# Nucleation of microtubules from mitotic centrosomes is modulated by a phosphorylated epitope

VICTORIA E. CENTONZE* and GARY G. BORISY

*Laboratory of Molecular Biology, University of Wisconsin-Madison, 1525 Linden Drive, Madison, WI 53706, USA*

* Author for correspondence

## Summary

**At the onset of mitosis a class of proteins appears that possess a phosphorylated epitope recognized by the monoclonal antibody MPM-2. Immunofluorescence staining shows that a subset of these proteins is associated with the centrosomes of the mitotic apparatus. The appearance of these proteins coincides with the increased microtubule nucleating capacity of the centrosomes. We have tested whether growth of microtubules from mitotic centrosomes in a lysed cell model is dependent on the availability of the phosphorylated epitope by blocking the epitope with a specific antibody or by modifying it by removal of the phosphate. Centrosomes incubated with purified tubulin nucleate microtubule asters. However, preincubating the centrosomes with MPM-2 blocks all microtubule nucleation. Pretreating mitotic centrosomes with alkaline phosphatase also inhibits nucleation. These data suggest that the phosphorylated epitope recognized by MPM-2 is important for microtubule nucleation.**

Key words: centrosomes, microtubules, nucleation, and MPM-2.

## Introduction

At the onset of mitosis an epitope shared by a subset of structural and soluble proteins becomes phosphorylated. This common phosphorylated epitope is specifically detected by the monoclonal antibody MPM-2 (Davis *et al.* 1983). While MPM-2 detects a large number of phosphoproteins on Western blots of whole mitotic cells, only four high molecular weight polypeptides are detected on blots of mitotic spindles isolated from synchronized CHO cells. Indirect immunofluorescence labeling with MPM-2 shows that the antibody stains the centrosomes, kinetochores and microtubule fibers of the mitotic spindle (Vandre *et al.* 1986). The MPM-2 epitope on the mitotic centrosome becomes phosphorylated immediately prior to nuclear envelope breakdown and remains phosphorylated through metaphase. Concurrent with the appearance of this epitope, the centrosome increases in volume (Rieder and Borisy, 1982) and exhibits an increased capacity to nucleate microtubules (Snyder and McIntosh, 1975; Kuriyama and Borisy, 1981). The phosphorylated state of the centrosome persists until the onset of anaphase. As the chromosomes separate, the phosphorylated epitope is lost from or greatly reduced in the centrosome (Vandre and Borisy, 1989), the microtubule nucleating capacity of the centrosome decreases to interphase levels (Snyder *et al.* 1982), and the pericentriolar material decreases in volume (Rieder and Borisy, 1982).

The temporal coincidence of the appearance and disappearance of the phosphorylated epitope and the increase and decrease in microtubule nucleating activity of the mitotic centrosome suggests a dependency relationship between these two properties. Centrosome phosphorylation is not dependent on the presence of microtubules, since the MPM-2 epitope appears on centrosomes of cells in the absence of polymerized microtubules (Engle *et al.* 1988; Vandre and Borisy, 1989). Therefore, the remaining possibilities are that the availability of the phosphorylated epitope is required for augmented microtubule nucleating activity on the centrosome, or that it is not required and is coincidental, for undetermined reasons. To distinguish between these possibilities we have prepared tubulin-depleted, lysed mitotic CHO cell cytoskeletons to provide an *in vitro* model of mitotic centrosomes. These cytoskeletons were treated to block or modify the phosphorylated epitope and then exposed to purified tubulin to test for microtubule nucleating capacity. Blocking the epitope with antibody or removing the phosphate from the protein markedly diminished microtubule nucleation. We conclude that phosphorylation greatly facilitates or is

required for the microtubule nucleating capacity of mitotic centrosomes.

## Materials and methods

### *Cell culture*

Chinese hamster ovary (CHO) cells were cultured as monolayers in Ham's F-10 medium supplemented with 10% fetal bovine serum.

