Regulation of the Microtubule Nucleating Activity of Centrosomes in *Xenopus* Egg Extracts: Role of Cyclin A-Associated Protein Kinase

Brigitte Buendia,* G. Draetta, and Eric Karsenti

EMBL, Cell Biology and *Differentiation Programs, Meyerhofstrasse 1, D-6900 Heidelberg, Germany

Abstract. Isolated centrosomes nucleate microtubules when incubated in pure tubulin solutions well below the critical concentration for spontaneous polymer assembly (~15 μM instead of 60 μM). Treatment with urea (2–3 M) does not severely damage the centriole cylinders but inactivates their ability to nucleate microtubules even at high tubulin concentrations. Here we show that centrosomes inactivated by urea are functionally complemented in frog egg extracts. Centrosomes can then be reisolated on sucrose gradients and assayed in different concentrations of pure tubulin to quantify their nucleating activity. We show that the material that complements centrosomes is stored in a soluble form in the egg. Each frog egg contains enough material to complement >6,000 urea-inactivated centrosomes. The material is heat inactivated above 56°C. One can use this in vitro system to study how the microtubule nucleating activity of centrosomes is regulated. Native centrosomes require ~15 μM tubulin to begin nucleating microtubules, whereas centrosomes complemented in interphase extracts begin nucleating microtubules around 7–8 μM tubulin. Therefore, the critical tubulin concentration for polymer assembly off native centrosomes is higher than that observed for the centrosomes first denatured and then complemented in egg extracts. In vivo, the microtubule nucleating activity of centrosomes seems to be regulated by phosphorylation at the onset of mitosis (Centonze, V. E., and G. G. Borisy. 1990. *J. Cell Sci.* 95:405–411). Since cyclins are major regulators of mitosis, we tested the effect of adding bacterially produced cyclins to interphase egg extracts. Both cyclin A and B activate an H1 kinase in the extracts. Cyclin A-associated kinase causes an increase in the microtubule nucleating activity of centrosomes complemented in the extract but cyclin B does not. The critical tubulin concentration for polymer assembly off centrosomes complemented in cyclin A-treated extracts is similar to that observed for centrosomes complemented in interphase extracts. However, centrosomes complemented in cyclin A treated extracts nucleate much more microtubules at high tubulin concentration. We define this as the "capacity" of centrosomes to nucleate microtubules. It seems that the microtubule nucleating activity of centrosomes can be defined by two distinct parameters: (*a*) the critical tubulin concentration at which they begin to nucleate microtubules and (*b*) their capacity to nucleate microtubules at high tubulin concentrations, the latter being modulated by phosphorylation.

In animal cells, the cytoplasmic environment is usually such that spontaneous microtubule assembly is not possible (Karsenti et al., 1984*a*). Microtubule organizing centers (MTOCs) are required for microtubule growth, and this appears to be essential for the assembly of polarized and organized networks in the cell (Bergen et al., 1980; Bornens and Karsenti, 1984; Dane and Tucker, 1986; McIntosh, 1983; Mogensen and Tucker, 1987; Mogensen et al., 1989; Tucker, 1984). The shape and localization of MTOCs change with cell differentiation and cell cycle phases. In metazoa, the major MTOC is the centrosome, which is composed of a pair of centriole cylinders surrounded by a fibrogranular material (Gould and Borisy, 1977; Karsenti, 1991; Mazia, 1984). Microtubules are not nucleated directly by the centriole cylinders. They originate from the fibrogranular material that surrounds them. In growing cells, the nucleating activity of centrosomes increases several fold during mitosis (Alvey, 1985; Kuriyama and Borisy, 1981*a*,*b*; Vorobjev and Chentsov, 1982). This is particularly striking in epithelial cells (MDCK) maintained in culture in which the nucleating activity associated with the centrioles is very poor or nonexistent in interphase and becomes prominent in prophase when the duplicated centrosome migrate around the nucleus (Bré et al., 1990). Although the microtubule nucleating material is usually associated with centrioles in fibroblasts and motile cells, this is not always the case. Centrioles may be absent or, if present, microtubules may not originate from them or at least not exclusively (Bré et al.,

1987, 1990; Buendia et al., 1990*b*; Mogensen and Tucker, 1987; Mogensen et al., 1989; Tassin et al., 1985*a*). This is probably due to dissociation of the pericentriolar material and relocalization on other cellular structures. Inversely, in many species, the sperm centriole which acts as a basal body for the flagellum, acquires the capacity to nucleate radial microtubules following fertilization, probably through the binding of pericentriolar material stored in the egg (Kuriyama and Kanatani, 1981).

These observations raise several important questions that had been barely addressed. We do not know how centrosomes nucleate microtubules, although assays have been developed to study this question (Bré and Karsenti, 1990; Kuriyama, 1984; Kuriyama and Borisy, 1981*a*,*b*; Mitchison and Kirschner, 1984). Although we use the term "nucleating activity" to mean the ability of centrosomes to nucleate a certain number of microtubules, we do not know what happens at the molecular level and how this nucleating activity is regulated during cell differentiation and cell division.

An understanding of how the intrinsic microtubule nucleating activity of centrosomes is regulated will require the characterization of the proteins involved in microtubule nucleation and how they interact with the centrioles and with tubulin. Several centrosomal proteins have already been identified (Baron and Salisbury, 1988; Buendia et al., 1990*a*; Gosti-Testu et al., 1986; Kellogg et al., 1989; Kuriyama, 1989; Maekawa et al., 1991; Moudjou et al., 1991; Sager et al., 1986; Salisbury et al., 1986), some of them have been cloned and characterized (Joswig et al., 1991; Kuriyama et al., 1990; Ohta et al., 1990), including γ-tubulin (Horio et al., 1991; Stearns et al., 1991; Zheng et al., 1991). Available data suggest that the microtubule nucleating activity associated with centrioles may be regulated both by changes in the amount or quality of material present in the pericentriolar material (Sager et al., 1986) and by posttranslational modifications, in particular phosphorylation at the onset of mitosis (Centonze and Borisy, 1990; Kuriyama, 1989; Riabowol et al., 1989; Vandre et al., 1984, 1985). Since many structural events in mitosis are regulated by phosphorylation under the control of cdc2 kinase, this enzyme may be directly or indirectly involved in the phosphorylation of centrosomal proteins (Bailly et al., 1989; Riabowol et al., 1989) and modulation of microtubule nucleating activity.

In this article, we describe a functional assay to study the regulation of the interaction between the centriole cylinders and the pericentriolar material and the role of phosphorylation in microtubule nucleation. Purified centrosomes isolated from various cells can be used instead of the sperm to "fertilize" *Xenopus* eggs (parthenogenesis; Tournier et al., 1989). We had previously examined the structural requirements of the centrosome in parthenogenesis (Klotz et al., 1990) and this investigation suggested that centrosomes inactivated by 2 M urea could be complemented functionally by the egg cytoplasm. Here, we show that components stored in the egg (in a soluble form) can modify the microtubule nucleating activity of exogenous centrosomes. Moreover, we find that centrosomes incubated in extracts treated with cyclin A have the highest microtubule nucleating activity. This correlates with a large increase in the amount of phosphorylated epitopes present on the centrosomes. This work opens the way to a dissection of the regulation of the interaction between the microtubule nucleating material and centriole cylinders and to a better understanding of the molecular events involved in "microtubule nucleation."

Materials and Methods

Preparation of Frog Egg Extracts

Eggs were obtained and activated by an electric shock as previously described (Karsenti et al., 1984*b*), incubated at room temperature (20–24°C) for 40 min and transferred to 4°C. After washing in acetate buffer (100 mM K-Acetate, 2.5 mM Mg-Acetate, 1 mM DTT, 10 mM EGTA, 10 μg/ml cytochalasin D [pH 7.2]), they were crushed by centrifugation at 10,000 *g*. An ATP regenerating system (1 mM ATP, 10 mM creatine phosphate, and 0.08 mg/ml creatine phosphokinase) was added to the supernatant which was then recentrifuged either at 100,000 *g* for 1 h, at 250,000 *g* for 2 h, or at 250,000 *g* for 2 h followed by a further spin at 150,000 *g* in a TLA 100 rotor (see Félix et al., 1989, 1990; Verde et al., 1990).

Activation of cdc2 Kinase by Adding Cyclins to Egg Extracts

Human cyclin A was produced in a soluble form in bacteria and purified by conventional procedures. Δ90 sea urchin cyclin B was produced in bacteria but was mostly insoluble. The inclusion bodies were washed, solubilized in urea, and the protein was renatured as described by Glotzer et al. (1991). The cdc2 kinase or cdc2-like kinase is present in an inactive state in 100,000 *g* extracts prepared from eggs arrested in interphase by inhibition of protein synthesis with cycloheximide. This kinase was activated by adding the bacterially produced cyclins to the extracts for 15 min at room temperature before doing complementation experiments with urea-treated centrosomes. The activity of cdc2 kinase during the complementation assays was assayed by taking aliquots and testing their H1 kinase activity as previously described (Félix et al., 1989). The kinase activity is expressed in picomoles of phosphate transferred per min per microliter of extract.

Preparation of Centrosomes

Centrosomes were prepared from human lymphoid cells in culture as previously described (Bornens et al., 1987). The nucleating activity of the centrosomes was inactivated by treatment with 2 M urea (Klotz et al., 1990). 1 vol of centrosomes (~10^7/ml) was mixed with 1 vol of 4 M Urea prepared in 10 mM K-Pipes (pH 7.2) (see Fig. 1).

