Microtubule nucleating activity of centrosomes in cell-free extracts from *Xenopus* eggs: involvement of phosphorylation and accumulation of pericentriolar material

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SUMMARY

We have studied the regulation of microtubule nucleating activity of the centrosome using cell-free extracts from *Xenopus* eggs. We found that the number of microtubules per centrosome increases dramatically with time during incubation of isolated centrosomes in interphase egg extracts prepared 20-30 minutes after electric activation of cytostatic factor (CSF)-arrested eggs. The increase in microtubule nucleation was still conspicuous even when KCl-treated centrosomes (centrosomes stripped of their microtubule nucleating activity by 1 M KCl treatment) were incubated in interphasic extracts. Electron microscopy and immunostaining by anti-γ-tubulin and 5051 human anti-centrosome antibodies revealed that pericentriolar material (PCM) was accumulated during the increase in microtubule nucleation from centrosomes in interphasic extracts, suggesting regulation of centrosomal activity by PCM accumulation. The ability of egg extracts to activate microtubule nucleation from centrosomes was also assumed to be regulated by phosphorylation, since addition of protein kinase inhibitors into interphasic extracts totally blocked the increase in microtubule nucleation from the KCI-treated centrosome. The ability of CSF-arrested mitotic extracts to increase microtubule nucleation from KCI-treated centrosomes was 3.5- to 5-fold higher than that of interphasic extracts, while PCM accumulation in mitotic extracts seemed to be similar to that in interphasic extracts. The increase in microtubule nucleation from KCI-treated centrosomes was strikingly enhanced by the addition of purified p34cdc2/cyclin B complex to interphasic extracts, but not by MAP kinase, which is activated downstream of p34cdc2/cyclin B. These results suggest two pathways activating centrosomal activity in egg extracts: accumulation of PCM and phosphorylation mediated by p34cdc2/cyclin B.

Key words: centrosome, pericentriolar material, cdc2 kinase, cyclin B, MAP kinase, microtubule, cell cycle

INTRODUCTION

Reorganization of the microtubule cytoskeleton during M phase is one of the most drastic changes in intracellular structure during the cell cycle in higher eukaryotes. Cytoplasmic microtubules formed in interphase disappear at the beginning of M phase, and are converted into highly dynamic and organized spindle microtubules.

It has been suggested that microtubule organizing centers (MTOCs), which govern the organization of cytoplasmic microtubules, are modified at the onset of M phase to have a character prerequisite for construction of the mitotic spindle. The centrosome of animal cells consists of a pair of centrioles and pericentriolar material (PCM) (reviewed by McIntosh, 1983; Mazia, 1984, 1987; Brinkley, 1985). PCM has been identified by electron microscopy as amorphous, electron-dense material around the centriole (Robbins et al., 1968), and it is responsible for nucleation of microtubules from the centrosome (Gould and Borisy, 1977). Mitotic centrosomes have a 5-fold higher competence for microtubule nucleation in vitro than interphase centrosomes (Kuriyama and Borisy, 1981). This high competence of the mitotic centrosomes for microtubule nucleation may be essential for the formation of numerous spindle microtubules. In addition, the full construction of spindle microtubules requires the existence of chromatin, which may be necessary for selective stabilization and generation of half-spindle microtubules (Karsenti et al., 1984; Swain and Mitchison, 1991). In fact, without chromatin, the centrosome can only form a small number of short astral...
microtubules in mitotic extracts from cytostatic factor (CSF)-arrested *Xenopus* eggs (Verde et al., 1990).

Little is currently known of the mechanisms regulating centrosomal microtubule nucleating potential during the cell cycle, but two hypotheses have been proposed. The first envisages a cell cycle-dependent variation in the amount of PCM, which is responsible for the microtubule nucleation on the centrosome. PCM has been shown to increase during M phase in mammalian cells (Rieder and Borisy, 1982), and microtubule-organizing granules (PCM in sea urchin eggs) accumulate at the spindle poles in a mitotic cycle-dependent manner (Endo, 1980). The increase in PCM during M phase may result in an increase in microtubule nucleation sites. However, it is quite unclear how the amount of PCM is regulated.

It is attractive to speculate that the cytoplasm has the ability to supply microtubule nucleating material to the centrioles or centrosomes in a cell cycle-dependent manner. It has been indicated that egg cytoplasm is able to supply microtubule nucleating material to the centriole or the centrosome in vivo. Kuriyama and Kanatani (1981) reported that sperm centrioles could form astral microtubules when they were injected into starfish eggs, while they failed to form astral microtubules in an in vitro system containing purified tubulin. This suggests that centrosomal proteins in the egg cytoplasm gather around the sperm centriole, thereby acquiring microtubule nucleating activity in vivo. Unfortunately, little is known about the kinetics of this process during the cell cycle.

The second idea is that activation of the centrosome is through the action of M phase-activated phosphorylation. Most simply, the microtubule nucleating potential of the centrosome is activated through the phosphorylation of the centrosomal material responsible for microtubule nucleation. This idea is supported by the recognition of centrosomes in mitotic cells of many species by monoclonal antibodies against M phase-specific phosphoproteins (Vandre et al., 1984; Kuriyama, 1989). In addition, Centonze and Borisy (1990) reported that one such antibody can block the microtubule nucleating activity of the centrosomes from mammalian cells.

In the G2/M transition, protein kinases are activated and protein phosphorylation becomes prominent (Maller et al., 1977; Karsenti et al., 1987). Maturation promoting factor (MPF) (Masui and Markert, 1971; Kishimoto and Kanatani, 1976; Kishimoto, 1988), which is a complex of p34<sup>cdc2</sup> kinase and cyclin B (Gautier et al., 1988; Dunphy et al., 1988; Labbé et al., 1988; Dreatta et al., 1989; Nurse, 1990), plays a central role in G2/M transition. MAP kinase, originally found as a mitogen-activated serine/threonine kinase (Ray and Sturgill, 1987, 1988; Hoshi et al., 1988; Gotoh et al., 1990), is also activated during M phase of *Xenopus* oocytes (Gotoh et al., 1991a). MAP kinase is activated downstream of p34<sup>cdc2</sup>/cyclin B (Gotoh et al., 1991b).

