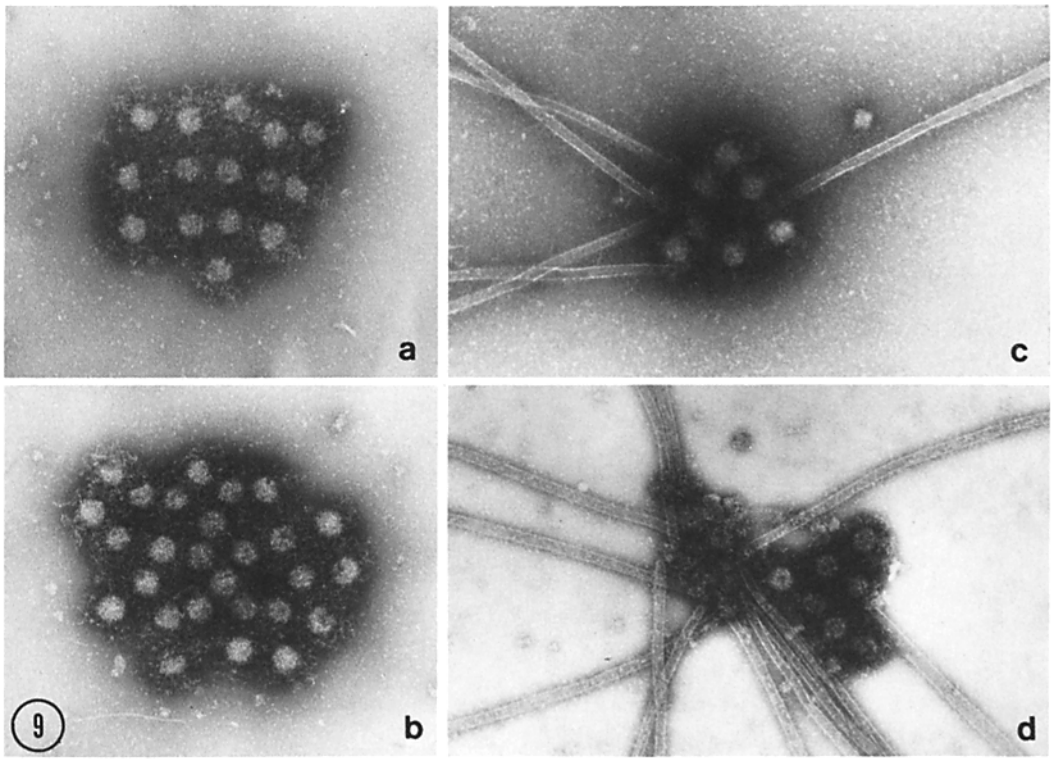

FIGURE 9 (*a* and *b*) Isolated patches of fibrous material from cells blocked in division with Colcemid. From the presence of the virus-like particles, the material is inferred to be pericentriolar material. (*c* and *d*) Similar fragments of putative pericentriolar material after incubation with tubulin in polymerization buffer. Microtubules have assembled onto the material. (*a–d*) × 53,800.

organelle in itself, which can be isolated intact at any stage of the cell cycle, and which consists of a pair of centrioles and pericentriolar material. Thus the early cytologists who first used the term "centrosome" (2, 7, 40) were quite right in hinting at its discrete nature. In recent years, the distinction between the centrioles and the larger centrosome of which they are a part has been blurred (32), and occasionally the two terms have been used interchangeably (18). However, the centrioles and pericentriolar material are two functionally distinct parts of the centrosome, and it is important to maintain the distinction between them.

Second, this report has also shown that the centrosomes from both dividing and interphase cells firmly anchor arrays of microtubules, even after cell lysis, under conditions that preserve native microtubules. Several recent studies with immunofluorescent staining have called attention to arrays of cytoplasmic microtubules that are present in mammalian cell lines during interphase (8, 24, 11). Many of these interphase microtubules, which are thought to play a role in maintaining cell shape, appear to originate at the centrosome (24). Although CHO cells have a diminished complement of cytoplasmic microtubules (8), we have nevertheless observed as many as two dozen microtubules attached to the centrosomes obtained from lysed cells, thus providing direct evidence that at least some interphase microtubules are anchored at the centrosome. These microtubules typically appear to originate from a region in the pericentriolar material close to the centrioles, but it has not yet been possible to determine unequivocally their exact origin.