Reisolation of Centrosomes on Sucrose Gradients after Urea Treatment and Incubation in Egg Extracts

After urea treatment, the centrosomes were separated from urea by centrifugation through a 20% (wt/wt) sucrose layer and recovered on a 70% (wt/wt) sucrose cushion (centrifugation was carried out for 20 min at 100,000 *g* in a SW60 rotor at 4°C; Fig. 1). The sucrose solutions were prepared in 10 mM K-Pipes buffer (pH 7.2). The centrosomes recovered on the 70% sucrose cushion (75 μl) were then incubated with 50 μl of undiluted extract, the volume of the reaction mixture being adjusted to 150 μl by adding 25 μl of acetate buffer, under the various experimental conditions specified in the figure legends (see Fig. 1). The complemented centrosomes were then diluted with 1 vol of acetate buffer (100 mM potassium acetate, 2.5 mM magnesium acetate, 10 mM EGTA [pH 7.2]) and loaded on a discontinuous sucrose gradient made of 0.5 ml 30% sucrose containing 0.1% Triton and protease inhibitors (10 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 1 mM PMSF), 2.5 ml 40% sucrose (containing protease inhibitors) and 0.6 ml of 70% sucrose. The last two layers did not contain Triton. The gradient was centrifuged at 100,000 *g* for 20 min at 4°C in an SW60 rotor. For mini-assays, the complementation was done with 25 μl of centrosomes and variable amounts of extract (as indicated in the figures), the final volume being adjusted to 50 μl with acetate buffer. For phosphorylation experiments, (in presence or in absence of either cyclin A or B or DMAP), 20 μl of a 100,000 *g* supernatant was preincubated with the appropriate drug for 10 min at room temperature in presence of nocodazole. The centrosomes were then added and incubated for a further 40 min at room temperature, the concentration of the drugs being kept constant in a final volume of 50 μl. This was then diluted with 1 vol of acetate buffer before loading on a mini gradient (0.16 ml of 30% sucrose, 0.32 ml of 40% sucrose, and 0.1

ml of 70% sucrose). These gradients were also centrifuged in a SW60 rotor using special adaptors at 100,000 *g* for 20 min at 4°C. In both cases, centrosomes were recovered at the 40–70% interface.

Assay of the Microtubule Nucleating Activity of Centrosomes in Pure Calf Brain Tubulin Solutions

Pure bovine brain tubulin purified on phosphocellulose (Mitchison and Kirschner, 1984) was kept frozen in liquid nitrogen at a concentration of 5.5 mg/ml (~55 μM). Under these storage conditions, it remained stable for at least one year. 10–15 μl of centrosomes were mixed with tubulin (final concentration 15 μM) in a volume of 100 μl, using the RG1 buffer (80 mM K-Pipes, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, and 1 mM GTP). It is important to adjust precisely the final K-Pipes and $MgCl_2$ concentrations in order to get proper regrowth. The tubulin is stored in a different buffer (Mitchison and Kirschner, 1984). Microtubule regrowth assays were carried out at 37°C for 10 min and the microtubule asters were fixed with 0.7% (final concentration) glutaraldehyde (E.M. grade), spun on coverslips for 10 min at 12,000 RPM in a HB4 rotor in a Sorvall centrifuge, postfixed with cold methanol (−20°C, 5 min), and processed for immunofluorescence (Bré and Karsenti, 1990; Mitchison and Kirschner, 1984). Microtubules were labeled with monoclonal antibodies, anti-α-tubulin and anti-β-tubulin (1/500 final, Amersham Corp., Arlington Heights, IL). The centrioles were revealed with a human anticentrosome serum (1/3,000 final). The secondary antibodies used were a Texas Red-labeled goat antimouse (diluted 1/100 final) and a fluorescein-labeled goat antihuman (diluted 1/70 final) from Dianova (Hambourg).

Detection of Phosphorylated Epitopes on Centrosomes

10 μl of centrosomes were diluted in 5 ml of 10 mM K-Pipes, 1 mM EDTA (pH 7.2), and spun onto a coverslip at 4°C at 12,000 rpm for 10 min in a HB4 rotor. The centrosomes were fixed with methanol at −20°C for 5 min and double immunofluorescence was performed using a human anticentrosome antibody (dilution 1/3,000) and a mouse mAb that recognizes phosphorylated epitopes (MPM2, 1/500 dilution (Centonze and Borisy, 1990; Davis et al., 1983). The secondary antibodies used were a fluorescein-labeled goat antihuman (1/70) and a Texas red-labeled Goat antimouse (1/100).

Microtubule Number per Centrosome

Microtubule asters labeled with an antitubulin and an anticentrosome antibody were photographed using a Zeiss Axiophot microscope equipped with a 63× planapo lens (aperture 1.25) and microtubule number counted directly on the negatives using a magnifying lens. The mean number of microtubules per centrosome was calculated from measurements carried out on ~50 asters in each case. The data were ordered in 13 classes of 5 microtubule increment steps. The last class comprised asters with 60 microtubules/centrosome or more.

Electron Microscopy

For asters containing more than 50–60 microtubules per centrosome, quantitation was made on electron microscope pictures of negatively stained preparations. The centrosomes were incubated with tubulin for 5 min at 37°C, fixed with 1% glutaraldehyde, and the samples sedimented onto ionized, carbon-coated grids placed in 0.6 ml SW 50.1 tubes containing a small grid holder at the bottom. Centrifugation was done at 12,000 RPM for 10 min in a SW 50.1 rotor. After washing on drops of RG2 buffer (RG1-GTP), the grids were stained either with 1% phosphotungstic acid or with 1% uranyl acetate. The samples were observed in a 400 Philips electron microscope.

Measurement of ATP Concentration

We used the luciferase-luciferin assay (Sigma Chemical GmbH, Deisenhofen, FRG-ref:L-033). 10 μl of egg extract was precipitated with 0.5 ml of 7% TCA for 10 min on ice and spun for 10 min in an Eppendorf centrifuge at 4°C. 0.4 ml of unbuffered Tris (1 M) were added to the supernatant. The same amount of TCA/Tris was added to all samples including the ATP standard reactions. The reaction was started by adding 5 ml of buffer (30 mM sodium arsenate, 13 mM $MgCl_2$, 10 mM NaH_2PO_4 [pH 7.4]) to 50 μl of luciferase and 50 μl of TCA/Tris containing the sample (increasing amounts). After quick shaking, the photons emitted in the reaction mixture were counted immediately in an LS 8100 Beckman machine on "single photon count."

Miscellaneous

Nocodazole (Sigma Chemical GmbH, Deisenhofen, FRG) was kept as a stock solution in DMSO at −20°C and diluted in the assays to 20 μM. Apyrase (Sigma Chemical GmbH) was used at 2.5 U/ml (final concentration).

Results

Reconstitution of the Microtubule Nucleating Activity of Urea-inactivated Centrosomes in Egg Extracts

We had previously shown that centrosomes isolated from human lymphoid cells and treated with 2 M urea still induced

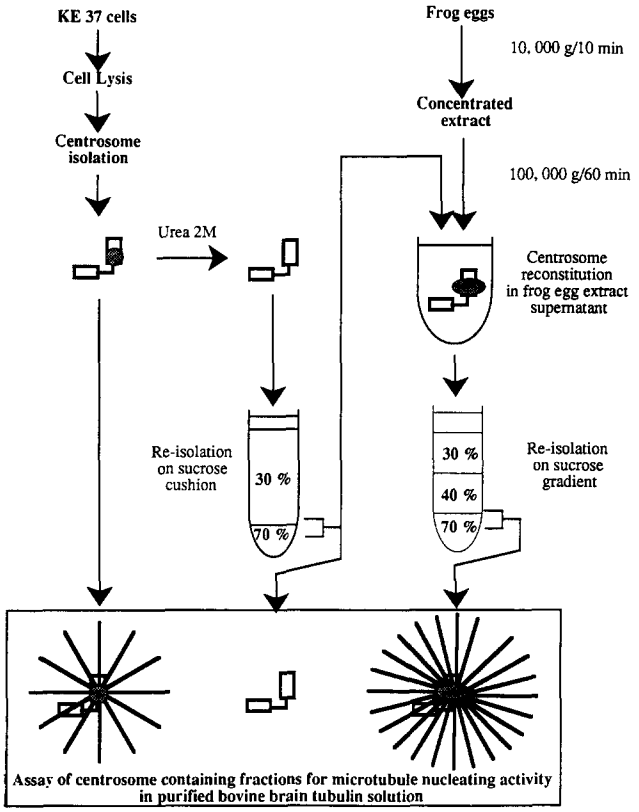

Figure 1. Complementation of inactivated centrosomes in Xenopus egg extracts and assay for microtubule nucleating activity. Centrosomes isolated from human lymphoid cells in culture (KE37 cells, left part of the figure) and treated with 2 M urea were centrifuged through 30% sucrose onto a 70% sucrose cushion (middle part of the figure) to remove the urea and extracted proteins. The 30–70% interface containing the cleaned inactivated centrosomes was then added to an egg extract (right part of the figure). After incubation, the centrosomes were again reisolated through a discontinuous sucrose gradient and the 40–70% interface collected. All centrosomes were assayed for microtubule nucleation by incubation in phosphocellulose-purified bovine brain tubulin devoid of MAPs. Centrosomes and microtubules were visualized by double immunofluorescence with antitubulin and anticentrosome antibodies. Centrioles are represented by open rectangles; microtubule nucleating material by a shaded circle on native centrosomes and a shaded oval on centrosomes complemented in egg extracts; microtubules are represented by thick black lines radiating from the nucleating material.