To isolate mitotic cells, cultures were partially synchronized in $G_1/S$ by incubation in 5.0 mM-thymidine for 13–16 h, then cultured in fresh medium for 2.5–3.0 h to release the thymidine block. Cells were accumulated in prometaphase by treatment with 0.04 $\mu g\,ml^{-1}$ nocodazole (Zieve *et al.* 1980) for 4 h. The rounded mitotic cells were shaken off into the medium, collected by centrifugation at 700 ***g*** and resuspended for 30 min in fresh medium containing 3.0 $\mu g\,ml^{-1}$ nocodazole. The higher concentration of nocodazole was employed to ensure complete depolymerization of all mitotic microtubules. While in nocodazole the cells were allowed to adhere to glass coverslips coated with a mixture of 0.5 $mg\,ml^{-1}$ concanavalin A and 25 $mg\,ml^{-1}$ 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. Coated coverslips were necessary because mitotic CHO cells are round and are easily washed off the coverslips during processing.

### *Cell extracts*

To prepare extracts of mitotic proteins, cells were collected as described above and resuspended in 2 volumes of cold Tris-buffered saline (25 mM-Tris–HCl, pH 7.4, 0.15 M-NaCl). The suspension was sonicated with a Sonifier Cell Disruptor (model W140-D, Heat Systems-Ultrasonics, Inc., Plainview, NY) set at 50% of the microtip maximum to disrupt the cells and then clarified by centrifugation at 12 000 ***g***, 4°C for 10 min. The supernatant was collected and stored at −70°C. Similarly, extracts of interphase proteins were prepared from cells that were scraped from the culture flasks with a rubber policeman.

### In vitro *nucleation of microtubules from centrosomes*

Mitotic cells on coverslips were rinsed in PEM (100 mM-Pipes, pH 6.9, 1 mM-EGTA, 1 mM-$MgCl_2$) for 30 s, lysed for 90 s in 0.07% Triton X-100 in PEM plus 1 $mg\,ml^{-1}$ tosyl-arginine methyl ester (TAME). Tubulin was purified by DEAE chromatography (Murphy *et al.* 1977) and desalted into PEM. Prior to incubation with tubulin, cytoskeletons underwent various pretreatments: antibodies diluted in Tris-buffered saline for 45 min, 1 unit $\mu l^{-1}$ bovine intestinal alkaline phosphatase (Sigma, St Louis, MO) in 10 mM-Tris–HCl, pH 8.4, for 90 min, or control buffers. After pretreatment, the cytoskeletons were rinsed in PEM plus TAME and incubated with 1.2–1.6 $mg\,ml^{-1}$ tubulin in PEM containing 1.0 mM GTP for 10 min at 37°C. Following a brief rinse in PEM plus 0.1 mM-GTP the cytoskeletons were fixed in 0.7% glutaraldehyde in PEM and processed for immunofluorescence.

### *Immunofluorescence*

Prior to the addition of antibodies, cytoskeletons were reduced in two changes of 1 $mg\,ml^{-1}$ $NaBH_4$ for 30 min and blocked with 5% normal goat serum in Tris-buffered saline. For double-label immunofluorescence the cytoskeletons were incubated for 45 min in each antibody in the following sequence: MPM-2, rhodamine-conjugated anti-mouse, rat anti-tyrosinated tubulin (Accurate, Westbury, NY), fluorescein-conjugated anti-rat. If the cytoskeletons had been pretreated with MPM-2 or one of the solutions of absorbed MPM then no additional primary antibody was added. The coverslips were mounted in 10% polyvinyl alcohol containing 1 $mg\,ml^{-1}$ *p*-phenylene diamine (Sammak *et al.* 1987). Immunofluorescence microscopy was performed on a Zeiss Universal microscope equipped with a 63× oil-immersion objective NA 1.3 (Carl Zeiss, Inc., Thornwood, NY). Micrographs were taken with Tri-X film (Kodak, Rochester, NY).