These M phase-activated kinases have been shown to play a crucial role in the conversion of microtubule dynamics at the onset of the mitosis (Verde et al., 1990; Gotoh et al., 1991a). It has also been suggested that they are involved in the activation of the centrosome during M phase. p34<sup>cdc2</sup> associates with the centrosome in animal cells (Riabowol et al., 1989; Bailly et al., 1989) and with the spindle pole body in yeast which is equivalent to the centrosome (Alfa et al., 1990). In addition, a B-type cyclin homologue of fission yeast p63<sup>cdc13</sup> is also associated with spindle pole body (Alfa et al., 1990), and cyclin B with mitotic spindles in human cells (Pines and Hunter, 1991) and the polar region of mitotic spindles in *Drosophila* embryos (Maldonado-Codina and Glover, 1992).

Using cell-free extracts from *Xenopus* eggs, we have studied the regulation of the microtubule nucleating potential of the centrosome, in the light of the variation in amount of PCM and the cell cycle-dependent control by protein phosphorylation, since cell-free extracts from *Xenopus* eggs have advantages for analysis of regulation of microtubule organization (Karsenti et al., 1984; Kohka and Maller, 1985; Gard and Kirschner, 1987a; Verde et al., 1990; Belmont et al., 1990; Gotoh et al., 1991a). We found that extracts from *Xenopus* eggs are able to cause a time-dependent increase in microtubule nucleation from isolated mammalian centrosomes. PCM around the centrosome increased in egg extracts in vitro with the activation in microtubule nucleation. The ability of the extracts to increase microtubule nucleation from the centrosome is probably controlled by phosphorylation, since the addition of protein kinase inhibitors into the extracts totally blocked the increase in microtubule nucleation from KCl-treated, inactive centrosomes. The activity of the extracts is regulated in a cell-cycle dependent manner, mitotic extracts having approximately a 3.5- to 5-fold greater microtubule nucleating potential of KCl-treated centrosomes than interphasic extracts. Furthermore, the ability of interphasic extracts was strikingly enhanced by adding p34<sup>cdc2</sup>/cyclin B to the extracts, but not by MAP kinase, a kinase downstream of p34<sup>cdc2</sup>/cyclin B. We propose that the microtubule nucleating potential of the centrosome is activated during the cell cycle by two different pathways: (1) accumulation of PCM to the centrosome and (2) phosphorylation of centrosomal materials, such as the microtubule nucleating material or the putative PCM accumulating factor.

**MATERIALS AND METHODS**

**Preparation of cell-free extracts**

Cytoplasmic extracts arrested in a mitotic state were prepared from *Xenopus* eggs arrested at metaphase II (the second meiotic metaphase) by CSF. Cell-free extracts were prepared according to the method of Verde et al. (1990) with some modifications. The eggs were washed twice with an extraction buffer (100 mM potassium acetate, 2.5 mM magnesium acetate, 60 mM EGTA, 5 µg/ml cytochalasin B, and 1 mM DTT, pH 7.2), and were crushed in a minimum volume of the extraction buffer by centrifugation at 30,000 g and 2°C for 10 min. The upper lipid layer was completely removed by aspiration and the middle, clear supernatant was collected. The supernatant was mixed with 1/100 volume of an ATP regeneration system (100 mM ATP, 1 M creatine phosphate and 8 mg/ml creatine phosphokinase (Sigma)), and again centrifuged in a Beckman TL-100 at 350,000 g and 2°C for 25 min. The lipid layer was aspirated and post-ribosomal high-speed supernatants were collected. For the preparation of extracts used, we centrifuged at higher speeds than the standard protocol to remove membranous and particulate materials. This enables real-time observation under dark field microscopy. "PA20-30 extracts"
were prepared from the eggs driven into an interphase state in MMR (0.1 M NaCl, 2 mM KCl, 1 mM MgSO4, 2 mM CaCl2, 5 mM HEPES, 0.1 mM EDTA, pH 7.8) by a 4-5 s electric shock (12 V, AC) followed by incubation for 20-30 min at 20°C. The electrodes were separated by 3 cm. This period corresponds to the stage when the sperm aster is growing, histone H1 kinase activity and MAP kinase activity have decreased and DNA replication has started. These extracts were stored at ~80°C until use.

**Microtubule growth from the centrosome in extracts**

Centrosomes were isolated from CHO cells or L5178Y cells as described previously (Mitchison and Kirschner, 1984; Bornens et al., 1987). The quality of centrosome preparations was analyzed by electron microscopy, aster formation in purified tubulin and double labeling with anti-centrosome antibody and anti-tubulin antibody along with phase contrast microscopy as described by Bornens et al. (1987). From the yield of centrosomes and the final protein content, purification was assumed to be approximately 103-fold when one cell contains one centrosome. Isolated centrosomes (0.5-1 µl, 107/ml) were added to 15 µl of the extracts. The mixture was mounted onto cleaned coverslips on a Parafilm sheet, and incubated in a moist chamber. Microtubules formed from centrosomes usually stick onto the coverslips during a brief incubation (less than 1-2 min), presumably through the action of microtubule-associated motor proteins in the extracts.

In the “extracts exchange assay”, the extracts containing 1 µl of KCI-treated L5178Y centrosomes (prepared as described later) were incubated on coverslips for the indicated time at 20°C. The extracts on coverslips were removed by a micropipet. Then we carefully added 100 µl of PEM1 (100 mM PIPES, 1 mM EGTA and 0.5 mM MgCl2, pH 6.8) containing 10 µM TATP at 0°C to the coverslips where the regrown asters were stuck, and very carefully washed by gently pipetting the solution up and down several times. Liquid on the coverslips was carefully aspirated, and the coverslips were washed once more. After the wash, 15 µl of fresh interphase extracts were quickly applied to the coverslips and incubated for 1-2 min at 20°C to form microtubules from the centrosomes. In the assay using purified tubulin, the experiment was done as described above, except that we used native CHO centrosomes and performed a second incubation at 37°C for 20 min in 80 mM PIPES, 1 mM MgSO4, 1 mM EGTA, 1 mM GTP, pH 6.8, containing DEAE-purified tubulin (3.8 mg/ml) prepared from porcine brains as described (Masuda et al., 1990).

