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Phosphorylation of NUMA occurs during nuclear breakdown and not mitotic spindle assembly

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SUMMARY

NuMA, the nuclear mitotic apparatus protein, is a component of the nuclear matrix at interphase that redistributes to the spindle poles at mitosis. While the function of NuMA is not known, it has been implicated in spindle organization during mitosis and nuclear reformation. Phosphorylation is thought to play a regulatory role in NuMA function. In this study, NuMA phosphorylation was examined through the cell cycle using highly synchronized cells. In intact cells labeled with ^{32}P-orthophosphate, NuMA appeared as a 250 kDa phosphoprotein in interphase that shifted to a higher apparent molecular mass in mitosis. The shift was due to phosphorylation as shown by reduction of the shifted band to interphase mobility by phosphatase treatment. This phosphorylation event occurred roughly at the G_2/M transition at the time of NuMA's release from the nucleus and its redistribution to the mitotic spindle. However, mitotic phosphorylation did not require spindle formation since the phosphorylated species was detected in nocodazole-treated cells lacking microtubule spindles. Dephosphorylation of NuMA occurred in two distinct steps, after lamin B assembled into the nuclear lamina, in early G_1 and at the end of G_1. Based on the timing of the phosphorylation and dephosphorylation observed in this study, we propose that they may play a role in nuclear events such as nuclear organization, transcription, or initiation of DNA replication at G_1/S.

Key words: NuMA, phosphorylation, cell cycle, nucleus, mitosis, spindle

INTRODUCTION

NuMA was first identified by Lydersen and Pettijohn (1980) using antisera raised against HeLa cell nuclear matrix proteins and was characterized in several subsequent studies (Van Ness and Pettijohn, 1983; Pettijohn et al., 1984; Price et al., 1984; Price and Pettijohn, 1986). It was found to be a 240 kDa protein present in a wide variety of species (Price and Pettijohn, 1986). By immunofluorescence microscopy through the cell cycle, NuMA appeared as granular staining in the nucleus at interphase, redistributed to the spindle poles at metaphase, and returned to the reforming nucleus in telophase (see Fig. 7; also see Lydersen and Pettijohn, 1980; Kallajoki et al., 1991; Tousson et al., 1991; Compton et al., 1992; Yang et al., 1992; Tang et al., 1993). The full length cDNA was cloned by several groups (Compton et al., 1992; Yang et al., 1992; Maekawa and Kuriyama, 1993; Tang et al., 1993) and the amino acid sequence predicted one of the largest known coiled-coil proteins flanked by non-coiled ends (Yang et al., 1992).

NuMA is a component of the nuclear matrix (Lydersen and Pettijohn, 1980; Compton et al., 1991; Kallajoki et al., 1991; Sparks et al., 1993). The nuclear matrix was first described by Berezney and Coffey (1974, 1977) as the residual nuclear protein structure remaining following digestion of chromatin with DNase I and extraction with 2 M NaCl. NuMA appears to be a component of fibers that form core filaments of the nucleus, as shown by immuno-gold electron microscopy (Zeng et al., 1994). It has been known for some time (Robbins and Gonatas, 1964) that the nuclear structural proteins dissociate at mitosis and become dispersed throughout the cell. In the later stages of mitosis, these nuclear proteins reassociate with newly segregated chromosomes that later coalesce to become the interphase nucleus. However, the mechanism by which these events occur is still unknown.

The function of NuMA has been addressed in previous studies that centered on its role at the spindle and the nucleus (reviewed by Compton and Cleveland, 1994). Microinjection of NuMA antibodies into somatic cells disrupts spindle structure and mitotic progression (Yang and Snyder, 1992) or induces the formation of aberrant nuclear structures such as micronuclei (Compton et al., 1992; Kallajoki et al., 1993). The results from these previous functional analyses suggest that the protein may perform multiple roles in the cell, perhaps stabilizing the mitotic spindle and nuclear structures.

NuMA function and distribution during the mitotic cell cycle may be regulated by phosphorylation. The entry of

eukaryotic cells into mitosis is regulated by a cascade of kinase activities initiated by phosphorylation of MPF, a complex of cyclin B and cdc2 kinase (see King et al., 1994). One of the first events as cells enter mitosis is the phosphorylation of lamin B which leads to the disassembly of the nuclear lamina. As cells exit mitosis, lamin B dephosphorylation drives its reassembly into reforming nuclei (Gerace and Blobel, 1980; Miake-Lye and Kirschner, 1985; Burke and Gerace, 1986; Heald and McKeon, 1990; Peter et al., 1990; Ward and Kirschner, 1990). An analogous redistribution of NuMA occurs during mitosis suggesting that the protein may be a substrate for the same mitotic kinases. In fact, NuMA is a phosphoprotein in interphase (Price and Pettijohn, 1986) and undergoes additional phosphorylation in mitosis (Compton and Luo, 1995). NuMA is predicted to have over 24 consensus sites for a variety of kinases (Yang et al., 1992). However, a functional role for phosphorylation has not been directly demonstrated and the dephosphorylation of NuMA has not been investigated.