### *Antibodies*

Preparation of MPM-2 has been described previously (Davis *et al.* 1983). MPM-2 ascites fluid was diluted 1:250 with Tris-buffered saline plus 1% bovine serum albumin. A sample of the diluted MPM-2 was used for all subsequent cytoskeleton pretreatments. To determine whether the effect of pretreatment with MPM-2 was antibody-specific, the remaining antibody solution was preabsorbed against either mitotic or interphase proteins, prior to being applied to cytoskeletons. MPM-2 should be selectively removed from solution by mitotic proteins but not by interphase proteins, since the antibody specifically recognizes a phosphorylated epitope that is present on cytoplasmic proteins of mitotic cells.

Duplicate columns of DEAE–Affigel (Biorad, Richmond, CA) were poured and equilibrated with 25 mM-Tris–HCl, pH 7.4 (Fig. 1). Mitotic proteins were applied to one column while interphase proteins were applied to a second column. The proteins were allowed to adsorb to the resin for 2 h. Approximately 2 mg of protein were bound per ml of packed resin. Unbound proteins were washed from the column and MPM-2 was applied. DEAE–Affigel was chosen as an appropriate matrix because cell proteins, including those that were recognized by MPM, would bind to the resin while MPM-2, an IgG antibody, would not bind. The eluate from the column of mitotic proteins was termed mitotic-absorbed MPM-2 while the

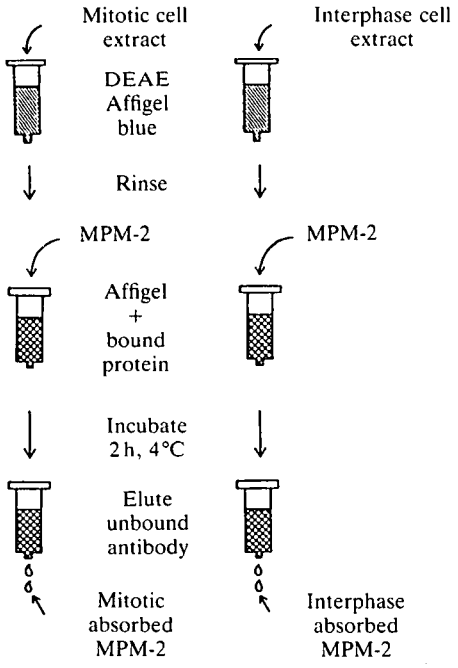



**Fig. 1.** Preparation of MPM-2 preabsorbed against mitotic or interphase proteins. Either mitotic or interphase proteins were adsorbed to duplicate columns of DEAE–Affigel. MPM-2 was then absorbed against the resin–protein matrix on each column and the unbound antibodies were collected. Mitotic absorbed MPM-2 and interphase absorbed MPM-2 were then used to pretreat cytoskeletons.

eluate from the column of interphase proteins was termed interphase-absorbed MPM-2. The original MPM-2 and the eluates from each column were tested for the presence of MPM-2 in a dot blot assay. These absorbed antibodies were used to pretreat cytoskeletons before incubation with tubulin.

*Dot blot assay*

Antibody solutions were deemed to contain MPM-2 if mitotic proteins could be detected. Nitrocellulose paper (Schleicher and Schuell, Keene, NH) was placed in a Biorad dot blot apparatus and 10 $\mu$g of mitotic proteins were added to each of three wells. The unabsorbed proteins were rinsed from the wells with Tris-buffered saline and blocking solution (10% horse serum in 25 mM-Tris–HCl, pH 7.4) was added for 15 min. The wells were again rinsed and one of the three antibody solutions was added to each well for 2 h at room temperature. After incubation with primary antibody the nitrocellulose paper was removed from the blotter and placed in blocking solution. Incubation with a peroxidase-labeled anti-mouse antibody followed by color development with 4-chloronaphthol (Sigma, St Louis, MO) was used to detect the MPM-2 bound to each dot of mitotic protein.