The major thrust of this work was to analyze and distinguish the respective roles of the pericentriolar material and the centrioles in nucleating microtubules during cell division. Whereas the centrioles were easily recognized in cell lysates, the pericentriolar material was more difficult to distinguish in many of these experiments and was chiefly identified by the numerous particles embedded in it.

These particles were first described by Wheatley (38), who suggested that they might be an unusual form of virus that specifically associated with the pericentriolar material in CHO so as to be automatically distributed to the daughter cells during division. Wheatley failed to observe these particles in twelve other cell lines examined and so concluded that the particles were specific to CHO cells. Brinkley (personal communication) has made similar observations but in addition has reported the particles to be present in the rat kangaroo cell line, PtK_1. Our observations of centrosomes in cell lysates confirm the presence of the particles in CHO and PtK_1 cells and their absence in seven other hamster, mouse, and human cell lines. Thus although the nature and function of these particles has not been established, they do not appear to be a universal component of the centrosome.

We have found that the particles in CHO displayed several staining patterns typical of viruses (15, 39): they had densely staining cores when positively stained with uranyl acetate; they were roughly spherical and often showed hexagonal outlines when the surrounding matrix was digested with trypsin and negatively stained; and they were observed either with or without cores. However, since the particles have not been shown to be infective, they only earn the appellation "virus-like."

There are several justifications for considering the virus-like particles to be markers for the pericentriolar material. First, in Wheatley's original observations on thin sections of CHO cells, the particles were found only in association with the centrosomes, and rarely more than 1 μm from the centrioles (38). Furthermore, they were always seen enveloped in a densely staining matrix that appeared identical to the pericentriolar material. Second, in the lysates of interphase CHO cells described in the present paper, the particles were found only in the pericentriolar material at each centrosome, and nowhere else. In lysates of Colcemid-treated mitotic cells, the particles were again found at the centrosomes, but also were occasionally found in small clusters embedded in fibrous material, isolated on the grid. We interpret these bits of material as fragments of pericentriolar material that were occasionally split off from the centrosomes during cell lysis.

We found only two conditions under which the virus-like particles were dissociated from the pericentriolar material. The first was by proteolytic digestion of the pericentriolar material with trypsin. The second was by polymerizing microtubules onto the centrosome. There was a roughly inverse correlation between the number of microtubules polymerized onto the centrosome and the number of particles remaining. Since the virus-like particles remained at the centrosome after incubation in assembly buffer lacking tubulin, it was apparently the formation of microtubules themselves, and not the ionic conditions of the incubation, that led to loss of the particles. One possible explanation is that polymerization of microtubules onto the centrosome altered the pericentriolar material sufficiently to allow some of the particles to dissociate during cell lysis.

Thus the virus-like particles appear to be a specific and stable marker for the pericentriolar material in CHO.

The pericentriolar material was shown to be capable of nucleating and anchoring microtubules both in vivo and in vitro based on several lines of evidence: (*a*) native microtubules converged on the pericentriolar material, but not on the centrioles, in cells recovering from Colcemid block.

In whole-mounts of centrosomes from dividing cells, the pericentriolar material was distributed predominantly at one end of the centriole pair, and when sedimented onto the electron microscope grid allowed a clear assessment of the origin of the microtubules. Although it is conceivable that the microtubules were nucleated at the centrioles and somehow were transferred to the pericentriolar material, the simplest explanation is that the microtubules were nucleated by and remained anchored at the pericentriolar material.

(*b*) Purified microtubule protein was polymerized in vitro onto intact centrosomes to form an astral array.

