Microtubule-nucleating Activity of Centrosomes in Chinese Hamster Ovary Cells Is Independent of the Centriole Cycle but Coupled to the Mitotic Cycle

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ABSTRACT   The nuclear-centrosome complex was isolated from interphase Chinese hamster ovary (CHO) cells, and, with exogenous brain tubulin as a source of subunits, the centrosome, while attached to the nucleus, was demonstrated to nucleate microtubule formation in vitro. We attempted to quantitate the nucleating activity in order to compare the activity of mitotic and interphase centrosomes. However, the proximity of the nucleus hindered these attempts, and efforts to chemically or mechanically remove the centrosome led to diminished nucleating activity. Therefore, the nuclear-centrosome complex was dissociated biologically through use of the cytochalasin B procedure for enucleation of cells. Cytoplasts were prepared that retained the centrosome. Lysis of the cytoplasts released free centrosomes that could nucleate microtubules in vitro. The nucleating activities of interphase and mitotic centrosomes were compared. In addition, through the use of whole-mount electron microscopy, the configuration of the centrioles was analyzed and the number of microtubules nucleated was determined as a function of the centriole cycle. Nucleating activity did not change discernibly throughout interphase but increased approximately fivefold at the transition to mitosis. Thus, we conclude that the nucleating activity of the centrosome is relatively independent of the centriole cycle but coupled to the mitotic cycle.

The centrosome has long been considered to be the organizing center for the astral fibers of the mitotic spindle (19). Experiments with lysed mitotic cells (12, 16) and with isolated centrosomes from mitotic cells (9, 18) have directly demonstrated that the centrosomes have the capacity to nucleate microtubule formation. Snyder and McIntosh (16) also did studies on lysed interphase cells and found greatly diminished levels of nucleating activity. They suggested that the centrosome "ripened" as the cell entered prophase and attained its greatest activity at metaphase. These conclusions were thought to be in accord with earlier electron microscope observations on the centriole cycle in cultured cells (15), which showed that the centrioles accumulated a cloud of pericentriolar material as the cell progressed towards mitosis. Thus, it seemed as though the activity of the centrosome was at a low level during interphase, and that its activity was greatly augmented in mitosis. In a recent study, Telzer and Rosenbaum (18) found that centriolar complexes capable of initiating microtubule assembly could be obtained only from populations containing mitotic cells. They

concluded that the pericentriolar material matures and only becomes competent as a microtubule-organizing center at the time of mitosis.

On the other hand, there is evidence that the centrosome of interphase cells does indeed nucleate microtubule formation. This is suggested by the pictures of asters in interphase cells as seen in Wilson (19). Electron microscope studies of thin sections also have indicated an array of microtubules focusing on the interphase centrosome (5, 7). McGill and Brinkley (12) observed, by thin sectioning, the assembly of microtubules onto the centriolar complex in interphase HeLa cells lysed in the presence of tubulin. In the last several years, the use of immunofluorescence techniques has convincingly demonstrated the existence of a cytoplasmic network of microtubules and that the interphase centrosome seemingly serves as an organizing center for at least part of this network (3, 6, 13, 17).

Thus, the available evidence indicates that the centrosome serves as a microtubule-organizing center throughout the cell cycle, but, without quantitative data to compare directly the

microtubule-nucleating activity of mitotic and interphase centrosomes, the concept of a ripening at mitosis would seem to benefit from further investigation.

In the accompanying paper (11) we have shown how electron microscopy of whole-mount preparations of lysed cells has permitted us to determine the structural changes in the centrioles as a function of the cell cycle. In this paper, we provide a quantitative comparison of the microtubule-nucleating activity of interphase and mitotic centrosomes. In addition, we investigate whether the nucleating activity of centrosomes depends upon position in the centriole cycle. Our results indicate that the centrosome has the capacity to nucleate microtubules throughout interphase and that the level of nucleating activity per centriole pair does not change discernibly during this period. At the transition to mitosis the level of nucleating activity increases significantly. The nucleating activity of the centrosome therefore appears to be relatively independent of the centriole cycle but coupled to the mitotic cycle.