## Results

The cytoskeleton of a mitotic CHO cell immunofluorescently labeled with both anti-tubulin (Fig. 2A) and MPM-2 (Fig. 2B) showed the normal distribution of microtubules and the mitosis-specific phosphorylated epitope. The bipolar array of metaphase microtubules emanated exclusively from the centrosomal material at each pole. Unlike the interphase centrosome, the material at the spindle poles possessed the mitosis-specific phosphorylated epitope and therefore was intensely stained by MPM-2. The intensity of MPM-2 staining at metaphase was similar in the presence or absence of cytoplasmic microtubules (data not shown). While MPM-2 also labeled kinetochores and spindle fibers, the fluorescent signal from these structures was obscured by the spherical geometry of the cell.

Prometaphase-arrested cells treated with 3 $\mu$g ml$^{-1}$ nocodazole contained no endogenous microtubules after lysis detectable by anti-tubulin immunofluorescence (Fig. 3A). However, after these microtubule-depleted cytoskeletons were incubated with DEAE-purified tubulin, centrosome-nucleated microtubules were formed rapidly (Fig. 3B). Under the conditions employed for microtubule nucleation, most (86%; Table 1) of the cytoskeletons contained one or two asters. The centrosomes from which the microtubules were nucleated retained their MPM-2 reactivity.

To determine if the MPM-2 epitope was involved in microtubule nucleation from the centrosome, antibody was applied to the microtubule-depleted cytoskeletons in an attempt to block tubulin-binding sites. The antibody-treated cytoskeletons were then incubated with purified tubulin. Dot blot analysis (Fig. 4A) verified that the original sample of diluted MPM-2 contained antibodies reactive with proteins in a mitotic cell extract. When this antibody solution was used to pretreat cytoskeletons, MPM-2 was specifically localized to the centrosomes

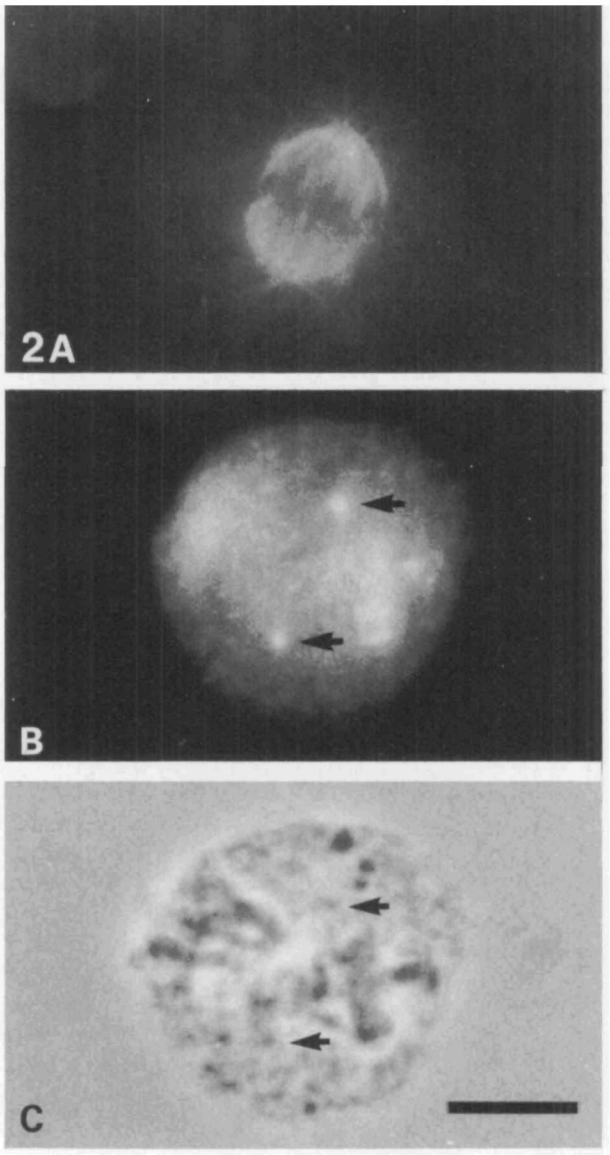
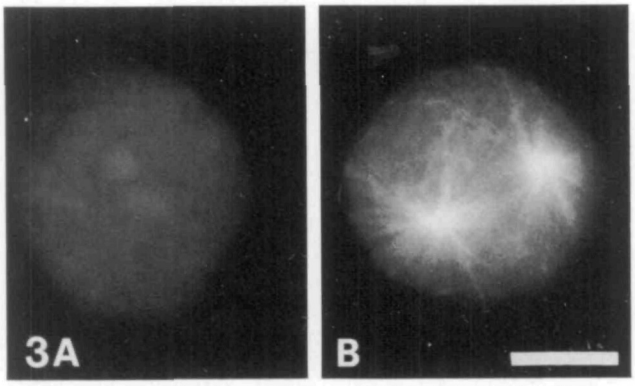