The microtubule protein used was prepared so as to be incapable of spontaneous self-assembly under the conditions of the assay, and to require nucleating centers for polymerization. No free, i.e., self-assembled, microtubules were ever observed in these experiments. Thus the centrosomes must have served as nucleating centers. The number of microtubules that polymerized (up to 250) was similar to the number of microtubules that would normally form at the centrosome in vivo (19), suggesting that the functional capacity of the centrosome was retained in vitro.

(*c*) When the pericentriolar material was removed, the centrioles alone did not give rise to an astral array of microtubules upon incubation with

tubulin, but instead nucleated a few microtubules in characteristic axoneme-like bundles.

These experiments were done with interphase centrioles and are therefore not directly comparable to the experiments done with dividing cells. Nevertheless, they show that the microtubules that assembled onto the ends of the centrioles were too few and too highly directional to account for the large, astral array that forms at the intact centrosomes as described above.

It is not surprising that microtubules can be polymerized from the ends of the centrioles, since the only proven function of the centriole is its role as a basal body, when it nucleates the axonemal microtubules of cilia and flagella (12, 25). In dividing cells, a few microtubules have been reported to arise directly from centriolar microtubules both in vivo (16) and in vitro (36). During interphase, the centrioles of some cell lines give rise to a short cylindrical bundle of microtubules that resembles an internal axoneme (37; R. R. Gould and G. G. Borisy, unpublished observations). The role, if any, of these centriole-derived microtubules is not known.

(*d*) Fragments of pericentriolar material from dividing cells gave rise to microtubules when incubated with purified microtubule protein.

These fragments were tentatively identified as pericentriolar material since they had the same densely staining fibrous appearance as pericentriolar material, and since they contained groups of the virus-like particles. Although it was not possible to prove directly, the simplest hypothesis is that these fragments were derived from the centrosome during cell lysis, as described above. Also consistent with the idea that the fragments were derived from the centrosome was the observation that they were uniformly associated with microtubules in lysates incubated with microtubule protein.

It is conceivable that a few microtubules were nonspecifically nucleated by the virus-like particles. However, most of the microtubules were not observed to end on the virus-like particles, in those favorable instances in which the ends of the microtubules could be determined. Furthermore, the microtubules remained firmly anchored at the centrosomes even in those cases in which no particles at all were discernible at the centrosome.

We conclude from the above evidence that the pericentriolar material from dividing CHO cells can nucleate and anchor microtubules both in vivo and in vitro.

This finding raises a number of intriguing questions about the mechanism by which the pericentriolar material nucleates spindle microtubules. On the one hand, it is easiest to picture ordered arrays of microtubules as arising from similarly ordered arrays of discrete nucleating sites (26). On the other hand, aside from the virus-like particles, the pericentriolar material appears to lack any obvious, discrete structures that might serve as nucleating sites. It is possible that the pericentriolar material is actually highly structured in vivo but that this structure is not preserved for electron microscopy. It is also possible that the material is itself amorphous, but that any portion of it is capable of nucleating microtubules roughly perpendicular to its surface with a given probability. On this view, the exact pattern of microtubules that resulted would be influenced by other factors, such as microtubule cross-bridges and interaction with other cell structures.

A second problem concerns the change in the centrosome's role as organizer of part of the cytoplasmic microtubule complex, during interphase, to its role as organizer of spindle microtubules during cell division. During much of interphase, there was considerable pericentriolar material at the centrosomes, yet only a fraction of it seemed to be associated with interphase microtubules. This suggests that as the cell enters division, along with a reorientation of the pericentriolar material, there may be either a proliferation of nucleating sites within the pericentriolar material, or an "activation" of preexisting material that allows it to nucleate microtubules. Alternatively, there might be several classes of microtubule initiators at the centrosome, e.g., one class to initiate interphase microtubules, one class responsible for centrosome separation, and a third class responsible for spindle microtubules.

Finally, it should not be assumed that centrioles remain inactive during cell division (25). They may well influence the initiation and patterning of microtubule formation by controlling, either directly or indirectly, the proliferation and functioning of the pericentriolar material. Understanding the detailed mechanism by which the centrosome nucleates microtubules poses a formidable challenge for the future.

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