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# Phosphorylation regulates the assembly of NuMA in a mammalian mitotic extract

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## SUMMARY

NuMA is a 236 kDa nuclear protein that is required for the organization of the mitotic spindle. To determine how NuMA redistributes in the cell during mitosis, we have examined the behavior of NuMA in a mammalian mitotic extract under conditions conducive to the reassembly of interphase nuclei. NuMA is a soluble protein in mitotic extracts prepared from synchronized cultured cells, but forms insoluble structures when the extract becomes non-mitotic (as judged by the inactivation of *cdc2/cyclin B* kinase and the disappearance of *mpm-2*-reactive antigens). These NuMA-containing structures are irregularly shaped particles of 1-2  $\mu\text{m}$  in diameter and their assembly is specific because other nuclear components such as the lamins remain soluble in the extract under these conditions. NuMA is dephosphorylated during this assembly process,

and the assembly of these NuMA-containing structures is catalyzed by protein dephosphorylation because protein kinase inhibitors enhance their formation and protein phosphatase inhibitors block their formation. Finally, immunodepletion demonstrates that NuMA is an essential structural component of these insoluble particles, and electron microscopy shows that the particles are composed of a complex interconnected network of foci. These results demonstrate that phosphorylation regulates the solubility of NuMA in a mammalian mitotic extract, and the spontaneous assembly of NuMA into extensive structures upon dephosphorylation supports the conclusion that NuMA serves a structural function.

Key words: NuMA, Nucleus, Mitosis, Nuclear Matrix

## INTRODUCTION

Nuclei of vertebrate cells undergo profound physical rearrangements during mitosis. These physical changes are necessary for the efficient segregation of the chromosomes and include the dissolution of the nuclear lamina, condensation of the chromatin into chromosomes, and vesicularization of the nuclear envelope (Newport and Forbes, 1987; Gerace and Burke, 1988). Each of these structural changes is completely reversible such that two daughter cell nuclei are assembled during telophase from preexisting subunits that were generated from the disassembly of the nucleus in prophase of the previous mitosis. Thus, the subunits generated from the disassembly of the nucleus at the onset of mitosis must be partitioned equally into each daughter cell to ensure efficient assembly of the daughter cell nuclei (Laskey and Leno, 1990; Fisher, 1987; Marshall and Wilson, 1997; Wiese and Wilson, 1993). To date, there have been three pathways described for the segregation of nuclear components into the daughter cells. Nuclear components may either associate with the condensed chromosomes, undergo random diffusion, or associate with the mitotic spindle, and examples of nuclear proteins that segregate during mitosis using each of these mechanisms are topoisomerase II (Gasser et al., 1986; Earnshaw and Heck, 1985), the nuclear lamins (McKeon, 1991; Nigg, 1992; Gerace and Blobel, 1980), and NuMA (nuclear-mitotic apparatus protein; Lydersen and

Pettijohn, 1980; reviewed by Compton and Cleveland, 1994; Cleveland, 1995), respectively.

NuMA is a 236 kDa protein that is required for the organization of microtubule minus ends at the mitotic spindle pole (Gaglio et al., 1995, 1996; Merdes et al., 1996; Yang and Snyder, 1992; Kallajoki et al., 1991). During interphase in both dividing and non-dividing cells, however, NuMA is localized uniformly throughout the interior of the nucleus as an insoluble component of the nuclear matrix (Price and Pettijohn, 1986; Compton et al., 1991, 1992; Kallajoki et al., 1991, 1992; Tousson et al., 1991; Yang et al., 1992; Maekawa et al., 1991; Tang et al., 1993; Zeng et al., 1994). Thus, there has been speculation that NuMA may be a structural component of the nuclear matrix. Experimental support for this proposal comes from the localization of NuMA to a subset of the core filaments of the nuclear matrix (Zeng et al., 1994), the perturbation of nuclear assembly following mitosis upon disruption of NuMA (Compton and Cleveland, 1993; Kallajoki et al., 1993), the finding that NuMA is capable of assembling into extensive filamentous structures (Saredi et al., 1996), and the fact that NuMA has an extensive  $\alpha$ -helical coiled-coil structure (Compton et al., 1992; Yang et al., 1992; Maekawa and Kuriyama, 1993; Tang et al., 1993; Harborth et al., 1995). Unfortunately, in the absence of any defined assay for specific nuclear functions, no role for the NuMA protein in the nucleus has been identified.

In addition to characterizing the specific function of the NuMA protein on the mitotic spindle and the proposed function inside the nucleus, there has been significant effort aimed at understanding how the dramatic mitotic redistribution of the NuMA protein is regulated. Phosphorylation is likely to regulate NuMA's function and/or distribution during mitosis and NuMA has been shown to be a phosphoprotein whose phosphorylation peaks during mitosis (Price and Pettijohn, 1986; Compton and Luo, 1995; Hsu and Yeh, 1996; Sparks et al., 1995). However, aside from a single report indicating that p34cdc2/cyclin B phosphorylation may be necessary for the accurate localization of NuMA during mitosis (Compton and Luo, 1995), all of the data regarding the regulation of NuMA during mitosis by phosphorylation are correlative in nature. Thus, the functional consequences of the phosphorylation of NuMA have not been thoroughly characterized.

In this article we use a cell free system derived from synchronized HeLa cells to examine the behavior of the NuMA protein under conditions that are conducive to nuclear assembly. The results presented here show that as an extract prepared from synchronized HeLa cells becomes non-mitotic, NuMA spontaneously assembles into large insoluble particles. These particles form through a specific assembly process that is strictly regulated by phosphorylation. Electron microscopy demonstrates that these insoluble, NuMA-containing particles are composed of an interconnected network of foci that resemble the structures formed by NuMA when it is expressed in the cell cytoplasm (Saredi et al., 1996), and immunodepletion demonstrates that NuMA is essential for their formation. These results demonstrate that NuMA's solubility in a mitotic extract is controlled by protein phosphorylation and supports the conclusion that NuMA is performing a structural function in the cell.

## MATERIALS AND METHODS

### Cell culture

The human HeLa cell line was maintained in DME containing 10% FCS, 2 mM glutamine, 100 i.u./ml penicillin, and 0.1 µg/ml streptomycin. Cells were grown at 37°C in a humidified incubator with a 5% CO<sub>2</sub> atmosphere.