In this study, we find that NuMA undergoes multiple mitotic phosphorylation events that occur with similar timing to lamin B phosphorylation. The mitotic phosphorylation occurs in the absence of intact microtubule spindles. Dephosphorylation of NuMA occurs in two discrete steps in G_1 after it has assembled into the reforming nucleus. Thus, the timing of NuMA dephosphorylation does not correlate with spindle organization and function but appears to occur during nuclear events in G_1.

MATERIALS AND METHODS

Cell culture and synchronization

Chinese hamster ovary (CHO) cells were maintained in a 5% CO_2 incubator at 37°C in Dulbecco's minimal essential media (DMEM, Gibco-BRL/Life Technologies, Gaitherburg, MD) supplemented with 10% fetal calf serum or iron-supplemented calf serum (Hyclone, Logan, UT), 50 μg/ml streptomycin, and 50 U/ml penicillin. Mitotic CHO cells were prepared using two methods. For most experiments described in this study, mitotic cells were obtained using a drug-free shake-off protocol described in detail elsewhere (Jostes et al., 1980) that typically produce populations with mitotic indices between 97 and 99% (data not shown). These mitotic populations were frozen and stored according to the method of Borrelli et al. (1987). Cells frozen in this way show no significant reduction in viability following thawing and plating and progress rapidly and synchronously through the cell cycle. It should be noted that the electrophoretic mobility of NuMA was unaffected by this procedure (Fig. 6B, lane I*). A second method used to obtain mitotic cells involved arresting them at mitosis with nocodazole (5 μg/ml) for 4 hours and collecting them by shaking manually. The nocodazole-blocked (N) mitotic cells consist of >90% prometaphase cells (data not shown; Zieve et al., 1980).

Preparation of cell extracts

Asynchronous CHO cells were grown to 95% confluency (5×10^6 cells per 100 mm plate) then washed three times in PBS to remove mitotic cells leaving >95% interphase (I) cells (see below). To obtain cells in various stages of the cell cycle, mitotic (M) cells (above) were thawed rapidly at 37°C, gently spun through 5 ml of ice-cold PBS at 30 ***g***, plated into 37°C medium, and pelleted at various periods of time after release (anaphase, 10 minutes; telophase, 25 minutes; G_{1E}, 60 minutes; and G_{1L}, 240 minutes). Samples of 5×10^6 cells were pelleted and lysed in a modified RIPA buffer (180 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0) with protease and phosphatase inhibitors (0.15 μg/ml phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml each of aprotinin, leupeptin, chymostatin, 5 mM EDTA, 2.5 mM beta-glycerol phosphate, 100 μM sodium vanadate) for ten minutes at 4°C. In some cases, phosphatase inhibitors were omitted (not shown). Relative protein concentrations were determined using a Coomassie assay (Bio-Rad, Hercules, CA).

Antibodies

Lamin B monoclonal, NuMA monoclonal and NuMA polyclonal antibodies were obtained from Matritech Inc. (Cambridge MA). Other antibodies were obtained from the following: tubulin monclonal antibody from Sigma Chemical Co. (DM1α, St Louis, MO), lamin B polyclonal antibodies from Dr H. Worman (Cance et al., 1992), pericentrin monoclonal antibodies from S. Doxsey and J. Burkhart (UCSF), and polyclonal pericentrin antibodies from S. Doxsey (Doxsey et al., 1994).

Orthophosphate labeling

CHO cells or ME180, human cervical carcinoma cells were grown in T-175 cm^2 flasks, washed twice in phosphate-free DMEM (Gibco-BRL) with 10% dialyzed serum, and incubated for 4 hours in phosphate-free DMEM with 10% dialyzed serum and ^{32}P-orthophosphoric acid (2 mCi/ml, Dupont/NEN Boston, MA). These cells were washed twice in cold PBS, and lysed at 4°C as described above. To obtain ^{32}P-labeled mitotic cells, cultures were treated with 5 μg/ml nocodazole for 4 hours, collected by mitotic shake-off, rinsed in cold PBS, and protein extracts were prepared from them as described above. An average of 10^4-10^5 mitotic cells was obtained as determined by cell counts done on unlabeled populations of CHO cells. Samples were immunoprecipitated and immunoblotted as described below. Blots were then exposed to reflection film (Dupont/NEN) at −70°C with an intensifying screen with exposure times varying between 6 hours for interphase sample lanes and 3 weeks for mitotic samples lanes.