Use of PA20-30 extracts for the second incubation provides several advantages over purified tubulin for detecting quantitative differences in the ability of extracts to increase microtubule nucleation from centrosomes. Interphase extracts (PA20-30 extracts) contain ‘XMAP’ (Gard and Kirschner, 1987b), which enables microtubules to grow very fast, so that we can detect microtubules long enough even in a very brief incubation (1-2 min). Owing to such a brief incubation, this system worked quite well for detecting the cell cycle dependence and effects of kinase addition to the extracts, in spite of the existence of phosphatase activities in PA20-30 extracts. In fact, using PA20-30 extracts, we obtained similar results to those using purified tubulin (Fig. 7). The brief incubation also prevents detachment of astral microtubules from coverslips, which was often seen in the experiment using purified tubulin. In addition, using PA20-30 extracts, we can easily distinguish individual astral microtubules, well expanded on coverslips against a very low fluorescence background, in contrast to the high fluorescence background in the experiment using purified tubulin, which made individual microtubules obscure. This advantage of PA20-30 extracts may be due to the existence of high concentrations of proteins other than tubulin, and motor proteins which ensure adhesion of the astral microtubules to coverslips.

After the incubation, excess extracts were very carefully removed by micropipet. The coverslips were slowly immersed in PEM1 containing 0.1% glutaraldehyde, and were incubated for 3 min at room temperature. After adequately removing the fixative, the coverslips were then postfixed in ~20°C methanol for 5 min. These steps were performed very carefully so as not to form aggregates of proteins in the extracts on coverslips and also not to damage microtubules. Fixed microtubules were then treated with freshly prepared 1 mg/ml NaBO3 in PBS (20 mM phosphate/KOH, 150 mM NaCl, pH 7.4) for 5 min at room temperature. Immunofluorescence labeling was performed with a monoclonal anti-α tubulin antibody (1/200 dilution, Amersham) and a human anti-centrosome antiserum (1/100 dilution, a gift from Dr. Y. Moroi (Moroi et al., 1983) as described in elsewhere (Ohita et al., 1988). Secondary antibodies used were a rhodamine-labeled goat anti-mouse IgG (1/300 dilution) and a fluorescein-labeled goat anti-human IgG antibody (1/100). Samples were mounted in Mowiol (Hoechst) and observed with a Nikon FX microscope. Pictures were taken on Kodak TriX film. Microtubule length was measured on prints by a digital curvimeter type “D” (Uchida Yohkoh, Tokyo, Japan). Microtubule numbers were counted under the microscope or on negatives enlarged by a low magnification binocular microscope. Growing microtubules at room temperature were directly observed under dark field illumination using a Nikon Optiphot microscope equipped with an oil immersion condenser lens and a mercury arc lamp.

**Preparation of KCl-treated centrosomes**

KCl-treated centrosomes were prepared as described by Klotz et al. (1990). 50 µl of the isolated L5178Y or CHO centrosome preparation (107/ml in 50% sucrose) were diluted with 50 µl of PEM2 (100 mM PIPES, 0.5 mM MgCl2, 1 mM EGTA, pH 6.8) containing 2 M KCl, and were incubated for 30 min at 0°C. The treated centrosomes were dialyzed against PEM2 for 1 h at 2°C. For the “extracts exchange assay”, we added 1 µl of the KCl-treated centrosomes into 15 µl of extracts, and incubated as described above.

**Electron microscopy**

CHO centrosomes (10 µl) were incubated in 90 µl of extracts for 7 and 30 min at 20°C in 1.5 ml microcentrifuge tubes. After the incubation, the centrosomes were fixed by adding 900 µl of PEM1 containing 0.25% glutaraldehyde and incubated for 5 min at room temperature. The fixed centrosomes were sedimented by a microcentrifuge at 30,000 g for 20 min. The precipitates were washed twice in PEM3 (20 mM PIPES, 0.5 mM MgCl2 and 1 mM EGTA, pH 6.8), and were postfixed with 1% OsO4 for 30 min. The precipitates were removed and dehydrated in ethanol and embedded in Rigolac mixture as described elsewhere (Endo, 1980). Thin sections (80 nm-thick) were stained with uranyl acetate and lead citrate, and were observed with a Hitachi H-7000 electron microscope.

**Detection of increase in PCM size by anti-γ-tubulin antibody and 5051 human anti-centrosome antibody**

2 µl of CHO centrosome preparation (107/ml) were added to 20 µl of extracts, and the mixture was incubated at 20°C. Then 88 µl of the extraction buffer, without cytochalasin B and DTT but containing 20 µM Nocodazole, were added to the reaction mixture. After incubating at 0°C for 30 min, the mixture was combined with 1.4 µl of 8% glutaraldehyde (EM grade, final 0.1%), and was incubated for 10 min at 20°C. Fixed samples were overlaid on 3 ml cushions of PEM3 containing 25% glycerol, and were
centrifuged onto round coverslips by a RPS40T-2 rotor (Hitachi) at 12,000 g for 15 min according to the method described by Mitchison and Kirschner (1984). Samples on coverslips were post-fixed in methanol at −20°C for 5 min, treated in PBS containing 1 mg/ml NaBO₃ for 3 min, and processed for immunofluorescence labeling by affinity-purified, rabbit anti-Schizosaccharomyces pombe γ-tubulin antibody (1:10 dilution) and 5051 human anti-centrosome antibody (1:200) (these antibodies were a gift from Dr. Masuda, The Institute of Physical & Chemical Research (RIKEN)). The anti-S. pombe γ-tubulin antibody used cross-reacts with γ-tubulin of frog and centrosomes from Chinese hamster and human cells. The secondary antibodies used were a fluorescein-labeled goat anti-human (1:100) and a rhodamine-labeled goat anti-rabbit (1:300). For comparison, we used the same exposure time for negatives and for prints in all cases.