### Preparation of mitotic extracts

HeLa cells were synchronized in the cell cycle by double block with 2 mM thymidine. After a release from thymidine block, the cells were allowed to grow for 6 hours, and then nocodazole was added to a final concentration of 40 ng/ml. The mitotic cells that accumulated during the next 5 hours were collected by mitotic shake off. We typically obtained between 10<sup>7</sup> to 10<sup>8</sup> cells from five T-150 cm<sup>2</sup> tissue culture flasks. The cells were collected by centrifugation at 1,500 rpm and washed at 4°C twice with KPM(-) buffer (50 mM KCl, 50 mM Pipes, pH 7.0, 10 mM EGTA, 2 mM MgCl<sub>2</sub>) and once with KPM(+) buffer (50 mM KCl, 50 mM Pipes, pH 7.0, 10 mM EGTA, 2 mM MgCl<sub>2</sub>, 1 mM DTT) as described by Suprynowicz and Gerace (1986). Cells were then Dounce homogenized at 4°C and at a concentration of 5×10<sup>7</sup> cells/ml in KPM(+) buffer containing 20 µg/ml cytochalasin B, 50 µg/ml phenylmethylsulfonyl fluoride, and 5 µg/ml each of chemostatin, leupeptin, antipain and pepstatin. The crude cell extract was then subjected to centrifugation at 10,000 g for 15 minutes at 4°C. The supernatant was recovered, supplemented with 1 µg/ml nocodazole, and incubated with or without the addition of 3 mM ATP at 30°C for various times as indicated in the text. After incubation, a

sample of 5 µl was taken for immunofluorescence microscopy. The remainder of the extract was subjected to sedimentation at 10,000 g for 15 minutes at room temperature and separated into soluble and insoluble fractions. Proteins from both the soluble and insoluble fractions were solubilized by boiling for 6 minutes in SDS-PAGE sample buffer.

For the depletion of NuMA, 100 µg of preimmune rabbit polyclonal IgG or anti-NuMA rabbit polyclonal IgG were adsorbed onto 25 µl of Protein A-conjugated agarose (Boehringer Mannheim, Indianapolis, IN) as described by Gaglio et al., 1995. The antibody-coupled Protein A-agarose was washed three times in KPM(+) buffer, and packed by centrifugation to remove the excess fluid. The agarose was then resuspended with the mitotic extract and incubated for 35 minutes at 4°C. After this incubation the agarose was removed from the extract by sedimentation at 15,000 g for 10 seconds. The process was repeated twice to achieve a depletion efficiency of more than 95%.

NuMA was radiolabeled in the cell extract by supplementing the extract with 60 µCi [<sup>32</sup>P]ATP (160 µM final concentration ATP) and preincubation at 30°C for 15 minutes. The ATP was removed by the addition of 0.1 units of apyrase and the assembly of the NuMA-containing structures induced by incubation at 30°C for 120 minutes. Following the assembly reaction, the soluble and insoluble fractions were collected and the proteins solubilized by boiling for 5 minutes in 2% SDS, 50 mM Tris-HCl, pH 6.8, 1 mM EDTA, 2 mM EGTA, 1 mM DTT, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate. The excess SDS was scavenged by diluting the samples 8 fold with 3.4% Triton X-100, 20 mM Tris-HCl, pH 7.4, 140 mM NaCl, and 10 mM sodium pyrophosphate. NuMA was immunoprecipitated, size fractionated on 5% SDS-PAGE, and processed for immunoblot analysis as described below. An autoradiographic exposure was obtained from the same immunoblot to determine phosphate incorporation into the NuMA protein as described by Compton and Luo (1995).

### Immunological techniques

Indirect immunofluorescence microscopy was performed essentially as described by Compton et al. (1991). After incubation, 5 µl of the extract was diluted into 20 µl of KPM(+) buffer, spotted onto a poly-L-lysine coated glass coverslip and fixed by immersion in -20°C methanol. Following fixation, primary antibodies were added to the samples and incubated for 30 minutes at room temperature in a humidified chamber. Coverslips were washed in TBS (10 mM Tris-HCl, pH 7.5, 0.15 M NaCl) containing 1% bovine serum albumin and the bound antibodies were detected with the appropriate Texas Red-conjugated secondary antibodies (Vector labs, Burlingame, CA). Coverslips were mounted in FITC-Guard (Testog, Chicago, IL) and observed with a Nikon Optiphot microscope equipped for epifluorescence.

For western blots, proteins were separated by size by SDS-PAGE and transferred to PVDF membrane (Millipore Corp., Bedford MA). This membrane was blocked in TBS containing 5% nonfat milk for 30 minutes at room temperature, and the primary antibody incubated for 4 hours at room temperature in TBS containing 1% nonfat milk. Non-bound primary antibody was removed by washing three times for 3 minutes each in TBS and the bound antibody was detected using horseradish peroxidase-conjugated Protein A or horseradish peroxidase-conjugated goat anti-mouse (Bio-Rad Co., Hercules, CA). Non-bound Protein A was removed by washing three times for 3 minutes each in TBS and the bound Protein A was detected using enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

### Electron microscopy

The extract was prepared, incubated and sedimented as described under preparation of mitotic extracts, and the pellet was fixed with 2% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.4, for 12 hours at 4°C. Following fixation, the pellet was rinsed in 0.1 M Na-cacody-

late buffer, post-fixed with 1% OsO<sub>4</sub> in 0.1 M Na-cacodylate buffer for 30 minutes at room temperature, and en-bloc stained in 2% aqueous uranyl acetate. The sample was then dehydrated through a graded series of ethanol and propylene oxide, and embedded in Epon (LX112). Sections (60-70 nm) were prepared and stained with 2% uranyl acetate in methanol for 20 minutes followed by 5 minutes in Reynold's lead citrate. Micrographs were taken at 80 or 100 kV on a JEOL 100CX.

For the immunogold electron microscopy, the extract was prepared, incubated and sedimented as described above, and the pellet was then fixed in 1% glutaraldehyde in phosphate-buffered saline (PBS) for 30 minutes. All the following washes were performed for 15 minutes: the pellet was washed twice with PBS and reduced by 3 washes with 0.5 mg/ml NaBH<sub>4</sub>. The pellet was further washed once with PBS and once with PBS containing 1% bovine serum albumin (PBS + 1% BSA). The anti-NuMA rabbit polyclonal IgG (Gaglio et al., 1995) was then added at a concentration of 0.4 µg/ml in PBS + 1% BSA and incubated for 12 hours. The sample was then washed three times with PBS + 1% BSA, and incubated for 75 minutes with a 1/50 dilution of goat anti-rabbit conjugated with 5 nm gold particles (Nanoprobes Inc., Stony Brook, NY) in PBS + 1% BSA. The sample was then washed once with PBS + 1% BSA, twice with PBS, and fixed with 2% glutaraldehyde in 0.1 M Na-cacodylate for 20 minutes. Following fixation, the sample was rinsed twice in 0.1 M Na-cacodylate buffer, post-fixed with 1% OsO<sub>4</sub> in Na-cacodylate buffer for 30 minutes at room temperature and embedded in Epon (LX112). Sections (60-70 nm) were prepared and stained with 2% aqueous uranyl acetate for 10 minutes. Micrographs were taken at 80 or 100 kV on a JEOL 100CX.