Immunoprecipitation

Cell lysates (above) were cleared of non-specific binding by incubation with 20 μl of Protein A agarose beads (Gibco-BRL) for 30 minutes at 4°C in an orbital shaker. After preclearing, 3 μg of each polyclonal antibody was added to 250 μl of a cell extract and incubated for one hour in the cold in an orbital shaker. Immune complexes were collected by incubation with 30 μl of Protein A or G beads for 3 hours in the cold and pelleting the bead-protein complex by centrifugation at 10,000 ***g*** for 15 seconds. Beads were washed three times with lysis buffer then resuspended in 30 μl of SDS sample buffer.

Immunoblotting

In order to detect mitotic shifts (Figs 1, 2, 3, 6), 5% polyacrylamide gels (Laemmli, 1970) were run on 11-12 cm resolving gels until the 200 kDa myosin marker reached the bottom of the gel. SDS-PAGE was followed by transfer to Immobilon-P nylon (Millipore Corporation, Bedford, MA) using a semi-dry blotting apparatus (Owl Scientific, Cambridge, MA). Blots were incubated in 5% blocking agent (Bio-Rad) in TBS (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl) then exposed to antibodies for one hour in TBS containing 1% blocking agent and 1% bovine serum albumin (BSA) at room temperature. After three washes in TBST (TBS, 0.05% Tween-20), blots were incubated in secondary antibodies (goat anti-mouse or goat anti-rabbit alkaline phosphatase conjugate; Bio-Rad) for one hour at room temperature. Blots were washed 3× in TBST, once in TBS then incubated for 5 minutes in Immunlite chemiluminescence substrate (Bio-Rad) and exposed to reflection film (Dupont /NEN) for 5 minutes to 3 hours at room temperature.

Phosphatase treatment

After immunoprecipitation, bead pellets were washed three times in

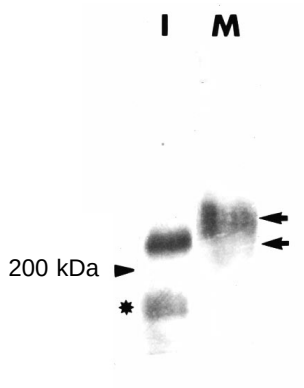


Fig. 1. Immunoprecipitation of NuMA from cells labeled with ^{32}P-orthophosphate. Protein extracts were prepared from interphase (I) and mitotic (M) CHO cells following incubation with ^{32}P-orthophosphoric acid. NuMA was immunoprecipitated, run out on 5% polyacrylamide gels, transferred onto Immobilon-P membranes and subjected to autoradiography. Arrows indicate electrophoretic shift in mobility of mitotic NuMA. Asterisk (*) indicates 180 kDa co-precipitating protein from interphase extracts.

phosphatase buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM DTT, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$) then incubated with 96 units of calf intestinal alkaline phosphatase (Sigma) at 37°C for 30 minutes (Cherniack et al., 1995). The pellets were washed three times in phosphatase buffer then resuspended in SDS sample buffer and analyzed by immunoblotting.

Immunofluorescence

CHO cells were cytospun onto poly-L-lysine coated coverslips or at 30 ***g*** for 10 minutes. Samples were fixed and extracted in 3.7% formaldehyde in CSK buffer (0.5% Triton X-100, 100 mM NaCl, 300 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM $MgCl_2$, 1 mM EGTA) for 15 minutes at room temperature or in –20°C methanol for 15 minutes. Isolated centrosomes were prepared as previously described (Doxsey et al., 1994). All subsequent steps were done at room temperature in a humid chamber. Coverslips were washed twice with PBS, and blocked in PBSAT (0.5% BSA and 0.1% Triton X-100 in PBS) for 5 minutes. Primary antibodies were diluted in PBSAT and incubated for