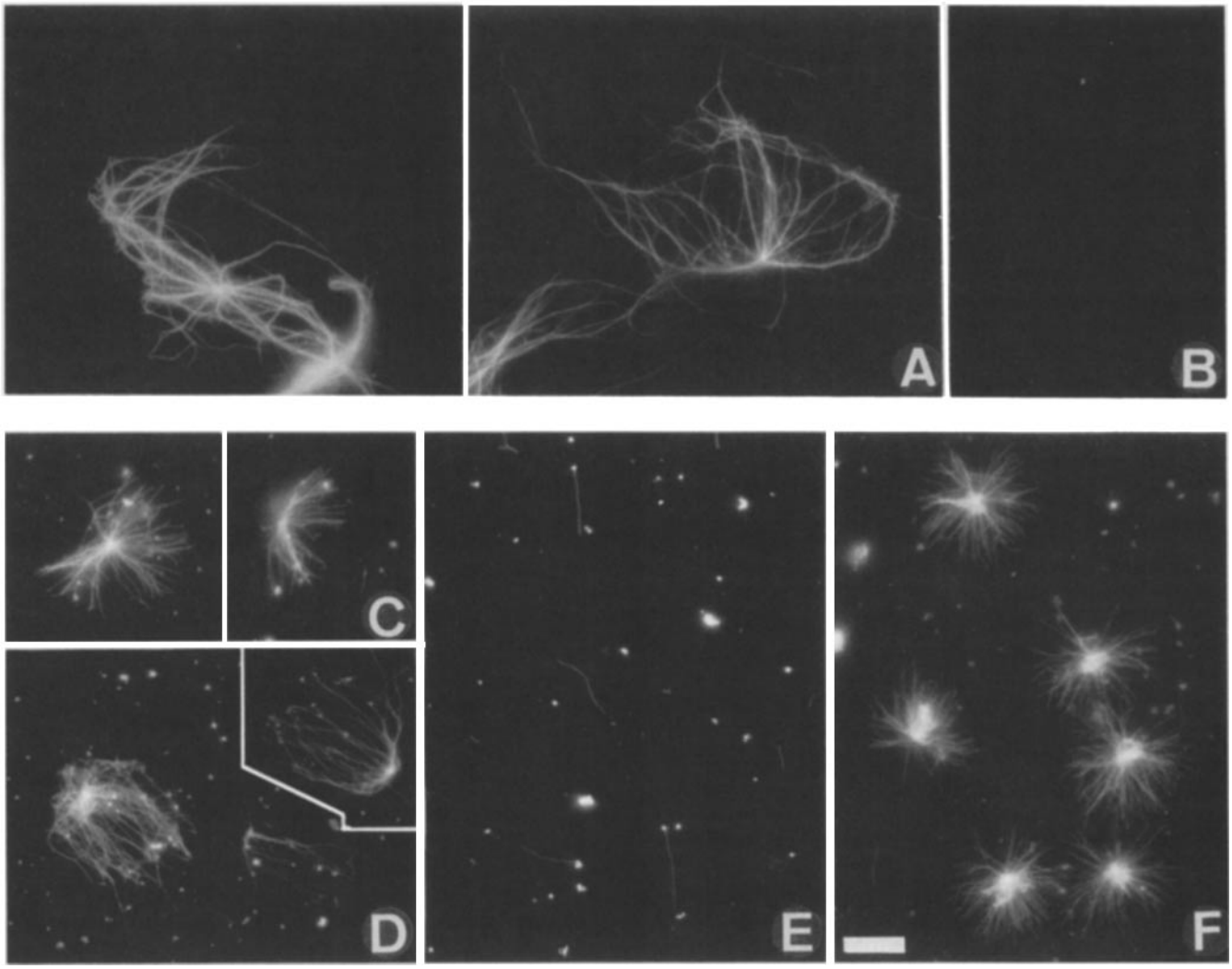

Figure 2. Microtubule nucleating activity of native, inactivated and inactivated centrosomes complemented in egg extracts. (*A*) Urea-treated centrosomes were added to a 250,000 *g* supernatant prepared from prophase eggs (40 min in the first cell cycle). The mixture was fixed by dilution in glutaraldehyde after 25 min of incubation at room temperature and processed for immunofluorescence as described in Materials and Methods. (*B*) Same experiment but in the presence of 20 μM nocodazole: microtubules are absent. (*C*) Native centrosomes were incubated in 20 μM tubulin or (*D*), in 40 μM tubulin for 10 min at 37°C, the samples fixed by dilution in glutaraldehyde and processed for immunofluorescence. (*E*) After urea treatment the same centrosomes did not nucleate microtubules in a solution of 30 μM tubulin. (*F*) Inactivated centrosomes preincubated in an egg extract and reisolated on a sucrose gradient recovered their activity and nucleated microtubules in a 15-μM tubulin solution. Bars, 10 μm.

cleavage when injected into frog eggs (Klotz et al., 1990). Since the elaboration of a mitotic spindle is a prerequisite for egg cleavages, these centrosomes must have been able to nucleate the frog egg endogenous tubulin although they had no microtubule nucleating activity in pure tubulin solutions (Klotz et al., 1990). This result suggested that the microtubule nucleating material was extracted from the centrosomes by treatment with urea and that frog eggs contained a similar material that could bind back to the injected centrosomes and reconstitute their nucleating activity. Given the fact that *Xenopus* egg extracts can support nuclear assembly on naked DNA (Newport, 1987) and even spindle assembly (Lohka and Maller, 1985), it seemed likely that the microtubule nucleating activity of urea-treated centrosomes (we will call these "inactivated centrosomes" throughout the text) could be reconstituted in such extracts. This was first attempted by mixing 1 vol of inactivated centrosomes prepared as described in Fig. 1 with an equal volume of egg extract (250,000 *g* supernatant prepared from eggs sampled at 40 min after activation by an electric shock). After 25 min of incubation at room temperature, the mixture was fixed by dilution in glutaraldehyde and centrifuged onto a coverslip. The presence of asters was searched for by immunofluorescence using an anti-tubulin antibody (Fig. 2). The inactivated centrosomes did not nucleate microtubule assembly in pure tubulin solutions (Fig. 2 *E*) but they did in *Xenopus* egg extracts (Fig. 2 *A*), as expected from the in vivo experiments (Klotz et al., 1990). No asters were found in the same extract incubated in the presence of centrosomes and 20 μM nocodazole, indicating that this drug efficiently blocked tubulin assembly in these extracts (Fig. 2 *B*). To examine whether the reconstitution of the microtubule nucleating activity of the centrosomes was stable, the complemented centrosomes were reisolated on a sucrose gradient and their

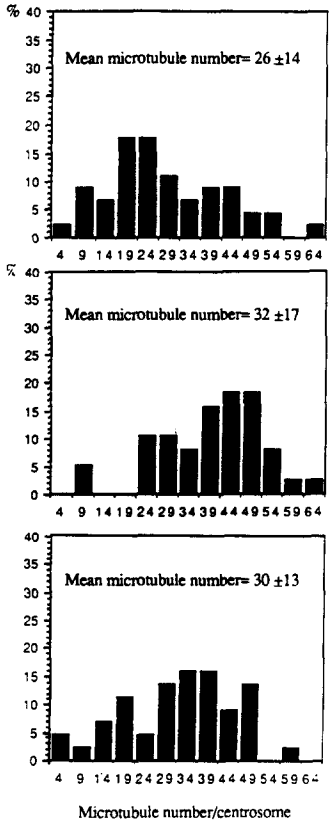

Figure 3. Microtubule number distribution per centrosome after urea inactivation and complementation in egg extracts. Inactivated centrosomes were incubated in a 250,000 *g* supernatant prepared from eggs sampled 40 min after activation, reisolated on a sucrose cushion (see Fig. 1) and assayed for their nucleating activity by incubation in 10 μM pure calf brain tubulin for 10 min at 37°C. The microtubule number distribution per aster is shown for three different complementation assays. The data is ordered in 13 classes with 5 microtubule increment steps. This figure shows the percentage of centrosomes (*ordinate*) nucleating between 4 and 64 microtubules (*abscissa*).

nucleating activity assayed in a solution of purified bovine brain tubulin (Fig. 1). Clearly, the intrinsic nucleating activity of the native centrosomes (Fig. 2, *C* and *D*) was inactivated by urea (Fig. 2 *E*) and reconstituted after incubation in the egg extract (Fig. 2 *F*). The complementation was done in an extract containing 20 μM nocodazole, demonstrating that microtubule assembly in the extract was not required for reconstitution of the microtubule nucleating activity of the centrosomes. We were concerned that some contaminating material from the extract could cosediment with the centrosomes in the gradient and cause microtubule nucleation in the solution of pure tubulin. To examine this possibility, we performed two controls. First, we tested the microtubule nucleating activity of the 40–70% sucrose interface of gradients run with the same amount of extract but in the absence of centrosomes. No asters were formed when the interface was added to pure tubulin despite the presence of contaminating proteins originating from the extract (data not shown). Second, inactivated centrosomes were mixed with the 40–70% interface from a gradient run with egg extract only and added to a solution of pure tubulin. These centrosomes were unable to nucleate microtubules (not shown). This set of experiments clearly demonstrates that some material present in egg extracts binds to or modifies the inactivated centrosomes, providing a functional complementation assay. Throughout this work, we have used centrosomes which are reisolated on a sucrose gradient after complementation in egg extracts as described above. This allows us to quantitate the intrinsic microtubule nucleating activity of these centrosomes by measuring the number of microtubules they nucleate in a solution of pure tubulin at a given concentration. The microtubule number distribution observed on complemented centrosomes incubated in 10 μM pure tubulin is given in Fig. 3 for three independent experiments performed on different days with different batches of extracts and centrosomes. Aster size ranged from very large (more than 55 microtubules/centrosome, 2–4% of the population) to very small (less than nine microtubules/centrosome, 6–14% of the population) with an average varying between 26 and 32 microtubules per centrosome between the different experiments. Given the microtubule number distribution per centrosome, these experiments appear to be reproducible.