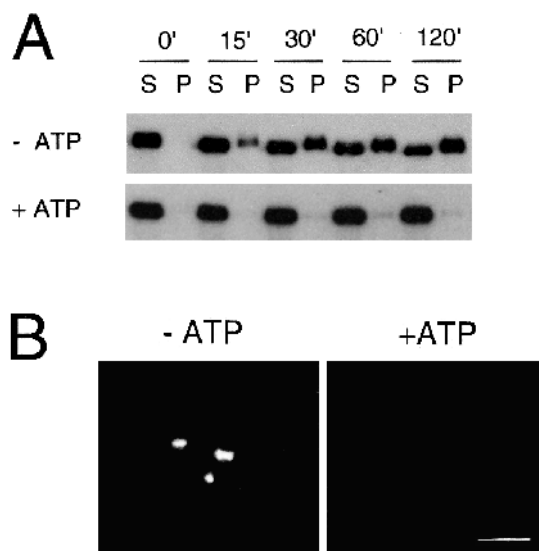
#### Histone H1 kinase assay

Histone H1 kinase activity was measured using 0.5 µl of the cell extract in a reaction containing 0.2 mg/ml of histone H1, 20 mM Tris-HCl, pH 7.2, 2 mM DTT, 1 mM EGTA, 10 µM ATP, and 2 µCi [ $\gamma$ -<sup>32</sup>P]ATP. Reactions were incubated at 30°C for 15 minutes and stopped by the addition of SDS-PAGE sample buffer. The histone was separated from the nonincorporated nucleotide on a 12.5% SDS-PAGE, the gel dried and exposed to X-ray film. Quantitation of the histone H1 kinase activity was performed by densitometric scanning of multiple different film exposures.

## RESULTS

#### Solubility properties of NuMA in a mitotic extract

Burke and Gerace (1986) have shown that under dephosphorylating conditions chromosomes will serve as substrates for the formation of structures resembling interphase nuclei in mitotic extracts prepared from synchronized CHO cells. Using synchronized HeLa cells, we have used a modification of their protocol to examine the behavior of the human NuMA protein under similar experimental conditions. NuMA is a soluble component of crude extracts (100,000 g supernatants) prepared from nocodazole-treated mitotic cells (Fig. 1A, time 0'; Gaglio et al., 1995; Kempf et al., 1994; Kallajoki et al., 1993; Maekawa et al., 1991). Upon incubation of this mitotic extract at 30°C, NuMA progressively shifts from the soluble fraction to the 10,000 g insoluble fraction. This dramatic change in the solubility of NuMA under these conditions is dependent on incubation time, is typically 50-60% efficient (ranging from 25% to 80% depending on the extract), and maximizes after approximately 120 minutes of incubation (Fig. 1A, -ATP). In addition, the conversion of NuMA from a soluble protein into an insoluble protein is dependent on temperature and the concentration of the mitotic extract because NuMA remains

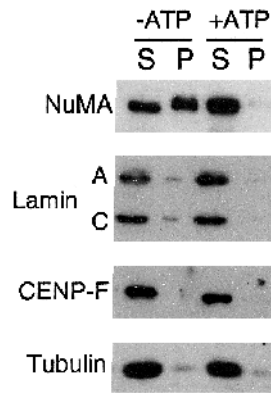


**Fig. 1.** The solubility of NuMA in a mitotic extract. (A) A mitotic extract was incubated at 30°C for the indicated times (minutes) either with (+ATP) or without (-ATP) the addition of 3 mM ATP. Following each incubation, the extract was separated into soluble (S) and insoluble (P) fractions and immunoblotted for NuMA. (B) Indirect immunofluorescence microscopy was performed on the samples with and without ATP using a NuMA-specific antibody at the final time point in the incubation. Bar, 8.7 µm.

in the soluble fraction if the extract is either maintained on ice or prepared at (or diluted to) 50% the concentration (data not shown). Finally, examination of the extract by immunofluorescence microscopy following incubation at 30°C for 90 minutes reveals that NuMA is present in numerous, readily detectable particles that have a somewhat irregular shape with an average diameter of 1-2 µm (Fig. 1B, -ATP).

Previously, Burke and Gerace (1986) demonstrated that addition of exogenous sources of ATP inhibited the formation of nuclei in CHO mitotic cell extracts. To determine if the conversion of NuMA from the soluble fraction to the insoluble fraction is inhibited by the addition of exogenous ATP we followed the solubility of NuMA by both immunoblot and immunofluorescence microscopy in the HeLa cell mitotic extract supplemented with ATP. Initially, the endogenous ATP concentration in the mitotic extract prepared under the conditions described here is 300 µM, and it diminishes to <30 µM within 15 minutes of incubation at 30°C (as determined by luciferase assay; data not shown). Addition of exogenous ATP to 3 mM is sufficient to maintain the ATP concentrations in the extract at >2.5 mM during incubation of the mitotic extract at 30°C for 120 minutes (data not shown). Examination of the solubility of NuMA by immunoblot reveals that the conversion of NuMA from the soluble fraction to the insoluble fraction is severely inhibited when the mitotic extract is supplemented with ATP (Fig. 1A, +ATP). Consistent with this result, no NuMA-specific signal was detectable by immunofluorescence microscopy (Fig. 1B, +ATP). Addition of ATP to the extract after the conversion of NuMA from the soluble fraction to the insoluble fraction led to the partial (~50%) reversion of NuMA from the insoluble fraction to the soluble fraction indicating that the process is partially reversible by ATP. The inhibitory

**Fig. 2.** Conversion of NuMA from the soluble fraction to the insoluble fraction in the extract is a specific process. The mitotic extract was incubated at 30°C for 120 minutes either with (+ATP) or without (-ATP) the addition of 3 mM ATP. The reactions were then separated into soluble (S) and insoluble (P) fractions and immunoblotted for NuMA, lamins A and C, CENP-F, and tubulin as indicated.



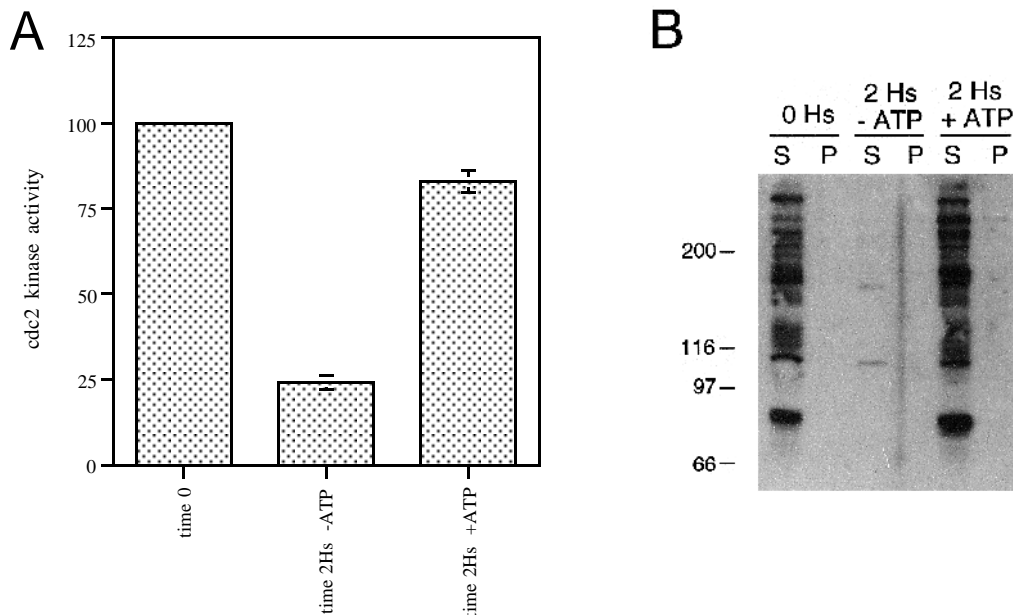
effect of ATP is most likely due to a requirement for ATP hydrolysis because addition of 3 mM AMP-PNP does not alter the conversion of NuMA from the soluble fraction to the insoluble fraction (data not shown). Taken together, these results show that large, insoluble, NuMA-containing particles form spontaneously in a mitotic extract upon incubation at 30°C, and that the formation of these particles is severely inhibited by the presence of ATP.