**Kinases**

p34<sup>cdc2</sup>/cyclin B was purified from starfish maturing oocytes using a suc1-conjugated Sepharose column described elsewhere (Brizuela et al., 1987; Labbé et al., 1989) with some modifications: this preparation contains cyclin B (Labbé et al., 1989; Tachibana et al., 1990), but not cyclin A (E. Okumura and T. Kishimoto, unpublished data). M phase-activated MAP kinase from Xenopus eggs was purified as described (Gotto et al., 1991a). We added 1.5 µl of purified p34<sup>cdc2</sup>/cyclin B (20.1 pmole phosphate transferred to histones/µl min<sup>−1</sup>) or purified MAP kinase (16 µg/ml, 5.2 pmole phosphate transferred to myelin basic protein/µl min<sup>−1</sup>) into 15 µl of interphase extracts with the centrosomes. Protein concentration was determined by the method of Bradford (1976) using γ-globulin as a standard. The effect of Stauroporine on kinase activity was examined as follows. Stauroporine (Kyowa Hakko) and/or 2 µl histone (Sigma, type III-S, 12 mg/ml) were added to 6.5 µl of interphase extracts containing 0.33 (−histone) or 1 (+ histone) µCi/µl of (γ-<sup>32</sup>P)ATP, and the mixture was incubated for 40 min (−histone) or 30 min (+ histone) at 20°C. The reaction was stopped by adding 7 µl of SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE followed by autoradiography (without addition of histone) or by counting Cherenkov radiation of <sup>32</sup>P in the histone H<sub>1</sub> bands. Histone kinase activity of the extracts was measured as described (Félix et al., 1989).

**RESULTS**

**Activation in microtubule nucleation from the centrosome in interphasic egg extracts**

We found that the microtubule number per centrosome was increasing dramatically during the incubation of isolated mammalian centrosomes in interphasic extracts prepared 20-30 min after electric activation of CSF-arrested Xenopus eggs. We call this type of extract “PA20-30 (post-activation 20-30 min) extract”. Fig. 1 shows the increase in number of astral microtubules during the incubation of isolated CHO centrosomes in PA20-30 extracts. We confirmed that each aster includes the centrosome, detected by a human autoimmune antisem against the centrosome from a scleroderma patient (Fig. 1D). Microtubule number per centrosome was increased from 53.4 ± 4.3 (s.e.) (6 min, n = 70) up to more than 200 (~270) (20 min, n = 70) during the incubation in the PA20-30 extracts. We also confirmed a similar increase in number of microtubules from a single centrosome in PA20-30 extracts under a real-time observation by dark field microscopy. Though the increase in microtubule number from the centrosome took place in vitro, it can be said to be reasonable, since in this period eggs are at the stage during which the sperm aster is growing and enlarging from the sperm centrioles which cannot form astral microtubules before their entry into the egg. Fig. 1E indicates time courses of microtubule number per centrosome and mean length of microtubules from centrosomes during the incubation. While microtubule number per centrosome was markedly increasing with time in PA20-30 extracts, mean microtubule length was generally constant after 5 min incubation in the same extracts, ruling out the possibility that the conditions of the extracts were becoming more advantageous for microtubule formation during the incubation.

Using centrosomes stripped of their microtubule nucleating activity by 1 M KCl treatment, we verified that the increase in microtubule number per centrosome is due to the addition of new microtubule nucleation sites on the centrosome. Klotz et al. (1990) reported that treatment of the centrosomes with high concentration of KCl or NaCl destroys the microtubule nucleating activity of PCM. We checked again that no microtubule could be formed from the 1 M KCl-treated CHO centrosomes in purified tubulin at 37°C. However, when we incubated KCl-treated centrosomes in PA20-30 extracts at 22°C, the microtubule nucleating activity recovered and increased rapidly after a 5 min lag (Fig. 2A). These observations suggest that the increase in microtubule number per centrosome in the extracts is due to the addition of new microtubule nucleation sites (activation of microtubule nucleation potential) on centrosomes.

**Increase in size of the centrosome and PCM in interphasic egg extracts**

To study whether or not centrosomes show qualitative or quantitative changes during the activation of microtubule nucleation, we examined the changes in signal intensity of the centrosome labeled by two centrosome markers: anti-S. pombe γ-tubulin antibody which cross-reacts with Xenopus γ-tubulin and mammalian centrosomes, and 5051 human anti-centrosome autoantibody from a scleroderma patient (Calarco-Gillam et al., 1983). γ-tubulin is a third member of the tubulin superfamily, and is a common component of spindle pole bodies of Aspergillus nidulans (Oakley and Oakley, 1989; Oakley et al., 1990) and S. pombe (Horio et al., 1991), and of centrosomes in Xenopus, Drosophila and mammalian cells (Zheng et al., 1991; Stearns et al., 1991). γ-tubulin and the antigen of the 5051 antibody are shown to be components of PCM of mammalian centrosomes (Zheng et al., 1991; Stearns et al., 1991; Calarco-Gillam et al., 1983). Based on genetic and immunochemical evidence, γ-tubulin was suggested to be involved in microtubule nucleation on the spindle pole body and centrosomes (Oakley et al., 1990; Joshi et al., 1992). Anti-γ-tubulin (Fig. 3B, E and I) and 5051 anti-centrosome antibody (Fig. 3A, D, G and H) specifically reacted with CHO centrosomes which had been treated for 0 min (A,B) and 25 min (D,E) in PA20-30 extracts. After 25 min incubation in PA20-30 extracts, signals of centrosomes became stronger (compare A, B with D, E). The size of the centrosome signal after the treatment is roughly estimated to be about twice as large (2 µm) as that without incuba-
Activation of centrosomes in vitro

Interestingly, the enhancement of signal intensity was also detected even when we added 20 µM of Nocodazole to PA20-30 extracts (Fig. 3G), indicating that the accumulation is not due to a retrograde transport of materials along the astral microtubules. We confirmed that few signals by 50S1 anti-centrosome (Fig. 3H) and anti-γ-tubulin antibody (Fig. 3I) can be seen without adding centrosomes to PA20-30 extracts.