MATERIALS AND METHODS

Cell Culture

Chinese hamster ovary (CHO) cells were used in all experiments and were grown as previously described (11). To obtain cells blocked in mitosis, we treated them first with 2–5 mM thymidine. After 10–15 h, the cells were resuspended in fresh medium for 3–5 h, exposed to 0.1 μg/ml Colcemid for an additional 3–5 h, and then collected by centrifugation in a tabletop centrifuge. In experiments where direct comparisons with cytoplasts were to be made, mitotic cells were exposed to cytochalasin B for 30 min to make the treatments of both cell populations equivalent.

Preparation of Nuclear-Centrosome Complexes from Interphase Cells and Free Centrosomes from Cytoplasts and Mitotic Cells

Interphase cells were harvested and nuclear-centrosome complexes prepared as described previously (11). Complexes were resuspended in extraction medium (10 mM PIPES, 1 mM EGTA, 0.5 mM MgCl₂, 0.25% Triton X-100, pH 6.7).

Free centrosomes were prepared from cytoplasts of interphase cells by a slightly different procedure. Pelleted cytoplasts were suspended in distilled water (10:1 vol/vol) and allowed to stand for 1–2 min at room temperature. Then, an equal volume of lysis solution (2 mM PIPES, 0.5% Triton X-100, pH 6.7) was added to lyse the cells at low ionic strength. After checking for cell lysis by phase-contrast microscopy, the sample was chilled to 0°C and 0.1 vol of a concentrated buffer solution was added to make the final composition of the sample equal to that of extraction medium.

For mitotic cells, two procedures were used to release free centrosomes. One was identical to that used for cytoplasts. The other consisted of the addition of twice-concentrated extraction medium to an equal volume of cells suspended in distilled water. The nucleating activity of centrosomes derived from mitotic cells by either procedure was the same.

Preparation of Microtubule Protein and Polymerization onto Centrosomes

Microtubule protein was purified from porcine brain as described previously (1, 10). Equal volumes of purified microtubule protein in polymerization buffer (100 mM PIPES, 1 mM EGTA, 0.1 mM MgCl₂, 1 mM GTP, pH 6.7) were added to test samples in extraction medium. Test samples included nuclear-centrosome complexes and lysates of interphase cells, mitotic cells, and cytoplasts. After incubation at 30°C for 5–20 min, polymerization was stopped by addition of 0.5 vol of 3% glutaraldehyde in 10 mM PIPES, 1 mM EGTA, 0.5 mM MgCl₂, pH 6.7.

Microscopy

Light and electron microscopy were performed as described previously (11).

RESULTS

To demonstrate the microtubule-nucleating activity of inter-

phase centrosomes in vitro, isolated nuclear-centrosome complexes were incubated with microtubule protein under polymerizing conditions. The source of the nuclear-centrosome complexes were exponentially growing CHO cells. Since the mitotic index of these populations was 4%, the overwhelming majority of the cells were interphase, and since only a minute proportion of the nuclei would have been derived from prophase cells, we considered the presence of a centrosome on a nucleus to be an indicator that it derived from an interphase cell. In these experiments, the complexes were first chilled at 0°C for 30 min to ensure depolymerization of native microtubules, and no microtubules were seen before the addition of the tubulin. Fig. 1 shows an electron micrograph of a whole-mount preparation of a nuclear-centrosome complex isolated from an interphase cell and clearly nucleating the formation of many microtubules. In preparations like this, the nucleus appeared as a dark sphere within which it was often difficult to see any detailed structure. However, when the centrosome was located at the edge of the nucleus, it could then be seen more clearly that the microtubules initiated by the complex originated from the centrosome. In the inset, the densely stained focal region of microtubules is shown in the same orientation but with greater beam exposure and is revealed to contain a pair of centrioles.

Although the nucleus provided a useful indicator for the interphase origin of the centrosome, its proximity hindered quantitation of the microtubules formed. Since we wished to provide a quantitative comparison of the nucleating activity of interphase and mitotic cells, attempts were made to dissociate the centrosome from the nuclear surface.