To determine if additional proteins undergo an alteration in solubility similar to NuMA under these conditions, we have performed immunoblot analysis of the 10,000 g soluble and insoluble fractions obtained following incubation of the extract at 30°C for 120 minutes with antibodies recognizing a variety of cellular proteins (Fig. 2). We have analyzed protein components of the nuclear matrix (CENP-F, Fig. 2; matrin 3, 4, and F, data not shown), coiled bodies (coilin, data not shown),

nucleolus (fibrillarin, B23, C23, data not shown), nuclear lamina (Fig. 2), and cytoskeleton ( $\alpha$ -tubulin, Fig. 2; vimentin, actin, Eg5, and  $\gamma$ -tubulin, data not shown). With the exception of actin (see below), we did not observe an alteration in the solubility of any of these proteins in the mitotic extract following incubation at 30°C in either the presence or absence of exogenously added ATP (Fig. 2). Actin was reproducibly converted from the soluble fraction to the insoluble fraction with an efficiency of ~40% in the presence or absence of ATP (data not shown). Thus, the formation of large, insoluble, NuMA-containing particles in this mitotic extract is a specific property of the NuMA protein, and does not represent the non-specific aggregation of proteins in the extract.

### NuMA's solubility is regulated by phosphorylation

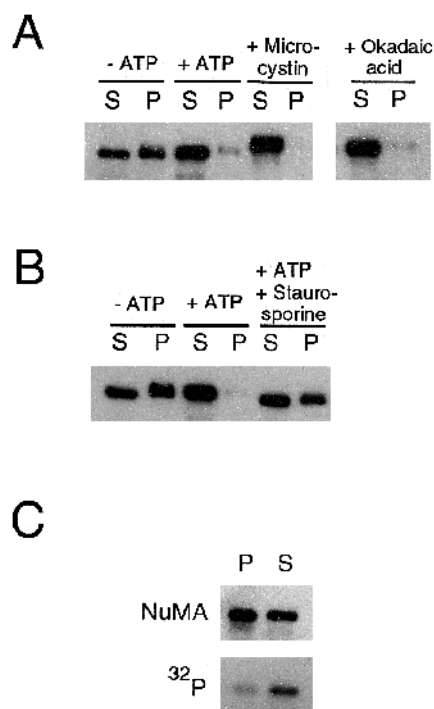
NuMA is an insoluble component of the nuclear matrix during interphase, and the formation of insoluble, NuMA-containing complexes under these conditions suggested that the mitotic extract may be exiting mitosis during the incubation. To test this directly we performed two different assays aimed at determining the mitotic state of the extract. First, we determined the relative activity of p34cdc2/cyclin B using histone H1 as a substrate (Fig. 3A). Initially, the activity of p34cdc2/cyclin B is high, consistent with the extract being prepared from synchronized mitotic cells. Following incubation at 30°C for 120 minutes, the p34cdc2/cyclin B activity in the extract supplemented with ATP remained high, but the p34cdc2/cyclin B activity of the extract lacking ATP was reduced approximately 4- to 5-fold. The second assay that we used to examine the cell cycle state of the extract was to immunoblot the soluble and insoluble proteins with the mpm-2 monoclonal antibody which



**Fig. 3.** The extract is becoming non-mitotic during the incubation. (A) Phosphorylation of histone H1 was used to determine the cdc2 kinase activity in the extract as it is initially prepared (time=0) or after 2 hours incubation at 30°C either with (+ATP) or without (-ATP) the addition of 3 mM ATP. The results were normalized to the relative value of 100 using the time 0 sample, and are the average of three independent experiments. (B) The mitotic extract was separated into soluble (S) and insoluble (P) fractions prior to any incubation (0 Hs) or following incubation at 30°C for 2 hours either with (2 Hs, +ATP) or without (2 Hs, -ATP) the addition of 3 mM ATP. These fractions were then immunoblotted using the mpm-2 monoclonal antibody which recognizes mitosis-specific phosphoepitopes. The migration position of myosin (200),  $\beta$ -galactosidase (116), phosphorylase B (97), and bovine serum albumin (66), are indicated on the left and are indicated in kDa.

recognizes mitosis-specific phosphoepitopes (Fig. 3B; Davis et al., 1983). Consistent with the results reported by Davis et al. (1983), a large variety of proteins are recognized by the mpm-2 monoclonal antibody in the mitotic extract (Fig. 3B, time=0). A majority of these antigens remain reactive with the antibody following incubation of the ATP-supplemented extract for 120 minutes, but nearly all of the antigens become undetectable upon incubation of the extract in the absence of exogenously added ATP. Thus, based on the inactivation of p34cdc2/cyclin B and the loss of the mitosis-specific phosphoepitopes recognized by the mpm-2 monoclonal antibody, we conclude that the extract has become non-mitotic during the incubation period.

The exit of the extract from mitosis coupled with the inhibition of the formation of the insoluble, NuMA-containing particles by ATP hydrolysis suggested that the formation of these structures may be regulated by phosphorylation. Specifically, the conversion of NuMA from the soluble fraction to the insoluble fraction in the extract appears to correlate with protein dephosphorylation. To test if protein dephosphorylation is required for the formation of the insoluble, NuMA-containing particles we determined the solubility of NuMA in the mitotic extract in the presence of the protein phosphatase inhibitors microcystin and okadaic acid (Fig. 4A). Immunoblot



**Fig. 4.** NuMA's solubility is regulated by phosphorylation. (A and B) The mitotic extract was separated into soluble (S) and insoluble (P) fractions following incubation at 30°C for 2 hours in the absence of ATP (-ATP) or in the presence of either 3 mM ATP, 1 μM microcystin, 2 μM okadaic acid, or 80 μM staurosporine and 3 mM ATP as indicated. These fractions were then immunoblotted for NuMA. (C) NuMA was radiolabeled by a brief incubation of the mitotic extract with [ $\gamma$ -<sup>32</sup>P]ATP, the reaction performed by incubation at 30°C for 120 minutes, and NuMA immunoprecipitated from either the soluble fraction (S) or the insoluble fraction (P). The identical gel was immunoblotted for NuMA and an autoradiographic exposure to film was taken to determine the relative <sup>32</sup>P content as indicated.