To examine the change in the fine structure of centrosomes during the incubation, we observed the centrosomes treated in PA20-30 extracts by electron microscopy. We found that incubation of CHO centrosomes with PA20-30 caused a marked increase in the amount of PCM around the centrioles (Fig. 4). After 30 min incubation (Fig. 4B,D), PCM had an average thickness of 127±29 nm (s.d., average of values on 8 well-defined centrioles in more than 200 random sections), having a value about double that after 7 min (56±24 nm, s.d., 4 centrioles) (Fig. 4A,C).

**Fig. 1.** Time-dependent increase of microtubules from the centrosomes in PA20-30 egg extracts. Microtubules were elongated from the isolated CHO centrosomes in PA20-30 extracts for 6 min (A) and 25 min (B,C and D) and visualized by an anti-tubulin antibody. (C and D) indicate a double labeling of regrown asters from the centrosome by anti-tubulin antibody (C) and human anti-centrosome antiserum (D) immunofluorescence labeling, as described in Materials and methods. Bars, 10 µm. (E) indicates time course of microtubule number per centrosome and mean length of microtubules from centrosome during the incubation of the centrosomes in PA20-30 extracts. The centrosomes were added to PA20-30 extracts, and incubated for the indicated time on coverslips to form microtubules. Microtubules were visualized by anti-tubulin immunofluorescence labeling. Microtubule (MT) numbers per centrosome (more than 25 centrosomes) were counted and the lengths of microtubules from the centrosomes (more than 100 microtubules) were measured. The data are plotted versus incubation time. Vertical bars in each time point indicate standard errors (= s.d./√n) of measurements.

**Activation of microtubule nucleating potential of centrosomes in egg extracts is controlled by protein phosphorylation**

We next studied the relationship between protein phosphorylation and the activation of microtubule nucleating potential of the centrosome, because many lines of evidence have suggested that phosphorylation is involved in the regulation of the centrosomal function. We examined the effect of protein kinase inhibitors on the increase in microtubule nucleating potential of KCl-treated L5178Y centrosomes. PA20-30 extracts had detectable kinase activities including...
low histone H1 kinase activity (1-4 pmole/µl min⁻¹). Stau-
rosporine (Tamaoki et al., 1986) and K252a (Kase et al.,
1987), first introduced as strong inhibitors for protein kinase
C, inhibit various other kinases at higher concentrations.
When increasing concentrations of Staurosporine or K252a
were added to PA20-30 extracts containing KCl-treated
centrosomes, the increase in microtubule nucleating poten-
tial of the KCI-treated centrosomes was inhibited in a dose-
dependent manner (Fig. 5A). Fig. 5B indicates that Stauro-
sporine actually inhibited bulk protein phosphorylation in
PA20-30 extracts in roughly the same dose-dependent
manner. Phosphate transferred to exogenous histone H1 in
the PA20-30 extracts decreased in the presence of 3 µM
Staurosporine to 36% of the control without Staurosporine.
Numerous free microtubules were formed in the presence
of inhibitors (data not shown), ruling out the possibility that
the kinase inhibitors blocked microtubule assembly in
PA20-30 extracts. Furthermore, addition of these kinase
inhibitors did not block the microtubule nucleation from
intact centrosomes in PA20-30 extracts, although it pre-
vented the time-dependent increase in microtubule number
per centrosome.

Activation of microtubule nucleating potential of
centrosomes in egg extracts is controlled in a cell
cycle-dependent manner
We examined the cell-cycle dependence of the ability of
extracts to increase the microtubule nucleating potential of
KCI-treated centrosomes. The number of microtubules
nucleated from a centrosome depends on both microtubule
nucleating potential (i.e. number of microtubule nucleation
sites) of the centrosome and the environmental conditions
for microtubule formation in solution. In fact, microtubule
formation is suppressed in mitotic extracts, but not in inter-
phasic extracts (Karsenti et al., 1984; Verde et al., 1990).
This point will result in a misleading underestimate of the
ability of mitotic extracts.

In order to compare accurately the intrinsic ability of

Fig. 2. Recovery and increase of microtubule nucleation from the KCl-
treated centrosomes in PA20-30 extracts. Centrosomes treated with 1
M KCI have no microtubule nucleating activity in purified tubulin in
vitro. KCI-treated CHO centrosomes were added to PA20-30 extracts,
and incubated for the indicated times on coverslips. Microtubules were
visualized by anti-tubulin immunofluorescence labelings (left panel).
Bar, 10 µm. Microtubule numbers per centosome (more than 25
centrosomes) were counted and plotted versus incubation time (graph).
Filled circles, native CHO centrosomes (partially the same data as in
Fig. 1); filled triangles, KCl-treated centrosomes. Bars indicate
standard errors.
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Interphasic and mitotic extracts to increase microtubule nucleating potential, we have developed an ‘extract exchange assay’ (Fig. 6). In this assay, we first incubated KCl-treated centrosomes in PA20-30 interphasic extracts or mitotic extracts, followed by a removal of the extracts and washing with a buffer solution. Then, to form microtubules under the same polymerization conditions, we incubated them in purified tubulin (Fig. 7E,F) or fresh PA20-30 extracts (Figs 6, 7A-D). Both experiments using purified tubulin and PA20-30 extracts gave similar results: mitotic extracts have a greater ability to activate microtubule nucleating potential from centrosomes than interphasic extracts (Fig. 7). Despite its simplicity, the experiment using purified tubulin was not good for quantitative analysis (e.g., counting the number of nucleated microtubules) mainly because of a high fluorescence background due to non-specific adsorption of purified tubulin to coverslips and also the tendency of asters to detach from coverslips during a rather long incubation (20 min instead of 1-2 min in PA20-30 extracts). Since the use of PA20-30 extracts for the second incubation can overcome these difficulties (see Materials and methods), and give similar results to those obtained with purified tubulin, we employed an ‘extracts exchange assay’ using PA20-30 extracts in the following experiment.