**Fig. 2.** Double-label immunofluorescence of a normal CHO cell in metaphase. Anti-tubulin (fluorescein) labeled the microtubule fibers of a typical metaphase spindle (A) while MPM-2 (rhodamine) was localized primarily to the centrosomes of the spindle (B, arrows). The centrosomes can be seen as faint dots by phase-contrast microscopy (C, arrows). Chromosomes appear somewhat fluorescent in the rhodamine channel because of autofluorescence induced by the glutaraldehyde. Bar, 10 $\mu$m.

**Table 1.** *Efficiency of microtubule nucleation*

| Preincubation treatment | % Centrosomes showing microtubule growth | *N* |
|---|---|---|
| PEM only | 86 | 99 |
| MPM-2 | 0 | 84 |
| Mitotic-absorbed MPM-2 | 81 | 64 |
| Interphase-absorbed MPM-2 | 0 | 72 |
| Alkaline phosphatase | 5 | 65 |
| Alkaline phosphatase+inhibitor | 82 | 107 |




(Fig. 4B). After incubation with purified tubulin, microtubule asters were not detected in these cytoskeletons (Fig. 4C). This indicates that MPM-2-blocked centrosomes have diminished accessibility for microtubule nucleation.

To prove that microtubule nucleation was blocked by MPM-2 and not non-specifically by the immunolabeling,

**Fig. 3.** Polymerization of microtubules onto centrosomes. Microtubule formation was assayed by anti-tubulin immunofluorescence of cytoskeletons before and after incubation with tubulin. A. After nocodazole treatment and lysis no microtubules were detected in the mitotic cytoskeleton. B. Cytoskeletons incubated with 1.2–1.6 mg $ml^{-1}$ tubulin for 10 min nucleated microtubule asters from the mitotic centrosomes. Bar, 10 $\mu$m.