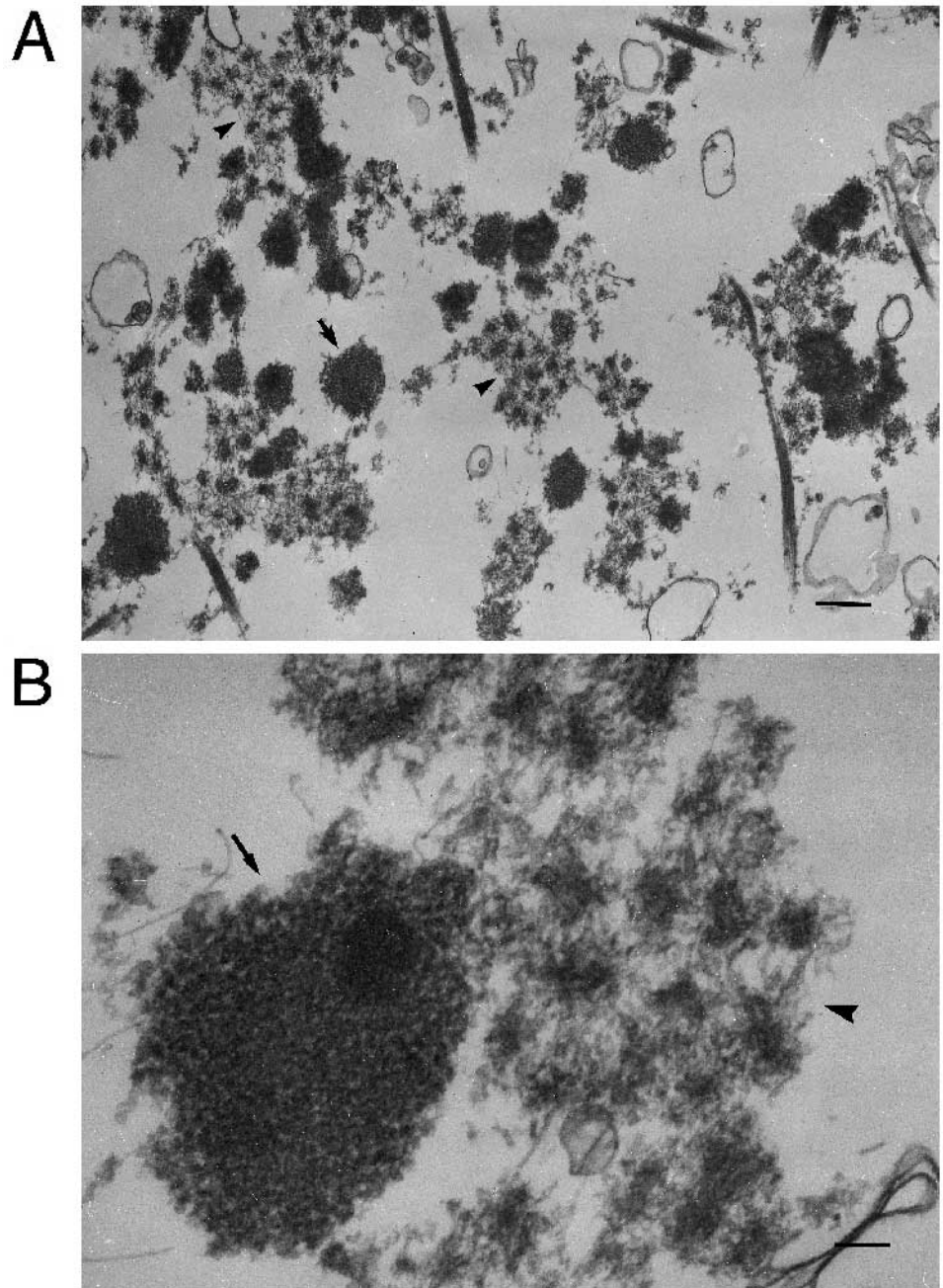
analysis of the soluble and insoluble fractions produced following incubation at 30°C for 120 minutes demonstrates that either 1 μM microcystin or 2 μM okadaic acid is sufficient to completely inhibit the formation of the insoluble, NuMA-containing particles. The inhibitory effect of these protein phosphatase inhibitors is similar to the inhibitory effect of ATP and occurs despite the fact that no exogenous ATP was added to these extracts. Thus, these results indicate that protein dephosphorylation is required for the formation of the insoluble, NuMA-containing particles in the extract under these conditions.

Based on the fact that protein dephosphorylation is required for the formation of the insoluble, NuMA-containing structures, we reasoned that protein kinases must be required to maintain NuMA in the soluble state. To test this idea we determined the solubility of NuMA in the mitotic extract in the presence of the protein kinase inhibitor staurosporine (Fig. 4B). Immunoblot analysis of the soluble and insoluble fractions produced following incubation demonstrates that addition of 80 μM staurosporine to the mitotic extract is sufficient to promote the formation of the insoluble, NuMA-containing particles. The promotion of the formation of the insoluble NuMA-containing structures by the inhibition of protein kinases using staurosporine occurred despite the presence of high concentrations of exogenously added ATP demonstrating that the inhibitory effect of ATP hydrolysis observed earlier is most likely due to phosphorylation reactions catalyzed by protein kinases. Thus, these data indicate that protein kinase activity is essential to maintain NuMA in a soluble state in a mitotic extract.

To determine if the NuMA protein is being dephosphorylated during the formation of the insoluble complexes we examined the relative phosphorylation state of NuMA in the soluble and insoluble fractions. NuMA was radiolabeled by a brief preincubation at 30°C in the presence of [ $\gamma$ -<sup>32</sup>P]ATP, the ATP was then removed by the addition of apyrase, the insoluble structures formed by incubation at 30°C for 120 minutes, and the NuMA protein was immunoprecipitated from either the soluble fraction or the insoluble fraction (Fig. 4C). Immunoblot analysis of the immunoprecipitates demonstrates that equal quantities of the NuMA protein have been immunoprecipitated from both soluble and insoluble fractions of the extract. Autoradiographic exposure of the immunoblot, however, shows that NuMA derived from the insoluble fraction contains approximately 3× less phosphate relative to NuMA derived from the soluble fraction. Thus, the NuMA protein is dephosphorylated during the formation of the insoluble NuMA-containing particles.

#### Ultrastructure of the NuMA-containing particles

To thoroughly characterize the insoluble structures formed by NuMA under these conditions, we examined their ultrastructure by transmission electron microscopy (TEM). For this analysis we fixed, embedded and sectioned the insoluble pellet fraction produced following incubation of the mitotic extract at 30°C for 120 minutes. Examination of this pellet at high resolution by TEM reveals four predominant structures (Fig. 5). First, there are membrane vesicles that are present to varying degrees depending on the particular extract and are probably contaminants from the 100,000 g centrifugation step used for the preparation of the mitotic extract. Second, there are