Fig. 3. Increase in size of centrosomes in egg extracts. CHO centrosomes were treated in egg extracts. Astral microtubules from centrosomes were then depolymerized in a buffer solution containing 20 µM of Nocodazole at 0°C for 30 min, and processed for indirect immunofluorescence labeling by two PCM markers: rabbit anti-γ-tubulin antibody and 5051 human anti-centrosome antibody, as described in Materials and methods. (A, D, G and H) 5051 anti-centrosome antibody; (B, C, E, F and I) anti-γ-tubulin. (A-C) treatment for 0 min in extracts; (D-I) for 25 min. (A, B, D, E, G, H and I) treatment in PA20-30 interphasic extracts; (C and F) treatment in CSF-arrested mitotic extracts; (G) treatment in PA20-30 interphasic extracts containing 20 µM Nocodazole. (H and I) extracts incubated in the absence of centrosomes. For comparison, we used the same exposure time for negatives and for prints in all cases. A and D are the same field of double labeling as B and E, respectively. Arrowheads indicate some of centrosomes. Arrows indicate duplicated centrosomes (adjacent double spots). Bar, 10 µm.

Fig. 4. Increase of PCM around the centrosome in egg extracts. Microtubules were formed from the added centrosomes in PA20-30 extracts (A-D) or CSF-arrested mitotic extracts (E and F), and the asters were sedimented and embedded. Sections (200-> 500 sections in each case) were observed by electron microscopy. Typical cross sections (A, B, E and F) and longitudinal sections (C and D) of the centrosomes are shown. PCM is defined as electron dense material around the centriolar cylinders. (A, C and E) 7 min after incubation; (B, D and F) 30 min after incubation. Arrowheads indicate the sites where microtubules attached to the PCM. Magnification of C and D is smaller than A, B, E and F. Bars, 100 nm.
Fig. 7 indicates the results of the extract exchange assay for cell-cycle dependence in the ability of the extracts to activate KCl-treated L5178Y centrosomes. Centrosomes preincubated in mitotic extracts (Fig. 7C,D) nucleated many more microtubules after the extracts exchange than centrosomes preincubated in PA20-30 extracts (Fig. 7A,B). Mean microtubule number per centrosome in mitotic extracts was roughly estimated to be 3.5-fold (118.4 ± 15.6: 33.8 ± 3.9, n = 25) after 5 min and about 5-fold (127 ± 11.9: ~600, n = 25) after 25 min, higher than that in PA20-30 extracts. The histone H1 kinase activity of mitotic extracts (14.9 pmole/µl min−1) was about 5-fold higher than that of PA20-30(55x139)\textit{pmole/µl min}^{-1}20-30 extracts. The quantitative difference in the ability of both types of extract to activate microtubule nucleation is generally in good agreement with the difference in microtubule nucleating activity in vitro between interphasic and mitotic centrosomes from CHO cells (Kuriyama and Borisy, 1981).

We also examined the accumulation of PCM in mitotic extracts by electron microscopy and immunofluorescence staining by anti-γ-tubulin and 5051 anti-centrosome antibodies. Accumulation of PCM in mitotic extracts is supported by the results of both the immunofluorescence (Fig. 3C,F) and the electron microscopy (Fig. 4E,F). Interestingly, the increase in signal intensity of centrosomes labeled by anti-γ-tubulin and 5051 anti-centrosome antibodies seemed to occur equally in both PA20-30 interphase extracts and CSF-arrested mitotic extracts (compare Fig. 3E,F). In addition, from the results of electron microscopy, it seems that there is little difference in the amount of PCM in mitotic extracts and in PA20-30 extracts after 30 min incubation. After 25 min in mitotic extracts, PCM had an average thickness of 131 ± 40 nm (s.d., average of values on 50 well-defined centrioles in more than 500 random sec-
Activation of centrosomes in vitro

Addition of p34^{cdc2}/cyclin B to PA20-30 extracts promotes the activation of microtubule nucleating potential of centrosomes

The results in Figs 5 and 7 suggest that the ability of the extracts to increase microtubule nucleating potential of the centrosome is under the control of phosphorylation mediated by M phase-activated kinases. We then added two major M phase-activated kinases, p34^{cdc2}/cyclin B and MAP kinase, to PA20-30 extracts, and examined the ability of the extracts to activate KCl-treated L5178Y centrosomes, by using the extracts exchange assay described above. MAP kinase is an M phase-activated kinase in the downstream of p34^{cdc2}/cyclin B (Gotoh et al., 1991b), and was suggested to be involved in the conversion of microtubule dynamics at the onset of M phase (Gotoh et al., 1991a) as well as p34^{cdc2}/cyclin B (Verde et al., 1990). We found that addition of purified p34^{cdc2}/cyclin B to PA20-30 extracts caused marked promotion of the ability of the extracts to increase microtubule nucleating potential; the microtubule number per centrosome was more than 200 (239±18.4, n = 25), in contrast to a control with the buffer solution alone (45.6±2.7, n = 25) (Fig. 8A,B), while MAP kinase had little effect (80.8±7.9, n = 30, Fig. 8D), compared with buffer solution alone (102.9±10, n = 30). The ability of p34^{cdc2}/cyclin B and the basal activity of PA20-30 extracts to increase microtubule nucleating potential of KCl-treated centrosomes was almost totally inhibited by 5 µM Staurosporine (Fig. 8C). When we added p34^{cdc2}/cyclin B or MAP kinase to a simple reconstitution system using purified tubulin and isolated centrosomes in the presence of ATP, little effect could be detected (data not shown). These results suggest that phosphorylation, presumably mediated by an indirect action of p34^{cdc2}/cyclin B but not by MAP kinase, is involved in the increase of microtubule nucleating potential of centrosomes in Xenopus mitotic extracts.