The Activity That Complements Inactivated Centrosomes Is Soluble and Denatured above 56°C

In a first attempt to characterize the material involved in the complementation of inactivated centrosomes, we examined whether it was stored in the eggs in a soluble form. A crude egg lysate was centrifuged at 100,000 *g* for 1 h, 250,000 *g* for 2 h or 250,000 *g* for 2 h followed by a further spin of a small volume of extract in the TLA100 centrifuge at 150,000 *g* for 35 min. This last procedure eliminated all particulate material having a sedimentation coefficient above 30S (Leiss, D., unpublished observations). To partially quantify the activity present in these various supernatants, we assayed the microtubule nucleating activity of inactivated centrosomes in 15 μM tubulin after complementation in increasingly diluted supernatants (see Materials and Methods). The level of complementation (as indicated by the average number of microtubules nucleated per centrosome) clearly decreased with increasing extract dilution and in parallel in the 100,000 *g* (40 mg protein/ml) and the 250,000 *g* (22 mg protein/ml) supernatants (Fig. 4 *a*). This suggests that there was no or little nucleating material associated with the material pelleted at 250,000 *g*. Further centrifugation in the TLA 100 rotor did not reduce the capacity of the final supernatant to complement the inactivated centrosomes (not shown).

The recovery of microtubule nucleating activity was also dependent on the concentration of centrosomes in the extract during the complementation reaction. For example, centrosomes incubated at a concentration of 0.4×10^6/ml in a 10-fold diluted extract nucleated, on average, 45 microtubules each. The same centrosomes, incubated at a concentration of 1.2×10^6/ml in the same 10-fold diluted extract, nucleated, on average, only 15–20 microtubules each (Fig. 4 *b*). When the extracts were diluted more than 20-fold, the microtubule nucleating activity was poorly restored (eight microtubules/centrosome for a regrowth carried out in 15 μM pure tubulin). The strong dependence of the complementation reaction both on the concentration of the centrosome and the dilution of the extract suggests that it involves at least one binding event between different components. The low complementation observed at high centrosome concentration indicates that the extract can be saturated by centrosomes.

Preincubation of extracts at 40°C for 2 min did not affect significantly their capacity to complement inactivated centrosomes. However, in extracts preincubated at 56°C or above, a precipitate formed and the supernatant recovered after centrifugation had little or no complementation activity. For example, inactivated centrosomes complemented by an extract preincubated at room temperature or at 40°C

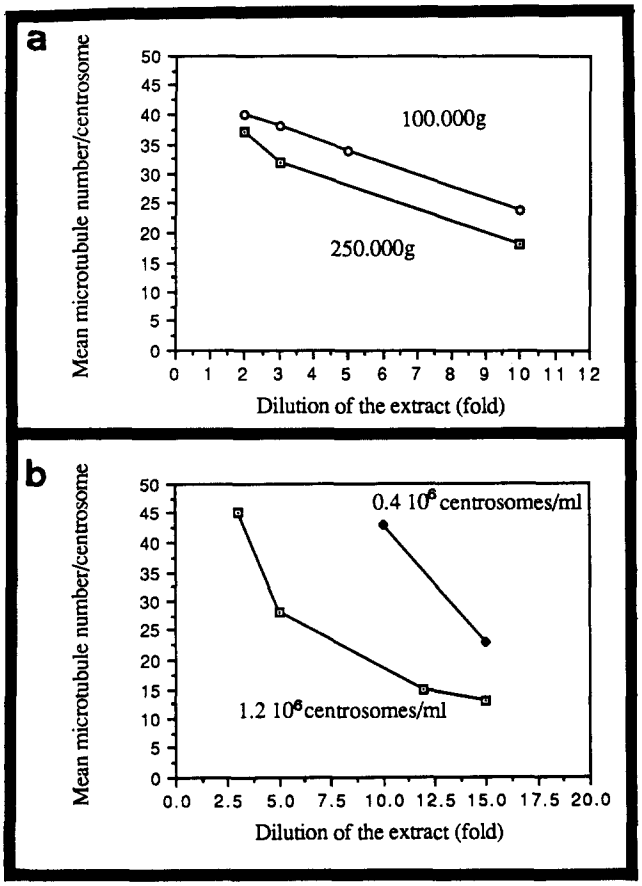

Figure 4. Effect of egg extract dilution and centrosome concentration on the complementation of inactivated centrosomes. (*A*) Inactivated centrosomes were incubated for 40 min at room temperature in 100,000-*g* (○—○) or 250,000-*g* (⊡—⊡) egg extract supernatants at increasing dilutions. The extracts were prepared from prophase eggs (40 min in the first cell cycle). The dilution ranged from 2-fold to 10-fold, corresponding to protein concentrations ranging from 20 to 4 mg/ml for the 100,000-*g* and from 13 to 2.6 mg/ml for the 250,000-*g* supernatant. After purification on a sucrose cushion, the centrosomes were incubated with 10 μM calf brain tubulin for 10 min at 37°C. The mean microtubule number nucleated per centrosome (*ordinate*) is plotted against the dilution of the extract used for complementing the centrosomes (*abscissa*). (*B*) In this experiment, urea-treated centrosomes were incubated at two concentrations, 1.2 × 10^6 centrosomes/ml (⊡—⊡) and 0.4 × 10^6 centrosomes/ml (◆—◆) in a 250,000-*g* supernatant at increasing dilutions. After purification on a sucrose cushion, the centrosomes were incubated with 15 μM calf brain tubulin for 10 min.

nucleated ~30–40 microtubules in 15 μM tubulin. After complementation in an extract preincubated at 56°C, they nucleated only 14 microtubules and no nucleation occurred after complementation of centrosomes in an extract preincubated at 95°C (not shown). Thus, the factor(s) involved in microtubule nucleation present in the extract is/are sensitive to temperatures higher than 56°C.

Effect of ATP Depletion on Centrosome Complementation by Frog Egg Extracts

To test whether ATP was required for centrosome complementation, we added apyrase in the extract (Fig. 5). This

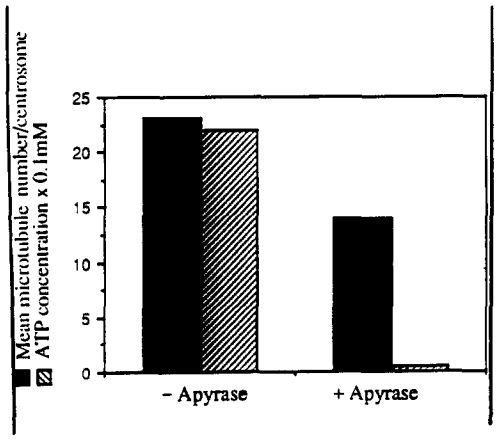

Figure 5. Effect of low ATP levels on the complementation of urea treated centrosomes by egg extracts. A 250,000-*g* supernatant prepared from eggs sampled at 40 min in the first cell cycle was preincubated for 10 min at room temperature in the presence or absence of apyrase (2.5 U/ml) before addition of the centrosomes that were further incubated for 40 min in the extract. The concentration of the apyrase was maintained constant during the complementation assay. The centrosomes were reisolated and tested for nucleating activity in pure tubulin (10 μM). Black bars represent the mean microtubule number per centrosome and shaded bars the ATP concentrations in the extracts during incubation with the urea treated centrosomes (× 0.1 mM). The mean microtubule number per centrosome in the absence or in the presence of apyrase was 23 ± 9 and 14 ± 5, respectively.

reduced the ATP concentration from 2.5 mM to 0.05 mM, but the centrosomes complemented in these extracts still recovered a basal level of microtubule nucleation (Fig. 5). These results show that physiological levels of ATP are not required for complementation of inactivated centrosomes in egg extracts although the presence of ATP increases the complementation efficiency.

Regulation of the Microtubule Nucleating Activity of Centrosomes by Phosphorylation

The experiments described above were carried out in extracts prepared from *Xenopus* eggs sampled at 40 min in the first embryonic cell cycle. This corresponds to a time when DNA replication has occurred and, therefore, to a G2-like phase. Since beginning of M-phase occurs at ~70 min, the time when the extracts were made corresponded to an early prophase. At this time, the accumulation of A and B cyclins has started but cdc2 kinase activity is still very low although above the background level found in eggs or egg extracts arrested in proper interphase by cyclin depletion (Félix et al., 1990; Karsenti et al., 1991; Minshull et al., 1990). We therefore compared the microtubule nucleating activity of centrosomes complemented in prophase (40 min after egg activation) to that of centrosomes complemented in interphase extracts (prepared from activated eggs incubated for 90 min in cycloheximide).

As shown in Table I, the centrosomes complemented in prophase extracts nucleated more microtubules than the centrosomes complemented in interphase extracts. Moreover, addition of 1 mM 6-dimethyl amino purine (6-DMAP, an ATP analogue inhibiting kinase activities; Félix et al., 1989;

Table I. Modifications of the Microtubule Nucleating Activity of Centrosomes by Phosphorylation Events

Extract used for the complementation assay	Histone kinase activity	Mean microtubule number per centrosome
	pmol/min/μl	
Prophase		41 ± 10
Prophase + DMAP		20 ± 16
Interphase		27 ± 10
Interphase + DMAP		28 ± 16
Interphase	1	25 ± 8
+ 15 nM cyclin A	1	28 ± 14
+ 25 nM cyclin A	4.5	40 ± 12
+ 50 nM cyclin A	8	45 ± 10
+ 100 nM cyclin A	16	46 ± 8
+ 100 nM cyclin A + DMAP		23 ± 10
+ 100 nM cyclin A + EDTA		17 ± 7

Urea-treated centrosomes were incubated in presence of different types of extracts as indicated in the first column. The histone kinase activity which was induced after addition of cyclin A to the extract is given in the second column (see Materials and Methods). After repurification on a sucrose cushion, the centrosomes were incubated with 10 μM calf brain tubulin for 10 min. The mean microtubule number nucleated per centrosome is indicated in the third column.