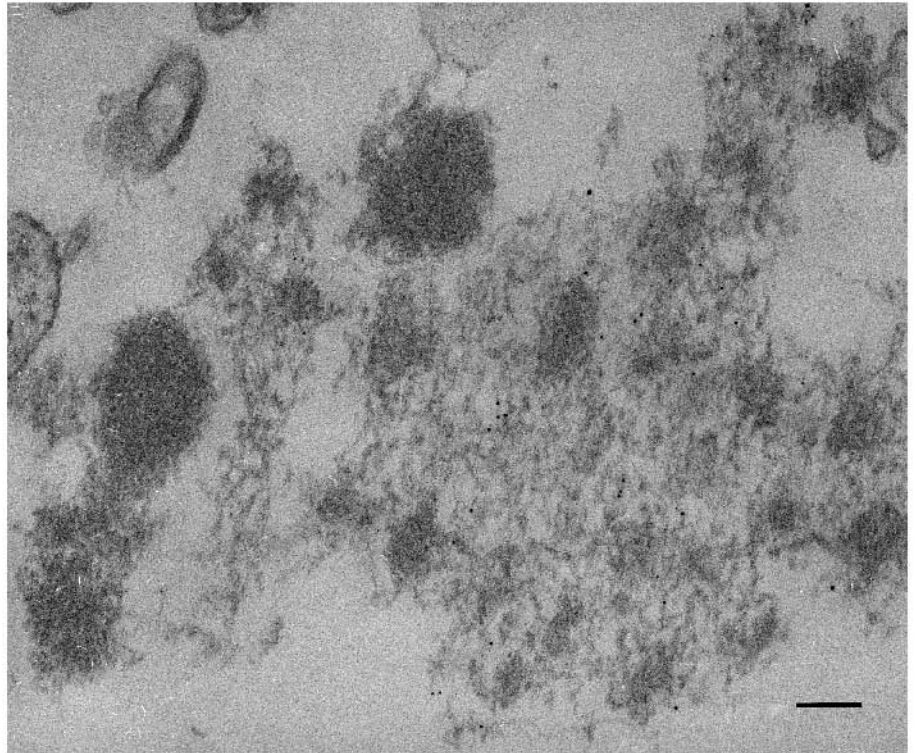


**Fig. 5.** Transmission electron microscopic analysis of the insoluble pellet formed following the incubation of the mitotic extract at 30°C for 120 minutes. Arrows point to the dense spherical particles and arrowheads point to the particles composed of an interconnected network of foci. Bars: 430 nm (A), 86 nm (B).

extensive bundles of filaments whose morphology is consistent with actin fibers. The observation of actin fibers by TEM agrees with our observation that, like NuMA, a significant proportion of actin is converted from a soluble form to an insoluble form during the incubation period. Finally, there are two different classes of particles that have diameters ranging from 0.5-2  $\mu\text{m}$ . One class of particle has a relatively uniform spherical shape and diameter ( $\sim 0.5 \mu\text{m}$ ) with a biphasic texture composed of a dense central core surrounded by a granular layer (Fig. 5A and B, arrows). These particles resemble pre-nucleolar bodies described in other systems (Bell and Scheer, 1996; Trimbur and Walsh, 1993) and will be referred to throughout this article as dense spherical particles. The second class of particle is less uniform in size and shape, has a more

open organization, and is composed of a complicated three-dimensional network of interconnected foci (Fig. 5A and B, arrowheads). The individual foci are approximately 70 nm in diameter and separated from the neighboring foci, on average, by 90 nm. Except for the contaminating vesicles all of these structures were resistant to extraction with Triton X-100 (data not shown).

To determine which of the particles in the insoluble fraction contain NuMA we performed immunogold electron microscopy using a rabbit antibody that is specific for the human NuMA protein. Fig. 6 shows that the gold particles are distributed relatively uniformly, and almost exclusively throughout the particles composed of the interconnected network of foci. Less than 1% of the gold particles were found



**Fig. 6.** NuMA is contained exclusively in the particles composed of interconnected foci. The insoluble pellet was formed by incubation of the mitotic extract at 30°C for 120 minutes, and NuMA was localized by immunogold electron microscopy using the NuMA-specific rabbit serum followed by 5 nm gold-conjugated goat anti-rabbit serum. Bar, 86 nm.

distributed in other areas of the section, and no significant decoration of either the vesicles, actin fibers, or dense spherical particles was observed. The gold particle decoration of the particles composed of interconnected foci by the NuMA-reactive antibody was specific because no significant decoration of any structure was observed if we eliminated the primary antibody from the reaction (data not shown). Thus, the NuMA protein is restricted to the particles that are composed of the interconnected array of foci.

To determine if the formation of these complex particles requires the NuMA protein, we have used the NuMA-specific antibody to deplete NuMA from the extract prior to incubation at 30°C (Fig. 7). As a control, we treated the extract in an identical manner using a preimmune antibody. Immunoblot analysis of the immune pellets and the subsequent supernatants following depletion with either the NuMA-specific antibody or the preimmune antibody indicates that >98% of the NuMA protein is depleted from the extract using the NuMA-specific antibody while the preimmune antibody has no effect on the distribution of NuMA (Fig. 7A). Three sequential immunodepletion steps are required to efficiently deplete NuMA from the extract, and each of these sequential immune pellets is analyzed separately in Fig. 7A. Following the depletion of the extract with the preimmune serum a significant fraction of NuMA was converted to an insoluble form upon incubation in the absence of ATP indicating that the three sequential depletion steps had not altered the activity of the mitotic extract (Fig. 7A, preimmune, lane -ATP). Furthermore, NuMA is the only protein depleted from these extracts because both we and others have demonstrated that NuMA is not in a pre-assembled protein complex in nocodazole-treated mitotic cell extracts (Gaglio et al., 1995; Kempf et al., 1994; Kallajoki et al., 1993).