DISCUSSION

Increase in microtubule nucleation sites of centrosomes in egg extracts

We found that the microtubule nucleating activity of isolated centrosomes is activated with time of incubation in Xenopus egg extracts. Most likely, the increase in microtubule nucleating activity is due to an increase in microtubule nucleating sites. Mean length of microtubules from

Fig. 7. Cell cycle dependence of the activation of centrosomes. Cell cycle dependence of the ability of the extracts to increase the microtubule nucleating potential of KCl-treated centrosomes was analyzed by the "extract exchange assay" described in Fig. 6. KCl-treated centrosomes were pre-incubated in PA20-30 interphase extracts (A and B) or CSF-arrested mitotic extracts (C and D), for 5 min (A and C) or 25 min (B and D). They were then washed with a buffer solution and incubated in PA20-30 extracts for 2 minutes at 20°C. Arrowheads indicate the centrosomes. (E and F) indicate the results of an experiment using purified tubulin for the second incubation, performed as described in Materials and methods. (E) pre-incubation in PA20-30 interphase extracts for 25 min; (F) in CSF-arrested mitotic extracts for 25 min. Bar, 10 µm.
the centrosomes in PA20-30 extracts was generally constant during the incubation (Fig. 1E). This rules out the possibility that improvement of the conditions for microtubule growth in the extracts caused an apparent increase in microtubule number per centrosome. The isolated centrosomes used here can nucleate 33.7 ± 1.5 (s.e., n = 80) microtubules in a simple system with purified brain tubulin even in the presence of brain MAP2 promoting microtubule assembly (Hoshi et al., 1992). Therefore, it is implausible that more than 200 microtubules could be formed from a single centrosome used here without increasing microtubule nucleation sites. In addition, the extracts were able to restore microtubule nucleating activity of centrosomes stripped of nucleating activity by KCl treatment. Increase in microtubule nucleation sites is also reinforced by the increase of PCM revealed by immunostaining with anti-γ-tubulin and 5051 anti-centrosome antibodies, and by electron microscopy.

**Activation of microtubule nucleating potential by both accumulation of PCM and phosphorylation**

From in vitro results, we can speculate that the microtubule nucleating potential of the centrosome is activated during the cell cycle through at least two different pathways: (1) accumulation of PCM to centrosomes and (2) phosphorylation of centrosomal materials. Correlation between the time-dependent increase in microtubule nucleation and accumulation of PCM in vitro suggests that the assembly of PCM around the centrioles from the cytoplasm plays an important role in the regulation of microtubule nucleating potential of the centrosome. Presumably, accumulation of PCM around the centrioles plays a role in the activation of the centrosome during M phase in vivo. Rieder and Borisy (1982) have shown that PCM of PtK2 cells increases in accordance with the increase in microtubule nucleating activity of the centrosome in the G2/M transition. Endo (1980) has shown that the microtubule-organizing granules (PCM in sea urchin eggs) accumulate at the poles in a mitotic cycle-dependent manner. However, from our results, the kinetics of accumulation of PCM seem similar in both type of extracts (Figs 3E,F and 4B,F). Possibly, the accumulation of PCM in egg extracts in vitro may not necessarily be controlled in a cell cycle-dependent manner. However, this issue should be examined further by other more quantitative assay systems.

The results also imply that centrosomes are activated during M phase through phosphorylation of centrosomal components. Addition of protein kinase inhibitors into interphase extracts and extracts driven into a mitotic state by the addition of p34cdc2/cyclin B totally blocked the recovery and increase in microtubule nucleating potential of KCl-treated centrosomes. On the other hand, addition of active p34cdc2/cyclin B kinase into interphase extracts promoted the increase in microtubule nucleation from KCl-treated centrosomes as described below. These are consistent with previous reports. Protein phosphorylation is activated during M phase in *Xenopus* eggs (Maller et al., 1977; Karsenti et al., 1987). Monoclonal antibodies to M phase-specific phosphoproteins recognize the mitotic centrosome (Vandré et al., 1984; Kuriyama, 1989), having maximal activity during the cell cycle (Kuriyama and Borisy, 1981). There should be at least two possibilities for the process regulated by M phase-activated phosphorylation: (1) tubulin interaction on centrosomes and (2) accumulation of PCM to the centrosome. If the former possibility is true, phosphorylated microtubule nucleating material on centrosomes is activated to bind tubulin stronger. The latter requires activation of putative PCM-accumulating factor, and is not necessarily supported by our results, since many more microtubules seem to emanate from the PCM in mitotic extracts than in the interphase extracts while there seems to be little difference in the amount of PCM in both