We first explored a variety of chemical and mechanical approaches but these met with only limited success. Dialysis of the complexes against a low ionic strength medium (1 mM PIPES, 0.1% Triton X-100, 0.1 mM EDTA, 0.1 mM dithiothreitol, pH 6.7) overnight at 0°C released some centrosomes, but also caused nuclear breakdown. Since heparin had been reported to solubilize chromatin (2), it was used at 350 μg/ml in the extraction medium, and, after several minutes at 0°C,
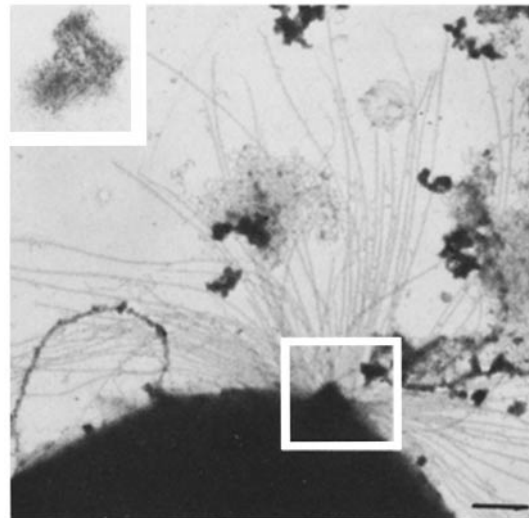

FIGURE 1   Electron micrograph of a whole-mount preparation of a nuclear-centrosome complex isolated from an interphase cell and incubated with porcine brain microtubule protein. Many microtubules radiate from the centrosome at the periphery of the nucleus. The inset shows the region bounded by the white square at higher electron beam illumination, revealing a pair of centrioles at the focus of the microtubule. Bar, 1 μm.

the nucleus was disrupted, freeing the centrosome. With both treatments the free centrosomes produced had diminished nucleating activity. Treatment with 0.1% β-mercaptomethanol in extraction medium for 30 min at room temperature only partially removed centrosomes. Sonic vibration (minimum setting, 20 s) in extraction medium at 0°C resulted in complete nuclear breakdown and liberation of centrioles in good yield; however, the pericentriolar material apparently was also removed and nucleating activity was low.

Since the chemical and mechanical procedures we explored did not seem very promising, we next investigated a biological method for dissociating the nuclear-centrosome complex. It has been described that enucleation of cells with cytochalasin B produces two cell fractions: the karyoplast and the cytoplast (4, 14). The cytoplast has been shown to contain the centrosome (8, 20). Therefore, by preparing cytoplasts and then making lysates, we expected that free centrosomes would be released and there would be no need to subsequently dissociate them from the nucleus.

Cytoplasts and karyoplasts were prepared from whole CHO cells by centrifugation at 37°C in a cytochalasin B–containing medium as described in the accompanying paper (11). As has been reported by others (8, 20), the karyoplast contained the nucleus surrounded by a thin shell of cytoplasm. The cytoplast lacked the nucleus but retained all of the cytoplasmic organelles including the centrosome from which microtubules clearly radiated (data not shown).

The conclusions drawn from electron microscopy of thin sections were also confirmed by analysis of whole-mount preparations. Lysis of karyoplasts in extraction medium released nuclei on which no centrosomes could be detected, whereas lysis of cytoplasts released centrosomes.

As described previously (11), the structural changes occurring in the centrioles as a function of the cell cycle can be readily determined by whole-mount electron microscopy. We have analyzed the centriole cycle in terms of six categories defined by the relative orientation and length of parent and daughter centrioles. Thus, by determining the number of microtubules nucleated by centriole pairs of different categories, we have been able to express nucleating activity as a function of the centriole cycle. In addition, since the centriole cycle is normally coupled to other cellular events, it is possible to assign the cycle position of a cell by inspection of its centriole configuration and relate nucleating activity to position in the cell cycle. For example, from the results of our previous study (11), category I centrioles are indicative of a cell in late M or early G_1 phase; category II centrioles are found in cells late in G_1 or in early S phase; categories III–VI are found in S, G_2, and M phases with the correlation that longer daughter centrioles are more likely to be found in cells the closer the cells are to M phase.