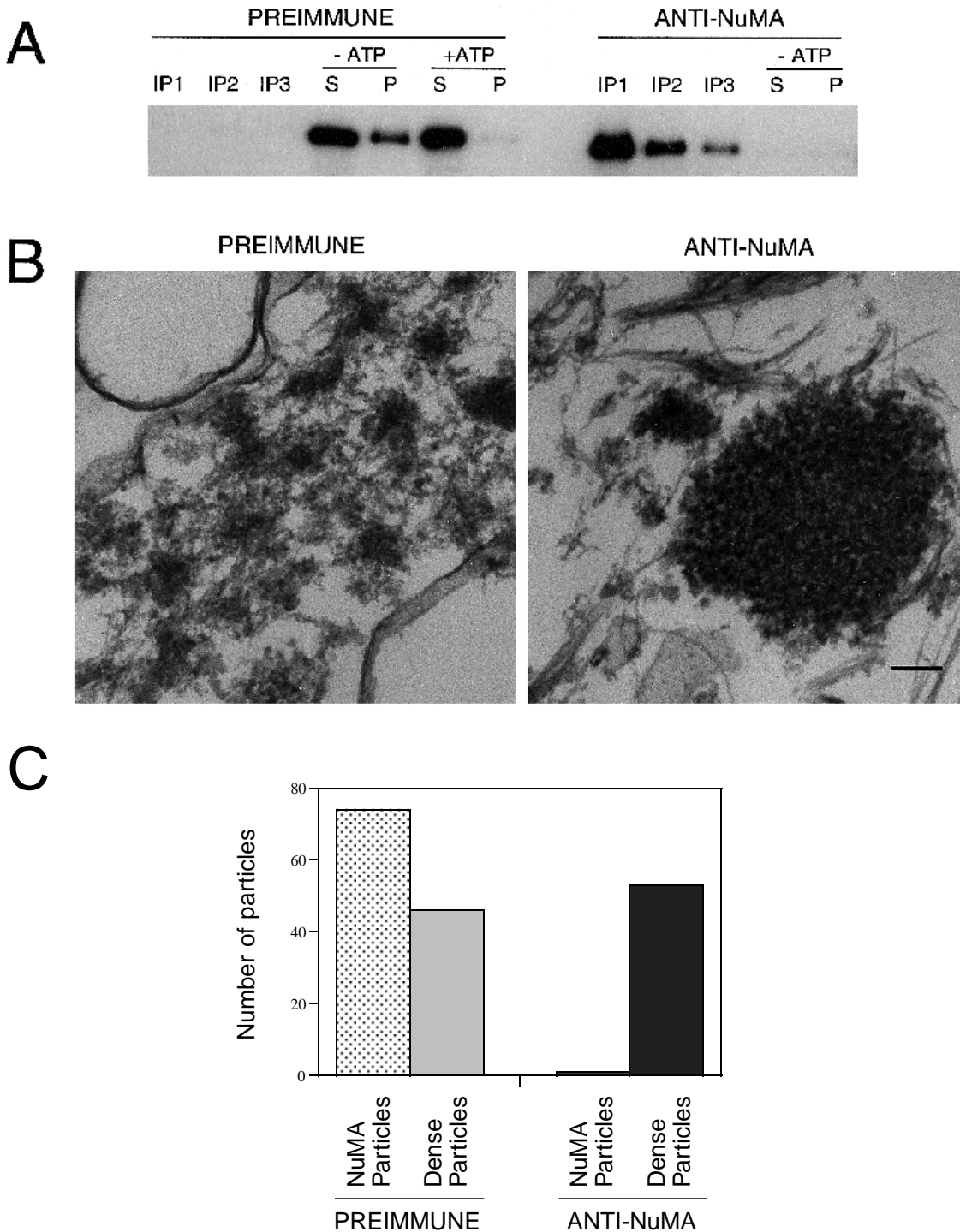
Following the immunodepletion steps we collected the

supernatant, incubated the samples at 30°C for 120 minutes, and performed TEM on the insoluble fraction (Fig. 7B). Immunodepletion with the preimmune antibody resulted in no detectable difference in the morphology of the actin filaments, the dense spherical particles, or the NuMA-containing particles. Following the depletion of NuMA, however, the particles composed of interconnected foci that contain NuMA were virtually absent (Fig. 7B). In contrast, the actin filaments and the dense spherical particles appeared to form normally and were morphologically indistinguishable from their counterparts formed in the extract that has been depleted using the preimmune antibody. To quantitate the effects of the depletion of NuMA from the extract, we compared the number of dense spherical particles to the number of NuMA-containing particles in the extracts depleted with either the preimmune antibody or the NuMA-specific antibody (Fig. 7C). Following the depletion of the mitotic extract with the preimmune antibody the NuMA-containing particles slightly outnumber the dense spherical particles (ratio = 1.6). In the absence of NuMA, however, there are approximately 70× fewer NuMA-containing particles relative to the dense spherical particles (ratio = 0.014). Thus, these data demonstrate that NuMA is an essential structural component of the particles composed of the complex, interconnected foci.

## DISCUSSION

The NuMA protein progresses through a complex series of solubility changes as it changes localization during mitosis (Fig. 8, *in vivo*). During interphase, NuMA is an insoluble component of the nuclear matrix. When the nucleus disassembles at the onset of mitosis, NuMA is rendered soluble and

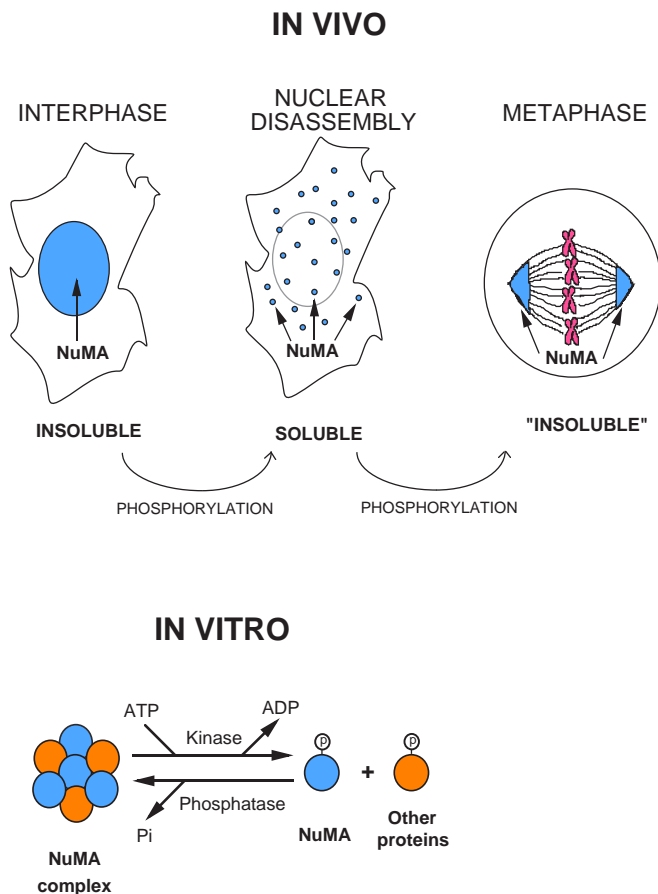




**Fig. 7.** NuMA is an essential structural component of the particles composed of interconnected foci. (A) The mitotic extract was depleted using either a preimmune antibody or the NuMA-specific antibody as indicated. Immunoblot analysis of the immune pellets derived from the three sequential immunodepletion (IP1, 2, and 3), and the soluble (S) and insoluble (P) fractions produced following incubation at 30°C for 120 minutes in the absence of ATP (–ATP) or presence (+ATP) of 3 mM ATP shows the distribution of NuMA and verifies that the depletion has been ~98% efficient. (B) Transmission electron microscopy of the insoluble pellet produced following the depletion of the mitotic extract with either the preimmune antibody or the NuMA-specific antibody as indicated. Bar, 86 nm. (C) The effects of NuMA depletion were quantitated from two separate experiments by counting the relative number of the NuMA-containing particles and the dense spherical particles following the depletion of the mitotic extract with either the preimmune antibody or the NuMA-specific antibody.

targets to the microtubules of the mitotic spindle where it becomes insoluble by virtue of its attachment to the micro-

tubules. If microtubule polymerization is prevented, however, NuMA remains soluble in mitosis following nuclear envelope



**Fig. 8.** The solubility of NuMA is regulated by phosphorylation. In vivo, NuMA progresses through at least two discrete phosphorylation events during mitosis as it moves from the nuclear matrix through a transient soluble intermediate to the mitotic spindle pole. The in vitro reaction described here demonstrates that the maintenance of NuMA in the soluble state requires active protein phosphorylation by protein kinases. On the other hand, if protein phosphatase activity predominates then NuMA and additional cellular proteins are converted through a specific process into large insoluble complexes. The formation of these complexes probably represents the conversion of NuMA from the soluble mitotic form to the insoluble interphase form.

break down and it is under these conditions that we prepare the mitotic extract. Surprisingly, we find that upon incubation in the absence of exogenously added ATP the extract becomes non-mitotic and NuMA spontaneously assembles into specific, insoluble particles. These results, in combination with the previous findings of Burke and Gerace (1989) demonstrating that several aspects of interphase nuclei form in somatic cell mitotic extracts under similar experimental conditions, lead us to conclude that this cell free system is mimicking the reaction by which NuMA changes from a soluble protein in mitosis to an insoluble protein in interphase (Fig. 8, in vitro).