Guerrier et al., 1990; Néant and Guerrier, 1988; Rime et al., 1989) to prophase extracts reduced their ability to complement inactivated centrosomes while it had no effect on the complementation ability of interphase extracts (Table I).

This indicated that part of the microtubule nucleating activity conferred to inactivated centrosomes by the prophase extract requires a phosphorylation event that does not take place in interphase extracts. Centrosomes complemented in prophase extracts are in fact more strongly labeled by the MPM2 antibody (which recognizes phosphorylated epitopes (Davis et al., 1983), a difference that is abolished when the complementation in prophase extracts is carried out in the presence of 6-DMAP (data not shown).

A Cyclin A-dependent Phosphorylation Event Increases the Nucleating Activity of Centrosomes

To test whether cyclin A or cyclin B–associated kinases could be responsible for the increased microtubule nucleating activity of centrosomes complemented in prophase extracts, we added bacterially produced cyclins to interphase extracts. This resulted in the stimulation of a cdc2 or cdc2-like kinase activity that could be assayed on histone H1 (Belmont et al., 1990; Buendia et al., 1991; Solomon et al., 1990). In these experiments, we used crude 100,000 *g* supernatants because we had characterized the mechanism of activation of cdc2 kinase by cyclins in these extracts (Clarke, P. R., D. Leiss, M. Pagano, and E. Karsenti, manuscript in preparation). As shown in Table I, the complementation efficiency of an interphase extract was enhanced by the addition of human cyclin A. At concentrations that produced the lowest level of measurable H1 kinase activity in the extract (4.5 pmol/min/μl), the microtubule nucleating activity of the complemented centrosomes was increased almost to a maximum level. This effect was completely inhibited both by 6-DMAP and EDTA (Table I), further suggesting that cyclin A had an effect through the activation of the H1 kinase and subsequent phosphorylation of substrate(s) in egg extracts or on the complemented centrosomes. The centrosomes complemented in cyclin A–treated extracts were more highly phosphorylated than those complemented in interphase extracts as judged by the increased labeling by the MPM2 antibody, which recognizes phosphorylated epitopes on mitotic centrosomes (Fig. 6, *A* and *B*). The labeling observed on centrosomes complemented in cyclin A–treated extracts in the presence of 6-DMAP or EDTA was comparable to that observed in interphase extracts, demonstrating further that these compounds inhibited the cyclin A–dependent phosphorylation of proteins belonging to pericentriolar material (Fig. 6, *C* and *D*).

Δ90 cyclin B added under conditions that produced a similar H1 kinase activity had a much reduced effect on microtubule nucleation (Fig. 7). Similarly, purified starfish cyclin B-cdc2 kinase had no effect (data not shown).

Taken together, these results show a causal relationship between the presence of cyclin A kinase activity, phosphorylation of centrosomal epitopes recognized by the MPM2 antibody and increased microtubule nucleating activity of centrosomes.

Cyclin A Kinase Increases the Microtubule Nucleating "Capacity" of Centrosomes

Throughout this investigation, the microtubule nucleating activity of complemented centrosomes has been routinely assayed in pure tubulin solutions at a fixed concentration (10 or 15 μM). In principle, microtubule nucleation could be regulated by a modulation of the "affinity" (taken in a very broad sense) of the nucleating material for tubulin or by a modulation of the "capacity" of the pericentriolar material to nucleate microtubules. A modulation of the affinity could be detected by a change in the critical tubulin concentration required for microtubule assembly off centrosomes; a modulation of the capacity could be detected by a change in the number of microtubules nucleated per centrosome at high tubulin concentrations, way above the critical concentration.

Fig. 8 shows how the number of microtubules nucleated per centrosome varied as a function of the tubulin concentration present in the regrowth assay. Centrosomes inactivated by urea treatment did not nucleate microtubules at any tubulin concentration. The first striking result was that the critical tubulin concentration for polymer assembly was twofold lower for centrosomes complemented in an interphase extract than that observed for native centrosomes (8 versus 15 μM). However, the slope of the curve representing the increase in mean microtubule number per centrosome as a function of tubulin concentration was roughly parallel for native centrosomes and centrosomes complemented in interphase extracts. The second striking result was that although the critical tubulin concentration for polymer assembly was very close for centrosomes complemented in interphase and in cyclin A–treated extracts, the slope of the curve representing the increase in mean microtubule number per centrosome as a function of tubulin concentration was twofold steeper in the latter case.

These results suggest that the intrinsic microtubule nucleating activity of centrosomes could be regulated at two levels: its affinity for tubulin and its capacity to nucleate high numbers of microtubules. We did not examine microtubule

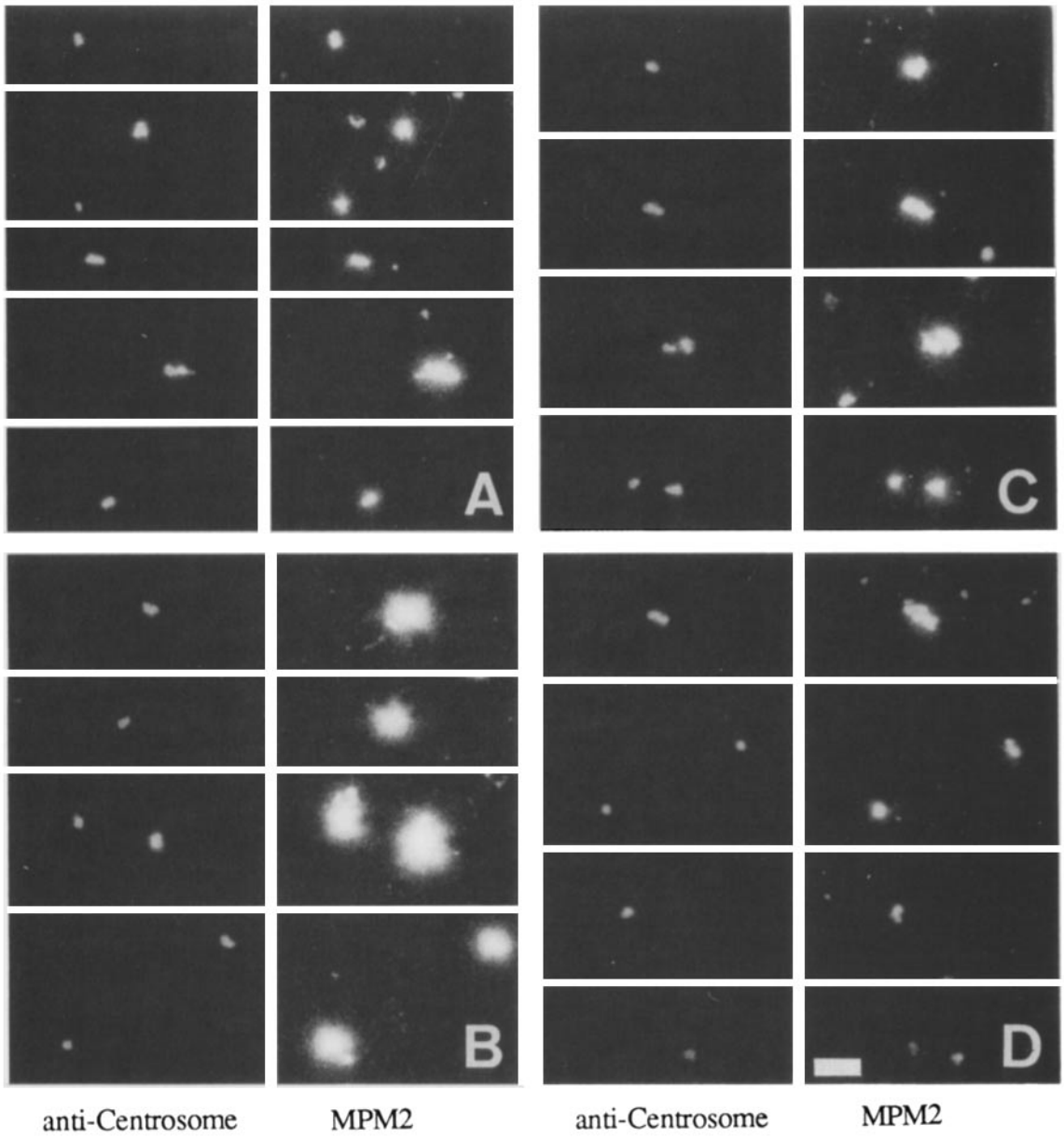

Figure 6. Presence of cyclin A in the extracts increases the phosphorylation level of the complemented centrosomes as detected by the MPM2 antibody. Urea-treated centrosomes were complemented in 100,000 *g* supernatants in the absence of cyclin A (*A*), in the presence of cyclin A alone (*B*), in the presence of cyclin A + DMAP (*C*), or in the presence of cyclin A + EDTA (*D*). After purification of the centrosomes on a sucrose cushion, an aliquot of 10 μl was diluted in a 10 mM K-Pipes buffer, centrifuged onto a coverslip, fixed with methanol at −20°C, and processed for immunofluorescence as described in Materials and Methods. The left panel in *A–D* shows the labeling with the anticentrosome antibody and the right panel shows the labeling with the MPM2 antibody. The exposure times of negatives and prints were the same in all cases.

nucleation above 40 μM tubulin because this approaches the critical concentration for spontaneous microtubule assembly (Bré and Karsenti, 1990).