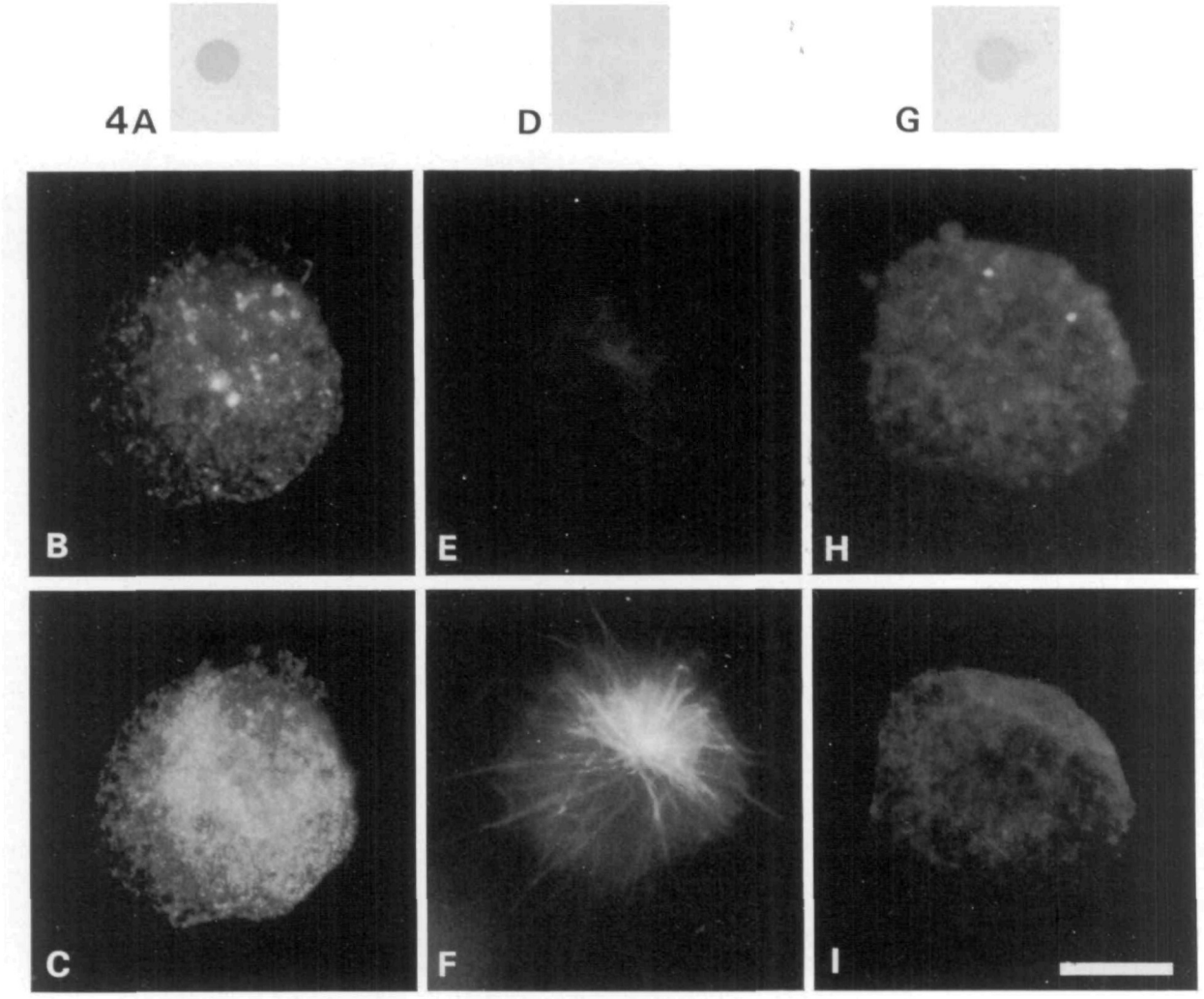



**Fig. 4.** Cytoskeletons pretreated with solutions containing MPM-2 do not nucleate microtubules. The original working solution of MPM-2 detected mitotic proteins in a dot blot assay (A). When this solution was applied to a cytoskeleton the antibodies localized specifically to the mitotic centrosome (B). The presence of these antibodies on the centrosomes blocked their ability to nucleate microtubules (C). Mitotic-absorbed MPM-2 did not contain antibodies reactive with mitotic proteins in a dot blot assay (D). When applied to a cytoskeleton no antibodies were detected at the centrosomes (E) and microtubule nucleation was not blocked (F). Interphase-absorbed MPM-2 did contain antibodies reactive with mitotic proteins in a dot blot assay (G). When applied to a cytoskeleton these antibodies were localized to the mitotic centrosomes (H) and also blocked microtubule nucleation (I). Bar, 10 $\mu$m.

preabsorbed antibody solutions were used to pretreat cytoskeletons. Mitotic-absorbed MPM-2 (Fig. 1), as established by dot blot analysis, no longer contained antibodies that recognized mitotic proteins (Fig. 4D). When this antibody preparation was applied to cytoskeletons, MPM-2 was not detected on the centrosomes by immunofluorescence (Fig. 4E). In contrast to MPM-blocked centrosomes, these centrosomes nucleated microtubule asters after incubation with purified tubulin (Fig. 4F). Preparations of interphase-absorbed MPM-2 (Fig. 1) contained antibodies that recognized mitotic proteins (Fig. 4G) and labeled centrosomes (Fig. 4H). When used to pretreat cytoskeletons, interphase-absorbed MPM-2 gave the same result as the original sample of MPM-2; microtubule nucleation was blocked. These experiments show that when MPM-2 is bound to the phosphorylated epitope it interferes with microtubule nucleation. Affinity absorption procedures that specifically remove mitotic-reactive antibodies also remove the blocking activity. This suggests that the MPM-2 epitope or a site adjacent to it greatly facilitates or is required for microtubule nucleation at the centrosome.