By manipulating the mitotic extract in this manner, we have generated evidence showing that the cell cycle-dependent changes in NuMA's solubility are regulated by phosphorylation (Fig. 8, in vitro). Both we and others have shown that NuMA is a phosphoprotein that undergoes (at least) two discrete phosphorylation events during the progression of

mitosis (Price and Pettijohn, 1986; Compton and Luo, 1995; Gaglio et al., 1995; Hsu and Yeh, 1996; Sparks et al., 1995). The sum of these data indicate that NuMA undergoes one phosphorylation event at the onset of mitosis concomitant with nuclear envelope breakdown and a second phosphorylation event upon association with the mitotic spindle (Gaglio et al., 1995; Hsu and Yeh, 1996). Following the completion of mitosis, protein phosphatases revert NuMA to its least phosphorylated state, but only after it has been imported into the nucleus in the subsequent G<sub>1</sub> phase of the cell cycle (Sparks et al., 1995). The correlation between NuMA's phosphorylation status and the timing of nuclear disassembly and reassembly combined with the present data demonstrating that dephosphorylation catalyzes the conversion of NuMA from a soluble protein to an insoluble protein indicates that there may be both spatial and temporal regulation to the cell cycle-dependent phosphorylation of NuMA. Indeed, our results would suggest that by spatially limiting the dephosphorylation of NuMA to the nuclear interior following the completion of mitosis, the possibility of accumulating the insoluble form of NuMA in the cell cytoplasm at the end of mitosis would be eliminated.

At present, the identity of the protein kinases and/or phosphatases responsible for the regulation of NuMA's dynamic behavior are unknown. NuMA is phosphorylated by p34cdc2/cyclin B (Hsu and Yeh, 1996), and mutant forms of NuMA lacking the p34cdc2/cyclin B phosphorylation sites do not target to the mitotic spindle suggesting that this phosphorylation event plays a key role in the targeting of NuMA (Compton and Luo, 1995). These mutant forms of NuMA, however, remain insoluble when dispersed throughout the cytoplasm of the mitotic cells as judged by extraction with Triton X-100. Thus, while p34cdc2/cyclin B-dependent phosphorylation appears important for some aspects of NuMA's mitotic regulation, there are probably additional protein kinases acting on NuMA that promote its release from the nuclear compartment when the nucleus breaks down at the onset of mitosis. The combination of the experimental system described here and the experimental system developed for NuMA's role in organizing the mitotic spindle (Gaglio et al., 1995) should permit a detailed investigation into the identity and role of protein kinases and phosphatases that modulate NuMA's activity.

### NuMA is a structural protein

The primary amino acid sequence of NuMA contains an extensive region predicted to fold into a  $\alpha$ -helical coiled-coil (Compton et al., 1992; Yang et al., 1992; Maekawa and Kuriyama, 1993; Tang et al., 1993) suggesting that NuMA performs a structural function in the cell. Consistent with this view we show here that NuMA is an essential structural component of large, insoluble particles that form spontaneously when the extract becomes non-mitotic. These particles are composed of a dense array of interconnected foci that form through a specific process because we could not identify any other proteins with similar solubility properties under these experimental conditions (actin undergoes a solubility change under these conditions, but it polymerizes independently of NuMA or ATP; see Fig. 5A). The organization of these particles from interconnected foci bears a remarkable resemblance to the structures that form when NuMA is expressed in the cell cytoplasm (Saredi et al., 1996), and

suggests that this type of organization may be the fundamental assembly pattern of the NuMA protein. The complex structures we observe using these two independent methods, however, differ markedly from the simple parallel, coiled-coil dimers that are formed by the recombinant NuMA protein expressed in bacteria and purified following denaturation with urea (Harborth et al., 1995). The most likely explanation for this discrepancy is that the parallel dimers formed by the purified recombinant protein are the first step in the assembly process for NuMA, and the more complex structures that we have observed under more physiological conditions (this report and that of Saredi et al., 1996) are derived from those simple dimeric molecules.

It is important to note, however, that the structures formed in the mitotic extract under the conditions described here and following the expression of NuMA in the cell cytoplasm are not identical. They share a common organization in that they are large, insoluble particles composed of interconnected foci, but they differ in fine detail. For example, the individual foci have larger diameters when NuMA is assembled by dephosphorylation in the mitotic extract compared to the foci formed by NuMA in the interphase cytoplasm in the presence of either taxol or nocodazole. Furthermore, the resolution of a filamentous network separating each of the foci which is clearly evident following the expression of NuMA in the cell cytoplasm is not well resolved in the structures described here. We attribute these structural differences to the presence of additional cellular proteins present within the NuMA-containing particles formed following dephosphorylation in the mitotic extract that are not present in the particles formed by NuMA when it is expressed in the cell cytoplasm. Support for this interpretation comes from the fact that the particles formed by NuMA when it is expressed in the cell cytoplasm are biochemically pure, and are composed exclusively of NuMA (Saredi et al., 1996). Unfortunately, we have not been able to determine if additional cellular proteins are associated with the NuMA particles produced in the mitotic extract because the polymerization of extensive actin-containing arrays renders the extract too viscous to successfully purify the NuMA-containing particles from the mitotic extract.

In summary, the results presented here support the conclusion that NuMA serves a structural role in the cell. Prevailing evidence indicates that one structural role that NuMA fulfills is for the organization of microtubule minus ends at the polar ends of the mitotic spindle (Gaglio et al., 1995, 1996; Merdes et al., 1996; Yang and Snyder, 1992; Kallajoki et al., 1991). The question as to whether NuMA provides an independent structural function within the nucleus during interphase is still unresolved. NuMA is expressed and localized in nuclei of terminally differentiated (non-dividing) cells (Kallajoki et al., 1992; Tang et al., 1993) suggesting a specific nuclear role, however, studies using both cultured cells and *Xenopus* egg extracts have found that nuclei assemble with grossly normal three-dimensional morphology in the absence of NuMA (Yang and Snyder, 1992; A. Merdes and D. W. Cleveland, personal communication). These data suggest that if NuMA serves a structural function within the cell nucleus during interphase it is either non-essential for the three-dimensional architecture of the nucleus or covered by an as yet unidentified redundant structural mechanism. Alternatively, NuMA may be sequestered in the cell nucleus during

interphase as part of its spatial and temporal regulation. Our current findings showing that NuMA forms insoluble structures upon dephosphorylation combined with our previous results showing that NuMA may perturb microtubule structure in the cytoplasm during interphase (Saredi et al., 1996) provides ample justification for sequestering NuMA in the nucleus during interphase.

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