Discussion

The Microtubule Nucleating Material Is Stored under a Soluble Form in Xenopus Eggs

In this work, we have taken advantage of a natural event, the transformation of the sperm basal body into a functional centrosome during fertilization, to develop an assay to study the regulation of microtubule nucleation by centrosomes in vitro. The nucleating activity of centrosomes is localized in the pericentriolar material which is still poorly defined in molecular terms although it is clear that proteins are essential components of it given the extreme sensitivity of the nucleating activity to the action of proteases (Kuriyama, 1984). Therefore, it is likely that during fertilization, the sperm basal body is transformed into a functional egg centrosome by association of proteins stored in the egg to the periphery of the centrioles originating from the sperm. We have used

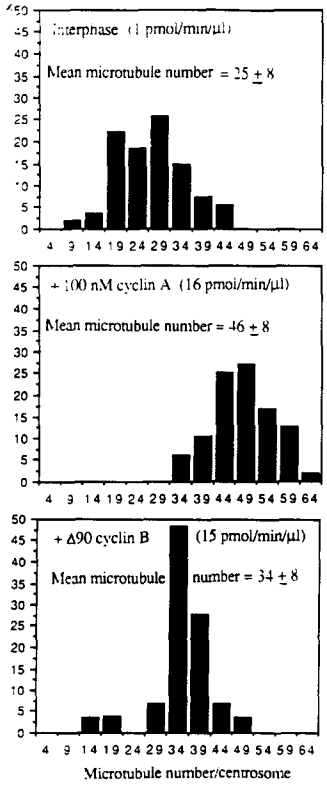

Figure 7. The microtubule nucleating activity of centrosomes complemented in interphase extracts can be increased by addition of cyclin A but not cyclin B. Urea-treated centrosomes were incubated in 100,000-*g* supernatants prepared from interphase eggs in the absence or presence of 100 nM cyclin A or Δ90 cyclin B (the extracts were preincubated with the cyclins for 10 min before addition of the centrosomes which were further incubated for 35 min). The histone H1 kinase activity present in the extract was determined on an aliquot of the reaction mixture at the end of the 35 min of incubation. This activity is expressed in picomole of phosphate transferred to histones per min per µl of extract and indicated in each panel. After reisolation, the centrosomes were assayed for microtubule nucleation in 10 µM pure tubulin. This figure shows the percentage of centrosomes (*ordinate*) nucleating increasing numbers of microtubules (*abscissa*) as described in Fig. 3.

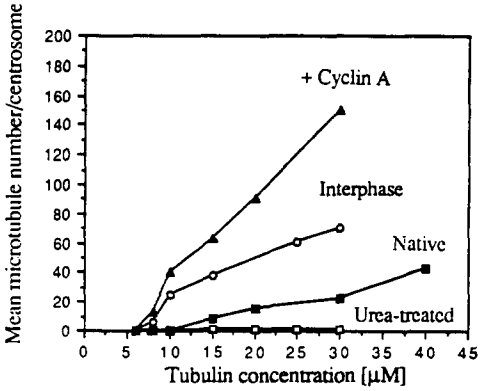

Figure 8. Centrosomes complemented in cyclin A–treated interphase extracts have a higher microtubule nucleating capacity. After reisolation, the centrosomes were assayed for microtubule nucleation in increasing concentrations of pure tubulin for 10 min at 37°C. The curves give the mean microtubule number nucleated per centrosome (*ordinate*) as a function of the tubulin concentration in which the regrowth was carried out. Native centrosomes (■—■); urea-treated centrosomes (□—□); centrosomes complemented in an interphase extract (○—○); centrosomes complemented in the same interphase extract containing 100 nM cyclin A (▲—▲). When the number of microtubules per aster was inferior to ~50, microtubules were counted on immunofluorescence pictures. Above this value, this became impossible and microtubules were counted on electron microscope pictures of glutaraldehyde-fixed aster centrifuged on carbon coated grids and negatively stained (see Materials and Methods). This was the case for the centrosomes complemented in the interphase extract and assayed in 30 µM tubulin and for the centrosomes complemented in the interphase extract in the presence of 100 nM cyclin A and assayed with 20 and 30 µM tubulin.

urea-inactivated centrosomes instead of sperm basal bodies for technical reasons, and frog egg extracts since they support the assembly of complex structures like nuclei or spindles. The inactivated centrosomes do not nucleate microtubules when incubated in pure tubulin solutions although the structure of the centrioles is only slightly affected (Klotz et al., 1990). Our previous investigation had shown that the treatment with 2 M urea did extract some proteins from the centrosomes but it was difficult to know whether any of the extracted proteins visible on silver-stained gels was involved in microtubule nucleation. Also, all attempts to renature the extracted centrosomes by dialysis in the presence of the extracted material was unsuccessful although the same urea extracted centrosomes could function when injected into frog eggs. This suggested that the centrioles were still functional and that the lack of renaturation in vitro was due either to an irreversible damage of the solubilized material or to the fact that this material was present at such a low concentration that it could not bind back to the centrioles.

In the present work, we find that inactivated centrosomes can be functionally complemented by frog egg extracts. Moreover, complementation occurs in extracts depleted of particulate material, after a 20-fold dilution of the extracts. It is not ATP dependent (at least for reconstitution of an interphasic level of microtubule nucleation) and is inhibited by temperatures higher than 56°C. Since after reisolation from the extract on a sucrose gradient, the centrosomes can nucleate microtubules in pure tubulin solutions, the complementation must be a stable modification of the centrosomes resulting from the binding of factors on the centrioles.

We have examined the effect of adding purified brain tau, MAP2 and MAP1 to inactivated centrosomes on their microtubule nucleating activity before and after reisolation on a sucrose gradient. This never conferred any nucleating activity to these centrosomes. We have also observed that when centrosomes were complemented in egg extracts at 4°C, they recovered microtubule nucleating activity at a four-fold lower rate than at room temperature (not shown).

We conclude from these experiments that soluble factors present in egg extracts probably bind to the inactivated centrosomes and restore their microtubule nucleating activity. This raises the question of how such factors shift from a soluble state when stored in the egg to an insoluble condition in the centrosome. If brain-like MAPs are involved in microtubule nucleation at the centrosome, they apparently cannot restore microtubule nucleating activity by simple binding to the inactivated centrosomes. We have attempted to identify proteins that bind specifically from the extract to the centrosomes during the complementation assay but so far, we have been unsuccessful at reaching a clear conclusion (limiting amounts of centrosomes and contamination from the extract). It would be interesting to examine whether γ-tubulin is one of these factors (Horio et al., 1991; Stearns et al., 1991; Zheng et al., 1991).

We find that the efficiency of complementation is strongly dependent not only on the dilution of the extract but also on the concentration of centrosomes added to the extract. This is particularly striking at high extract dilutions. Recovery of the nucleating activity is barely detectable after complemen-

tation of inactivated centrosomes in extracts diluted 20-fold under assay conditions where 7 μl of extract (~180 μg of proteins) were added to ~90,000 centrioles. Considering that one egg represents ~0.5 μl of high speed supernatant, we estimate (very roughly) that one egg contains enough material to complement 6,000 centrioles. This is in the same order of magnitude as the number of cells present at the mid-blastula transition (Kirschner et al., 1985), indicating that there is enough material stored in the egg to produce functional centrosomes until the mid-blastula transition (Gard et al., 1990). This situation seems to be common to many other cellular structures.

Protein Phosphorylation and the Regulation of the Microtubule Nucleating Activity of Centrosomes by Cyclin A Kinase in Egg Extracts

Kinase inhibitors (Apyrase, 6-DMAP or EDTA) reduce the microtubule nucleating activity of centrosomes complemented in prophase extracts but not in interphase extracts. This indicates that interactions between the microtubule nucleating material and centrioles does not require protein phosphorylation, although phosphorylation may modulate positively the nucleating activity.

The nucleating activity of centrosomes complemented in extracts can be increased by human cyclin A but not by Δ90 sea urchin cyclin B, or purified starfish cyclin B-cdc2 kinase (Labbé et al., 1989). Given the level of conservation between *Xenopus* and human cyclin A (Buendia et al., 1991), it is likely that the cyclin A kinase effect is physiological, especially because this cyclin is produced in a soluble form in bacteria and therefore does not require a renaturation step. The lack of effect of cyclin B kinase should be taken with more caution although cyclin B cdc2 kinase complexes purified in a native form have also no effect while both reagents have strong effects on microtubule dynamics (Belmont et al., 1990; Verde et al., 1990) and inhibition of endosome fusion (Tuomikoski et al., 1989). Although cyclin A and B kinases produce similar overall patterns of protein phosphorylation in egg extracts, there are subtle differences and this is probably where we should look for specific effects of each of these molecules (Minshull et al., 1990). How the specificity is provided is entirely unclear, but this is likely to be due to a targeting process involving sequences which are specific of A and B type cyclins and conserved among species for each type of molecule (Buendia et al., 1991).

The Notion of Microtubule Nucleating Activity

Throughout this work, we have used the term "microtubule nucleating activity" to refer to the number of microtubules nucleated by a given population of centrosomes, assayed at a given tubulin concentration and for a given time of regrowth. Obviously, changing these parameters affects the number of microtubules nucleated per centrosome. As previously shown for native centrosomes, we find that after 10 min of regrowth the number of microtubules per centrosome increases with the concentration of tubulin (Bré and Karsenti, 1990; Kuriyama, 1984; Mitchison and Kirschner, 1984). Apparently, for all centrosomes tested, the number of nucleated microtubules does not reach a plateau, even at tubulin concentrations close to the critical concentration for spontaneous assembly. This suggests that, the number of nucleating sites is not limiting and probably in very large excess over the number of microtubules that can be nucleated in a solution of pure tubulin (on a centrosome complemented in a cyclin A-treated extract, we have counted up to 260 microtubules on an electron micrograph). This is at variance with previous results obtained with microtubule proteins containing MAPs (Kuriyama et al., 1984). In this case, the centrosomes can be saturated with microtubules (Kuriyama et al., 1984) probably because the MAPs stabilize microtubules and change the conditions for nucleation at the surface of the centrosome (Bré and Karsenti, 1990).