Fig. 2 shows a free centrosome that was obtained from a lysed interphase cytoplast and incubated with brain tubulin in vitro. Microtubules were nucleated by the structure, and, under high-beam illumination in the electron microscope, centrioles were identified at the center of the microtubule array. To compare the nucleating activity of mitotic and interphase cells, centrosomes were prepared from mitotic cells under the same conditions as for interphase cytoplasts. Fig. 2 b shows an example of a mitotic centrosome nucleating microtubules, and it is evident from direct visual inspection of the two figures that the mitotic centrosome nucleated significantly more microtubules than did the interphase one.
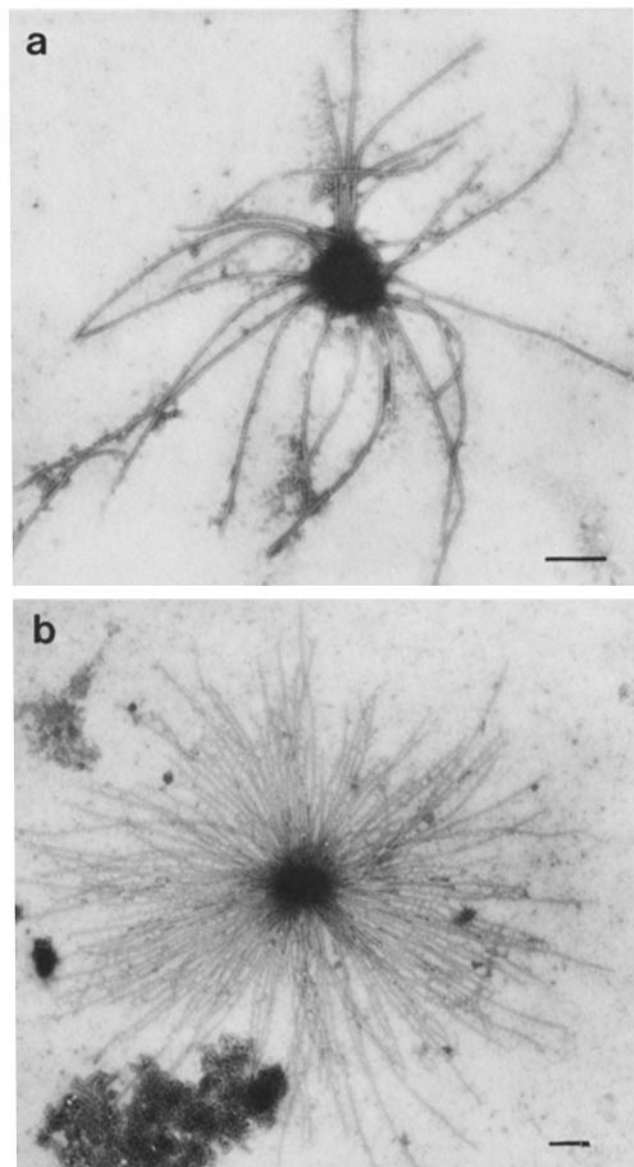
FIGURE 2   Free centrosomes incubated with porcine brain microtubule protein and nucleating microtubules in vitro. Centriole pairs present at the focus of the microtubules can be seen at higher beam illumination. (a) Interphase centrosome obtained from a lysed cytoplast. Approximately 25 microtubules radiate from this centrosome. (b) Mitotic centrosome. Approximately 140 microtubules radiate from this centrosome. Bar, 1 μm.

To determine whether the nucleating activity of the centrosome was augmented specifically in mitotic cells or increased uniformly as the centriole cycle progressed, we compared the nucleating activity of interphase and mitotic centrosomes having the same category of centriole configuration.

The relative frequencies of centriole categories were of course different in interphase and mitotic cells (Fig. 3). The most abundant centriole configurations in mitotic cells were those represented by categories V, VI, and I; however, occasionally category IV and rarely category III configurations were also found. No category II configurations were found in mitotic cells. However, all category configurations were found in centrosomes released from interphase cytoplasts. Thus, nucleating activity of interphase and mitotic centrosomes could be readily compared for categories IV, V, VI, and I, and the quantitative
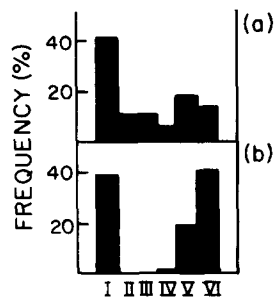
FIGURE 3 Frequency histogram of centriole configurations in (a) interphase and (b) mitotic cells. The centriole category is defined in reference 11; also see Fig. 4 of this paper. The mitotic cells were collected using a Colcemid block as described in Materials and Methods. The interphase histogram was calculated by subtracting the contribution of mitotic cells (4% of an exponentially growing population) from the centriole profiles seen in an exponentially growing population.
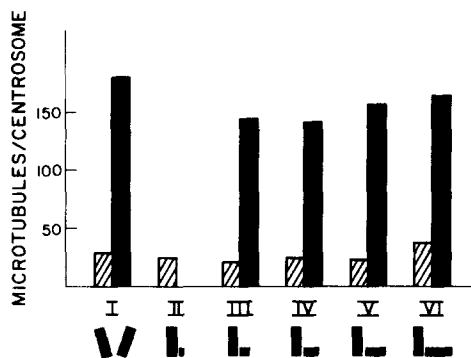