Is the phosphate on the MPM-2 epitope important for microtubule nucleation? Treatment of the cytoskeletons with 1 unit $\mu l^{-1}$ alkaline phosphatase completely removed MPM-2 reactivity (Fig. 5A). When phosphatase-treated cytoskeletons were then incubated with purified tubulin, the centrosomes were not able to nucleate microtubules (Fig. 5B). High concentrations of alkaline phosphatase were required to remove MPM-2 reactivity completely, raising the possibility that contaminating enzymes such as proteases might have been responsible for the loss of nucleating activity. At lower concentrations of phosphatase, not all of the MPM-2 reactivity was abolished and the centrosomes were able to nucleate small microtubule asters (data not shown). Phosphatase activity is specifically inhibited by alpha-naphthyl phosphate. When a mixture of phosphatase and inhibitor was used to pretreat the cytoskeletons, MPM-2 reactivity was not removed (Fig. 5C) and the centrosomes retained their microtubule-nucleating activity (Fig. 5D). Since the inhibitory activity of the phosphatase was antagonized by a phosphatase-specific inhibitor, we conclude that contaminating enzymatic activities were not responsible for the loss of nucleation capacity. A summary of the nucleating capacity of mitotic centrosomes after both antibody and enzyme pretreatments is shown in Table 1.

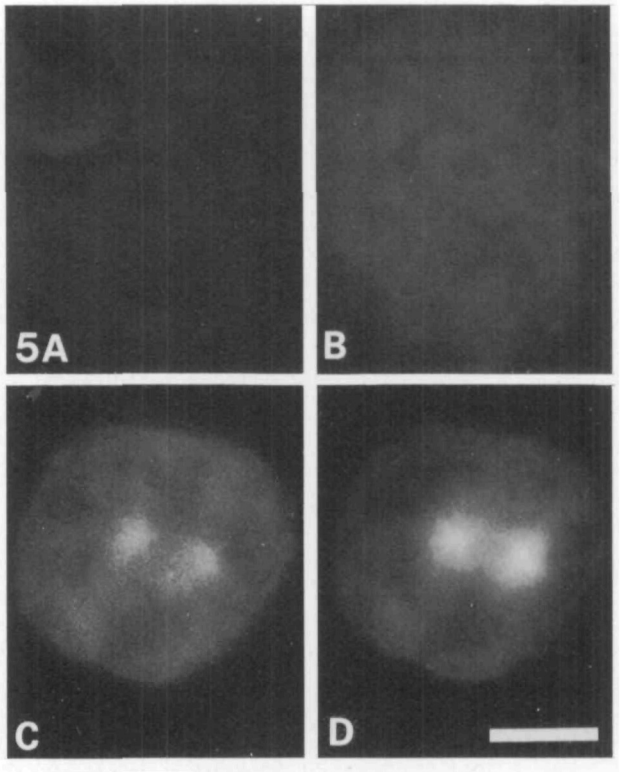



**Fig. 5.** Alkaline phosphatase removes the microtubule nucleating capacity of mitotic centrosomes. Double-label immunofluorescence micrographs of cytoskeletons pretreated with alkaline phosphatase (A and B) or alkaline phosphatase plus its inhibitor, alpha-naphthyl phosphate (C and D). Dephosphorylation abolished reactivity with MPM-2 (A) and mitotic centrosomes no longer nucleated microtubules (B). After treatment with alkaline phosphatase plus alpha-naphthyl phosphate MPM-2 reactivity was preserved (C) and the centrosomes retained their microtubule nucleating activity (D). Bar, 10 $\mu$m.