We find that the critical tubulin concentration for polymer assembly off native centrosomes is twofold higher than that measured for centrosomes complemented in an interphase extract. Presently, we tentatively explain this result by saying that the "affinity" of the pericentriolar material of the complemented centrosomes for tubulin is higher. But this may be more complicated and should be examined further. We were very surprised to find that the critical tubulin concentration for polymer assembly was similar for centrosomes complemented in interphase and cyclin A-treated extracts. This may mean that the centrosomes complemented in cyclin A-treated extracts do not have a better "affinity" for tubulin than the centrosomes complemented in an interphase extract. Since the number of microtubules nucleated per centrosome complemented in cyclin A-treated extracts increases more steeply as a function of tubulin concentration, it seems that these centrosomes have a higher "capacity" of microtubule nucleation. This may mean that they have more individual nucleating sites or that the organization or the physical properties of the nucleating material are different. It seems that the "affinity" of the nucleating material for tubulin is not regulated by phosphorylation, whereas its "capacity" to nucleate microtubules is.

Implications for the Regulation of Microtubule Nucleating Activity of Centrosomes In Vivo

In vivo, the apparent microtubule nucleating activity of centrosomes increases in prophase, when the duplicated centrosomes start to migrate around the nucleus. This is particularly striking in MDCK cells where the centrosomes nucleate very few microtubules in interphase (Bré et al., 1990). We have seen that centrosomes complemented in cyclin A-treated extracts have a higher capacity to nucleate microtubules and that this is regulated by phosphorylation whereas their affinity for tubulin is not very much changed. It is therefore likely that in prophase cells, the microtubule nucleating capacity in centrosomes is increased by a cyclin A-dependent phosphorylation event of centrosomal proteins. Cyclin A has been reported to be mainly localized in the nucleus during the G1-S transition period, but it becomes partially cytoplasmic and localized at centrosomes in prophase exactly when their microtubule nucleating activity increases (Pagano et al., 1992). Similarly, $p34^{cdc2}$ becomes localized at centrosomes in prophase (Bailly et al., 1989; Rattner et al., 1990; Riabowol et al., 1989). Activation of cyclin A-associated kinase occurs during the S-G2 period, before that of the cyclin B-associated kinase (Minshull et al., 1990; Pagano et al., 1992; Pines and Hunter, 1990; Whitfield et al., 1990), when centrosome phosphorylation begins to occur (Centonze and Borisy, 1990) and their nucleating

activity increases. This is consistent with the finding that cyclin A kinase increases the nucleating activity of centrosomes in frog egg extracts. The fact that in our in vitro experiments cyclin B does not increase the microtubule nucleating capacity of centrosomes is surprising, since in vivo at metaphase, the activity of the cyclinB-cdc2 is high and centrosomes still have a very high microtubule nucleating activity. In any case, cyclin A is not degraded before the onset of metaphase, and dephosphorylation of centrosomal antigens may not occur before the onset of anaphase possibly requiring a phosphatase activated only at this moment. Therefore, a cyclin B–dependent phosphorylation of centrosomes may not be essential, while we can not exclude the possibility that in vivo the cdc2-cyclin B kinase could phosphorylate centrosomal antigens, but only after a previous phosphorylation step due to the cyclinA-cdc2 complex. It remains to be examined, whether cyclin A–dependent phosphorylations regulate the microtubule nucleating activity of centrosomes at the level of the binding of nucleating material to the centrioles or by changing the conformation of the pericentriolar material. This will require purified cyclin A–associated kinase and careful biochemical and ultrastructural analysis.

We thank J. Domingez and J. Avila for their generous gift of human autoimmune antibody against centrosomes; P. Rao for the MPM2 antibody; M. Dorée for the gift of purified cdc2 kinase; M. Glotzer for bacteria producing the Δ90 sea urchin cyclin B; and M. Pagano for human cyclin A. We also thank M. Cyrklaf for help with EM and P. Clarke and M-A. Félix for critical reading of the manuscript.

This work was supported in part by a Human Frontier Science Program grant.

Received for publication 1 October 1991 and in revised form 21 November 1991.

References

Alvey, P. L. 1985. An investigation of the centriole cycle using 3T3 and CHO cells. *J. Cell Sci.* 78:147–162.

Bailly, E., M. Dorée, P. Nurse, and M. Bornens. 1989. P34cdc2 is located in both nucleus and cytoplasm; part is centrosomally associated at G2/M and enters vesicles at anaphase. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3985–3995.

Baron, A. T., and J. L. Salisbury. 1988. Identification and localization of a novel, cytoskeletal, centrosome-associated protein in PtK2 cells. *J. Cell Biol.* 107:2669–2678.

Belmont, L. D., A. A. Hyman, K. E. Sawin, and T. J. Mitchison. 1990. Real-time visualization of cell cycle-dependent changes in microtubule dynamics in cytoplasmic extracts. *Cell.* 62:579–589.

Bergen, L. G., R. Kuriyama, and G. G. Borisy. 1980. Polarity of microtubules nucleated by centrosomes and chromosomes of Chinese hamster ovary cells in vitro. *J. Cell Biol.* 84:151–159.

Bornens, M., and E. Karsenti. 1984. The Centrosome. *In* Membrane Structure and Function. Bittar, E. E., editor. John Wiley and Sons, New York. 99–171.

Bornens, M., M. Paintrand, J. Berges, M. C. Marty, and E. Karsenti. 1987. Structural and chemical characterization of isolated centrosomes. *Cell Motil. Cytoskeleton.* 8:238–249.

Bré, M. H., and E. Karsenti. 1990. Effects of brain microtubule-associated proteins on microtubule dynamics and the nucleating activity of centrosomes. *Cell Motil.* 15:88–98.

Bré, M. H., T. E. Kreis, and E. Karsenti. 1987. Control of microtubule nucleation and stability in Madin-Darby canine kidney cells: the occurrence of non centrosomal, stable detyrosinated microtubules. *J. Cell Biol.* 105:1283–1296.

Bré, M. H., R. Pepperkok, A. M. Hill, N. Levilliers, W. Ansorgue, E. H. K. Stelzer, and E. Karsenti. 1990. Regulation of microtubule dynamics and nucleation during polarization in MDCK II cells. *J. Cell Biol.* 111:3013–3021.

Buendia, B., C. Antony, F. Verde, M. Bornens, and E. Karsenti. 1990*a*. A centrosomal antigen localized on intermediate filaments and mitotic spindle poles. *J. Cell Sci.* 97:259–271.

Buendia, B., M. H. Bré, G. Griffiths, and E. Karsenti. 1990*b*. Cytoskeletal control of centrioles movement during the establishment of polarity in MDCK cells. *J. Cell Biol.* 110:1123–1135.

Buendia, B., P. Clarke, M. A. Félix, E. Karsenti, D. Leiss, and F. Verde. 1991. Regulation of protein kinases associated with cyclin A and cyclin B and their effect on microtubule dynamics and nucleation in Xenopus egg extracts. *Cold Spring Harbor Quant. Symp. Biol.* 56:523–532.

Centonze, V. E., and G. G. Borisy. 1990. Nucleation of microtubules from mitotic centrosomes is modulated by a phosphorylated epitope. *J. Cell Sci.* 95: 405–411.

Dane, P. J., and J. B. Tucker. 1986. Supracellular microtubule alignments in cell layers associated with the secretion of certain fish scales. *J. Cell Sci. Suppl.* 5:273–291.

Davis, F. M., T. Y. Tsao, S. K. Fowler, and P. N. Rao. 1983. Monoclonal antibodies to mitotic cells. *Proc. Natl. Acad. Sci. USA.* 80:2926–2930.

Félix, M.-A., J. Pines, T. Hunt, and E. Karsenti. 1989. A post-ribosomal supernatant from activated Xenopus eggs that displays post-translationally regulated oscillation of its cdc2+ mitotic kinase activity. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3059–3069.

Félix, M. A., P. Cohen, and E. Karsenti. 1990. Cdc2 H1 kinase is negatively regulated by a type 2A phosphatase in the Xenopus early embryonic cell cycle: evidence from the effects of okadaic acid. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:675–683.

Gard, D. L., S. Hafezi, T. Zhang, and S. J. Doxsey. 1990. Centrosome duplication continues in cycloheximide-treated Xenopus blastulae in the absence of a detectable cell cycle. *J. Cell Biol.* 110:2033–2042.

Glotzer, M., A. W. Murray, and M. W. Kirschner. 1991. Cyclin is degraded by the ubiquitin pathway. *Nature (Lond.).* 349:132–138.

Gosti-Testu, F., M. C. Marty, J. Berges, M. Maunoury, and M. Bornens. 1986. Identification of centrosomal proteins in a human lymphoblastic cell line. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:2545–2550.

Gould, R. R., and G. G. Borisy. 1977. The pericentriolar material in chinese hamster ovary cells nucleates microtubule formation. *J. Cell Biol.* 73:601–615.

Guerrier, P., P. Colas, and I. Neant. 1990. Meiosis reinitiation as a model system for the study of cell division and cell differentiation. *Int. J. Dev. Biol.* 34:93–109.

Horio, T., S. Uzawa, M. K. Jung, B. R. Oakley, K. Tanaka, and M. Yanagida. 1991. The fission yeast γ-tubulin is essential for mitosis and is localized at microtubule organizing centers. *J. Cell Sci.* 99:693–700.

Joswig, G., C. Petzelt, and D. Werner. 1991. Murine cDNAs coding for the centrosomal antigen centrosomin A. *J. Cell Biol.* 98:37–43.