FIGURE 4 Dependence of microtubule-nucleating activity on the centriole cycle. The ordinate gives the number of microtubules nucleated per centriole pair in a centrosome. Most centrosomes contained one pair of centrioles. Some centrosomes contained two pairs of centrioles. For these, the number of microtubules nucleated was divided by two. The data are presented for six categories of centriole configuration defined in terms of the orientation and relative length of parent and daughter centrioles (see text and reference 11). Category I represents centriole pairs in late M or G_1. The parent is full-sized, and the daughter is also full-sized, or nearly so, but not in an orthogonal orientation. Categories II, III, IV, V, and VI represent stages in the elongation of the daughter centrioles and correspond to ratios of daughter to parent centriole length of 0.0–0.2, 0.2–0.4, 0.4–0.6, 0.6–0.8, 0.8–1.0, respectively. Hatched bars: nucleating activity of interphase centrosomes; the numbers of centrosomes analyzed for categories I–VI were 40, 39, 80, 103, 70, and 25, respectively. Solid bars: nucleating activity of mitotic centrosomes; the numbers of centrosomes analyzed for categories I–VI were 40, 0, 8, 50, 91, and 47, respectively.

data are presented in Fig. 4. For each of these categories, the mitotic centrosomes nucleated approximately five times as many microtubules as the interphase centrosomes. Category III centrosomes were only rarely observed (eight instances) in mitotic cells and so the data set is too small for us to draw firm conclusions, but these centrosomes also nucleated more microtubules than did their interphase counterparts. Thus, we conclude that nucleating activity is apparently not dependent upon the elongation of the daughter centriole but, instead, appears to correlate with the entry of the cell into mitosis.

DISCUSSION

The results show that the centrosome of interphase cells can nucleate microtubules. Moreover, as judged by analysis of nucleating activity in comparison with the centriole cycle, the activity of the centrosome does not change discernibly during interphase. Thus, our conclusions are in agreement with the inferences drawn from immunofluorescence studies on the reformation of tubules in cultured cells after treatment with low temperature or colchicine to induce depolymerization (3, 6, 13, 17). However, our results are in disagreement with the recent study of Telzer and Rosenbaum (18), which failed to find activity in interphase cells. They prepared lysates of synchronized cells and examined nucleating activity by dark-field light microscopy. Assembly was observed only in lysates of mitotic cells. It is not clear how to account for the difference in results.

We have demonstrated a nuclear-centrosome complex in CHO cells (11) and have preliminary evidence that, under our conditions, a similar complex exists in HeLa. Since Telzer and Rosenbaum (18) generally centrifuged their lysates to sediment nuclei and measured nucleating activity in the supernatants, it seems possible that they sedimented the centrosome as well. However, they considered this possibility and reported that no centriolar complexes could be observed adhering to the nuclei, nor did the nuclei display any microtubule initiating activity. Nevertheless, since the nucleus in HeLa cells is larger than CHO cells, it is possible that the centrosome may have been missed.

Our results are qualitatively similar to those of Snyder and McIntosh (16), who examined nucleating activity in rat kangaroo cells lysed into solutions containing tubulin. Although they did not attempt to quantitate their nucleating activity, they described their results as indicating a "ripening" or maturation of the centrosome at the onset of mitosis. We too find an enhancement in the number of microtubules initiated per centrosome, indicating that in addition to a substantial basal level of activity present in interphase, the activity of the centrosome is significantly augmented at the transition to mitosis.

From previous studies (9), it has been shown that the activity of the centrosome responsible for nucleating microtubules resides in the pericentriolar cloud. It cannot be decided from our data alone whether the increased activity at the transition to mitosis is a result of an increase in the amount of the pericentriolar cloud or in its specific nucleating activity. However, it is clear that the amount of the pericentriolar material must double with each cell cycle and one question here is whether the accumulation of the material at the centrosome increases abruptly or throughout the cell cycle. Another question is whether the pericentriolar material is first accumulated and later activated, for example, by a posttranslational modification. Answers to these questions require the identification of the molecular nature of the nucleating activity and the changes occurring in it at the transition to mitosis.

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