## Discussion

We have shown that binding of MPM-2 antibody to the pericentriolar material of mitotic centrosomes, or removal of phosphate from these centrosomes blocks the nucleation of microtubules. This evidence indicates that a phosphate moiety promotes microtubule nucleation, suggesting that phosphorylation and dephosphorylation of centrosomal proteins is an important mechanism by which the cell can regulate the number of microtubules nucleated by the centrosome. The cell requires a network of microtubules to maintain its shape and to support intracellular particle motility. However, during mitosis, the cell forms a compact bipolar spindle. Since fewer but longer microtubules exist in the interphase network than in the mitotic spindle, only a basal number of nucleation sites need be available. During interphase, the centrosome could be maintained in a dephosphorylated or low phosphorylated state. As the cell makes the transition from interphase to mitosis nucleation sites might be added to the centrosome and phosphorylation could play an important regulatory role.

Phosphorylation of the centrosome at mitosis may result from: (1) unmasking of pre-existing phosphorylated epitopes in the pericentriolar material; (2) accumulation at the centrosome of molecules phosphorylated in the cytoplasm; and (3) phosphorylation of proteins in the pericentriolar material by a specific kinase. Unmasking of a phosphorylated epitope is the least likely possibility, since the epitope is not detectable in SDS-containing gels of proteins from synchronized interphase cells (Davis *et al.* 1983). Newly phosphorylated proteins in the cell

cytoplasm may be recruited and accumulated at the centrosome. The phosphorylated epitope appears on the centrosome in cells treated with microtubule inhibitors at the $G_2$/M transition (Vandre and Borisy, 1989) and on the spindle pole body in the *nimA* mutant of *Aspergillus*, which never forms a spindle (Engle *et al.* 1988). This suggests that if the phosphorylated material is accumulated at the centrosome it arrives there by a mechanism that does not require microtubules. This could be accomplished by diffusion and binding. The third possibility is that the pericentriolar material is phosphorylated *in situ*. The addition of phosphate to the protein may contribute directly to the formation of a microtubule nucleation site, or phosphorylation may elicit a reversible, conformational change in the protein, thus exposing a site of nucleation elsewhere.

Since the onset of mitosis is accompanied by a profound increase in protein kinase activity, resulting in the phosphorylation of histones (Lagan, 1982; Matsukawa *et al.* 1985), nuclear lamins (Gerace and Blobel, 1980; Miake-Lye and Kirschner, 1985; Ottaviano and Gerace, 1985; Burke and Gerace, 1986), microtubule-associated proteins (Vandre *et al.* 1990) and intermediate filaments (Evans and Fink, 1982; Celis *et al.* 1983), it is likely that the MPM epitope is also specifically phosphorylated at this time. A highly conserved M-phase promoting factor, MPF, which has as an active component cdc2 protein kinase (Gautier *et al.* 1988; Dunphy *et al.* 1988; Labbe *et al.* 1989) initiates a cascade of events leading to nuclear envelope breakdown, chromosome condensation and formation of the mitotic spindle. Recently, Kuang *et al.* (1989) found that the appearance of MPM-2 antigens was coincident with the increase in MPF activity during progesterone-induced maturation of *Xenopus* oocytes. MPM-2 antibody blocked MPF activity when injected into oocytes prior to induction and immunodepleted MPF activity in extracts of both mature oocytes and mitotic HeLa cells. These results suggest that the appearance of the MPM epitope is closely linked to the kinase activity of MPF. Therefore the kinase activity of MPF or a kinase that becomes active during mitotic induction downstream from MPF may be responsible for phosphorylating the pericentriolar material at the MPM epitope, thus creating microtubule nucleation sites. Once phosphorylated, the pericentriolar material is capable of nucleating enough microtubules to form the mitotic spindle, but whether or not the spindle is formed also depends upon additional factors, since the *nimA* mutant of *Aspergillus* contains a phosphorylated spindle-pole body without a spindle (Engle *et al.* 1988).

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