Karsenti, E. 1991. Mitotic spindle morphogenesis in animal cells. *Seminars Cell Biol.* In press.

Karsenti, E., S. Kobayashi, T. Mitchison, and M. Kirschner. 1984*a*. Role of the centrosome in organizing the interphase microtubule array: properties of cytoplasts containing or lacking centrosomes. *J. Cell Biol.* 98:1763–1776.

Karsenti, E., J. Newport, R. Hubble, and M. Kirschner. 1984*b*. Interconversion of metaphase and interphase microtubule arrays, as studied by the injection of centrosomes and nuclei into Xenopus eggs. *J. Cell Biol.* 98:1730–1745.

Karsenti, E., F. Verde, and M. A. Félix. 1991. Role of type 1 and type 2A protein phosphatases in the cell cycle. *Adv. Prot. Phosph.* 6:453–482.

Kellogg, D. R., C. M. Field, and B. M. Alberts. 1989. Identification of microtubule-associated proteins in the centrosome, spindle, and kinetochore of the early Drosophila embryo. *J. Cell Biol.* 109:2977–2991.

Kirschner, M. W., J. Newport, and J. C. Gerhart. 1985. The timing of early developmental events in *Xenopus*. *Trends Genet.* 1:41–47.

Klotz, C., M.-C. Dabauvalle, M. Paintrand, T. Weber, M. Bornens, and E. Karsenti. 1990. Parthenogenesis in *xenopus* eggs requires centrosomal integrity. *J. Cell Biol.* 110:405–415.

Kuriyama, R. 1984. Activity and stability of centrosomes in chinese hamster ovary cells in nucleation of microtubules in vitro. *J. Cell Sci.* 66:277–295.

Kuriyama, R. 1989. 225-Kilodalton phosphoprotein associated with mitotic centrosomes in sea urchin eggs. *Cell Motil. Cytoskelton.* 12:90–103.

Kuriyama, R., and G. G. Borisy. 1981*a*. Centriole cycle in chinese hamster ovary cells as determined by whole mount electron microscopy. *J. Cell Biol.* 91:814–821.

Kuriyama, R., and G. G. Borisy. 1981*b*. Microtubule-nucleating activity of centrosomes in CHO cells is independent of the centriole cycle, but coupled to the mitotic cycle. *J. Cell Biol.* 91:822–826.

Kuriyama, R., and H. Kanatani, 1981. The centriolar complex isolated from starfish spermatozoa. *J. Cell Sci.* 49:33–49.

Kuriyama, R., G. Keryer, and G. G. Borisy. 1984. The mitotic spindles of Chinese Hamster Ovary cells isolated in taxol-containing medium. *J. Cell Sci.* 66:265–275.

Kuriyama, R., P. Savereide, P. Lefebure, and S. Dasgupta. 1990. The predicted amino acid sequence of a centrosphere protein in dividing sea urchin eggs is similar to elongation factor (EF-1a). *J. Cell Sci.* 95:231–236.

Labbé, J. C., J. P. Capony, D. Caput, J. C. Cavadore, J. Derancourt, M. Kaghdad, J. M. Lelias, A. Picard, and M. Dorée. 1989. MPF from starfish oocytes at first meiotic metaphase is a heterodimer containing one molecule of cdc2 and one molecule of cyclin B. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3053–3058.

Lohka, M., and J. Maller. 1985. Induction of nuclear envelope breakdown chromosome condensation, and spindle formation in cell-free extracts. *J.*

Cell Biol. 101:518–523.
Maekawa, T., R. Leslie, and R. Kuriyama. 1991. Identification of a minus end-specific microtubule-associated protein located at the mitotic poles in cultured mammalian cells. *Eur. J. Cell Biol.* 54:255–267.
Mazia, D. 1984. Centrosomes and mitotic poles. *Exp. Cell Res.* 153:1–15.
McIntosh, J. R. 1983. The centrosome as an organiser of the cytoskeleton. *Mod. Cell Biol.* 2:115–142.
Minshull, J., R. Golsteyn, C. S. Hill, and T. Hunt. 1990. The A- and B-type cyclin associated cdc2 kinases in Xenopus turn on and off at different times in the cell cycle. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:2865–2875.
Mitchison, T., and M. Kirschner. 1984. Microtubule assembly nucleated by isolated centrosomes. *Nature (Lond.).* 312:232–237.
Mogensen, M. M., and J. B. Tucker. 1987. Evidence for microtubule nucleation at plasma membrane-associated sites in *Drosophila. J. Cell Sci.* 88: 95–107.
Mogensen, M. M., J. B. Tucker, and H. Stebbings. 1989. Microtubule polarities indicate that nucleation and capture of microtubules occurs at cell surfaces in Drosophila. *J. Cell Biol.* 108:1445–1452.
Moudjou, M., M. Paintrand, B. Vigues, and M. Bornens. 1991. A human centrosomal protein is immunologically related to basal body-associated proteins from lower eucaryotes and is involved in the nucleation of microtubules. *J. Cell Biol.* 115:129–140.
Néant, I., and P. Guerrier. 1988. 6-dimethylaminopurine blocks starfish oocyte maturation by inhibiting a relevant protein kinase activity. *Exp. Cell Res.* 176:68–79.
Newport, J. 1987. Nuclear reconstitution in vitro: Stages of assembly around protein-free DNA. *Cell.* 48:205–217.
Ohta, K., M. Toriyama, M. Miyazaki, H. Murofushi, S. Hosoda, S. Endo, and H. Sakai. 1990. The mitotic apparatus-associated 51-KDa protein from sea urchin eggs is a GTP-binding protein and is immunologically related to yeast polypeptide elongation factor 1 alpha. *J. Biol. Chem.* 265:3240–3247.
Pagano, M., R. Pepperkok, F. Verde, W. Ansorge, and G. Draetta. 1992. Cyclin A is required at two points in the human cell cycle. *EMBO (Eur. Mol. Biol. Organ.) J.* In press.
Pines, J., and T. Hunter. 1990. Human cyclin A is adenovirus E1A-associated protein p60 and behaves differently from cyclin B. *Nature (Lond.).* 346: 760–763.
Rattner, J. B., J. Lew, and J. H. Wang. 1990. p34cdc2 kinase is located to distinct domains within the mitotic apparatus. *Cell Motil. Cytoskeleton.* 17: 227–235.
Riabowol, K., G. Draetta, L. Brizuela, D. Vandre, and D. Beach. 1989. The cdc2 kinase is a nuclear protein that is essential for mitosis in mammalian cells. *Cell.* 57:393–401.
Rime, H., I. Neant, P. Guerrier, and R. Ozon. 1989. 6-Dimethylaminopurine (6-DMAP), a reversible inhibitor of the transition to metaphase during the first meiotic cell division of the mouse oocyte. *Dev. Biol.* 133:169–179.
Sager, P. R., N. L. Rothfield, J. M. Oliver, and R. D. Berlin. 1986. A novel mitotic spindle pole component that originates from the cytoplasm during prophase. *J. Cell Biol.* 103:1863–1872.
Salisbury, J. L., A. T. Baron, D. E. Coling, V. E. Martindale, and M. A. Sanders. 1986. Calcium-modulated contractile proteins associated with the eucaryotic centrosome. *Cell Motil.* 6:193–197.
Solomon, M. J., M. Glotzer, T. H. Lee, M. Philippe, and M. W. Kirschner. 1990. Cyclin activation of p34cdc2. *Cell.* 63:1013–1024.
Stearns, T., L. Evans, and M. Kirshner. 1991. γ-Tubulin is a highly conserved component of the centrosome. *Cell.* 65:825–836.
Tassin, A. M., B. Maro, and M. Bornens. 1985. Fate of microtubule organizing centers during in vitro myogenesis. *J. Cell Biol.* 100:35–46.
Tournier, F., E. Karsenti, and M. Bornens. 1989. Parthenogenesis in Xenopus eggs injected with centrosomes from synchronized human lymphoid cells. *Dev. Biol.* 136:321–321–329.
Tucker, J. B. 1984. Spatial organisation of microtubule-organising centers and microtubules. *J. Cell Biol.* 99:55s–62s.
Tuomikoski, T., M.-A. Félix, M. Dorée, and J. Gruenberg. 1989. Inhibition of endocytic vesicle fusion in vitro by the cell-cycle control protein kinase cdc2. *Nature (Lond.).* 342:942–945.
Vandre, D., F. Davis, P. Rao, and G. Borisy. 1984. Phosphoproteins are components of mitotic microtubule organizing centres. *Proc. Natl. Acad. Sci. USA.* 81:4439–4443.
Vandre, D. D., P. Kronebusch, and G. G. Borisy. 1985. Interphase-mitosis transition: microtubule rearrangements in cultured cells and sea urchin eggs. *In* Molecular Biology of the Cytoskeleton. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 3–16.
Verde, F., J. C. Labbé, M. Dorée, and E. Karsenti. 1990. Regulation of microtubule dynamics by cdc2 protein kinase in cell-free extracts of Xenopus eggs. *Nature (Lond.).* 343:233–238.
Vorobjev, I. A., and Y. S. Chentsov. 1982. Centrioles in the cell cycle. I. Epithelial cells. *J. Cell Biol.* 98:938–949.
Whitfield, W. G., C. Gonzalez, C. G. Maldonado, and D. M. Glover. 1990. The A- and B-type cyclins of Drosophila are accumulated and destroyed in temporally distinct events that define separable phases of the G2-M transition. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:2563–2572.
Zheng, Y., M. K. Jung, and B. R. Oakley. 1991. γ-Tubulin is present in Drosophila melanogaster and Homo sapiens and is associated with the centrosome. *Cell.* 65:817–823.