**Fig. 8. Effects of purified p34cdc2/cyclin B and MAP kinase added to PA20-30 extracts on the activation of KCl-treated centrosomes.** Purified p34cdc2/cyclin B or MAP kinase was added to PA20-30 interphase extracts, and the increase in microtubule nucleating activity of KCl-treated centrosomes in the extracts was analyzed using the "extract exchange assay" as described in Fig. 6. For the experiment using purified p34cdc2/cyclin B, PA20-30 extracts containing purified p34cdc2/cyclin B were pre-incubated for 15 min at 20°C. For the experiment using purified MAP kinase, we added MAP kinase and KCl-treated centrosomes at the same time, because prolonged incubation causes a decrease of MAP kinase activity in PA20-30 extracts. Histone H1 kinase activity of PA20-30 extracts with purified p34cdc2/cyclin B (8.1 pmole/µl min⁻¹), was 2.8-fold higher than that of PA20-30 extracts without purified p34cdc2/cyclin B. For the buffer control, the same volume of the buffer solution used for the preparation of p34cdc2/cyclin B or MAP kinase was added to PA20-30 extracts. (A) buffer control; (B) + p34cdc2/cyclin B; (C) + p34cdc2/cyclin B + 5 µM Staurosporine; (D) + MAP kinase. Bar, 10 µm.
The centrosome during M phase, but MAP kinase is not. Opposite effects caused by M phase-activated phosphorylation on microtubule stability and microtubule nucleating sites on the centrosome. M phase-activated protein phosphorylation (probably mediated by MAP kinase and p34\(^{cd2/cyclin}\) B) decreases stability of microtubules. On the other hand, it (probably mediated by p34\(^{cd2/cyclin}\) B) activates the increase of microtubule nucleation sites on the centrosome. These opposite effects of microtubule stability and microtubule nucleation on the centrosome result in the highly centrosome-dependent microtubule formation during M phase, which is a preferential site for microtubule nucleation in animal cells. It is also suggested that p34\(^{cd2/cyclin}\)s are responsible for the regulation of microtubule nucleation sites on the centrosome during M phase, but MAP kinase is not.

The proposed activation mechanism of the centrosomal activity by M phase-activated phosphorylation seems rather self-contradictory, because phosphorylation has been shown to suppress microtubule assembly during M phase (Verde et al., 1990; Gotoh et al., 1991a). However, these apparently opposite effects of phosphorylation reactions can explain well the fact that in vivo the microtubule organization in mitotic phase is highly dependent on the centrosomes, which are preferential sites for microtubule nucleation (see Fig. 9). Activated centrosomes during M phase are supposed to be competent for the construction of numerous spindle microtubules if the chromatins are in the vicinity of the centrosome, since the existence of chromatin is necessary for the selective stabilization and the generation of half-spindle microtubules (Karsenti et al., 1984; Sawin and Mitchison, 1991).

**Role of p34\(^{cd2/cyclin}\) B complex in the activation of the centrosome**

We demonstrated that the increase in microtubule nucleating potential is strikingly enhanced by the addition of p34\(^{cd2/cyclin}\) B to interphasic extracts. This idea is consistent with previous data that the microtubule nucleating activity in vitro reaches a maximum at M phase (Kuriyama and Borisy, 1981), where the kinase activity of MPF (p34\(^{cd2/cyclin}\) B) is also maximal and p34\(^{cd2/cyclin}\) B is localized in the mitotic centrosome and spindle pole body (Vandre et al., 1984; Kuriyama, 1989; Riabowol et al., 1989; Bailly et al., 1989; Alfa et al., 1990). In addition, cyclin B associates with spindle pole body (Alfa et al., 1990) and with the polar region of mitotic spindles in *Drosophila* embryos, whereas cyclin A mainly associates with chromatins during M phase (Maldonado-Codina and Glover, 1992). In addition, most recent work by Masuda et al. (1992) revealed that interphase extracts from *Xenopus* eggs gained an ability to convert inactive, interphase spindle pole body of fission yeast to a competent state for microtubule nucleation when the interphase extracts were incubated with \(\Delta\)90 sea urchin cyclin B1. However, purified p34\(^{cd2/cyclin}\) B did not directly activate microtubule nucleation from centrosomes in a purified in vitro system (data not shown). Therefore, centrosomes should be activated through a downstream pathway regulated by p34\(^{cd2/cyclin}\) B, as claimed by Masuda et al. (1992).

In contrast to our results, Belmont et al. (1990) reported that there was no increase in microtubule number from centrosomes incubated in extracts driven into M phase by the addition of \(\Delta\)90 sea urchin cyclin B1 into interphase extracts prepared by a complete destruction of cyclins. In addition, Buendia et al. (1992) have claimed that addition of bacterially produced cyclin A into cyclin-depleted interphase extracts caused approximately a 1.8-fold increase in the microtubule nucleating activity of the centrosomes complemented in the extracts, whereas the addition of \(\Delta\)90 sea urchin cyclin B1 or purified starfish p34\(^{cd2/cyclin}\) B complex did not, although they had mentioned previously that the addition of p34\(^{cd2/cyclin}\) B in interphase extracts from electrically activated *Xenopus* eggs brought about a promotion of apparent microtubule nucleating activity of the centrosome (Verde et al. 1990). They concluded that a cyclin B-dependent phosphorylation of centrosomal antigens may not be essential in the activation processes of the centrosome during M phase.

The reason for the discrepancy is not clear at present. It is possible that the preparation of interphasic extracts is of prime importance in the activation assay of the centrosome by p34\(^{cd2/cyclin}\) B. Judging from the time when we prepared the PA20-30 extracts (20-30 min after the electrical activation), our PA20-30 extracts could have trace amounts of cyclins. However, the interphase extracts prepared by Belmont et al. (1990) and Buendia et al. (1992) lack cyclin A and B completely after the destruction of cyclins and inhibition of protein synthesis by cycloheximide. So it is possible that cyclin-depleted interphase extracts are not competent for the full activation of microtubule nucleating activity of the centrosome by p34\(^{cd2/cyclin}\) B. And it is also possible that p34\(^{cd2/cyclin}\) B can cause full activation of the centrosome only with the action of cyclin A-associated kinase in cell-free extracts. This point should be examined by adding cyclin A and B together in the cyclin-depleted interphase extracts.

In contrast to the p34\(^{cd2/cyclin}\) B, the addition of MAP kinase, an M phase-activated kinase downstream of p34\(^{cd2/cyclin}\) B (Gotoh et al., 1991b), had little effect on the ability of interphase extracts to recover and increase microtubule nucleating potential of the centrosome. Therefore, the pathway by MAP kinase only acts in converting microtubules to a dynamic state in the microtubule reorganization at G2/M transition.

The mechanism regulating microtubule nucleation of the


(Received 18 June 1992 - Accepted 29 September 1992)