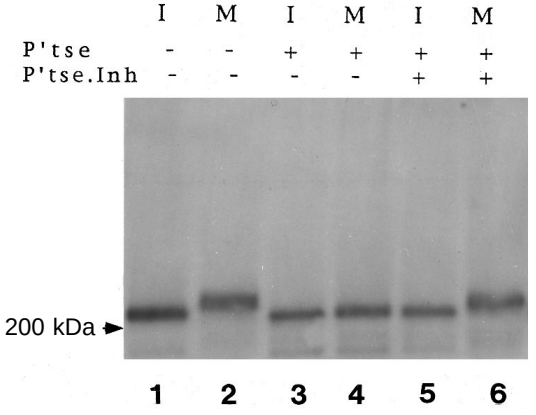


Fig. 2. Phosphatase sensitivity of NuMA. NuMA immunoprecipitates from interphase (I) and mitotic (M) CHO cells were treated under various conditions, run on SDS gels, transferred to Immobilon, and detected by chemiluminescence. Lanes 1 and 2, untreated samples; lanes 3 and 4, alkaline phosphatase-treated; lanes 5 and 6, treated with alkaline phosphatase in the presence of phosphatase inhibitors.

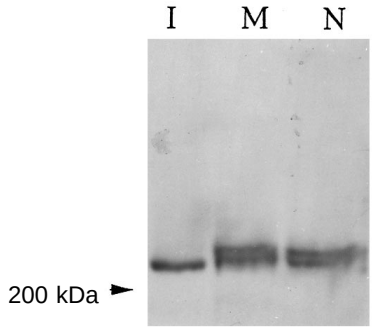


Fig. 3. NuMA phosphorylation in the absence of mitotic spindles. NuMA was immunoprecipitated from extracts prepared from interphase CHO cells (I), cells prepared by mitotic shake-off (M) and nocodazole-treated mitotic cells (N). Samples were blotted and probed as described for Fig. 2.

30 minutes. Coverslips were washed 3× in PBSAT and incubated in secondary antibodies, goat anti-mouse, rabbit, or rat conjugated to fluorescein or rhodamine (Jackson ImmunoResearch Labs, West Grove, PN) for 20 minutes. Coverslips were washed for one minute twice in PBSAT, once in PBS, once in PBS with 4′-6 diamidino-2-phenylindole (DAPI, 2 μg/ml, Sigma) to detect DNA, twice in PBS and mounted in glycerol with phenyline diamine (100 μg/ml, Sigma). Microscopy was done on an Axioplan immunofluorescence microscope (Carl Zeiss, Oberkochen, Germany) with a Photometrics 200 series CCD camera (Tuscon, Az).

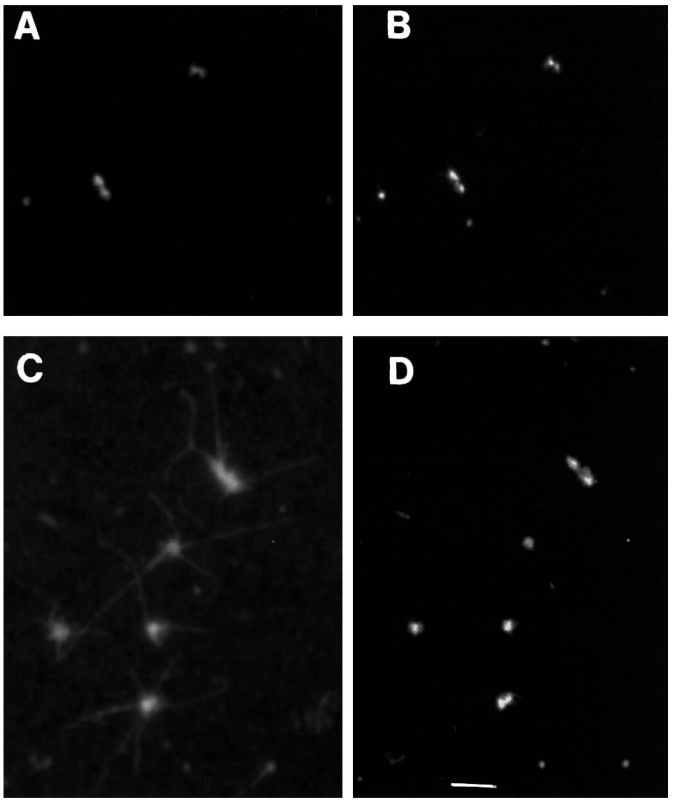


Fig. 4. A portion of NuMA is present in centrosomes at all cell cycle stages. Centrosomes isolated from log-phase CHO cells (Doxsey et al., 1994) were spun onto coverslips and costained with pericentrin (A) and NuMA (B). Isolated centrosomes with regrown microtubules (Doxsey et al., 1994), were stained for tubulin (C) and NuMA (D). Bar, 5 μm.

RESULTS

NuMA is phosphorylated as cells enter mitosis

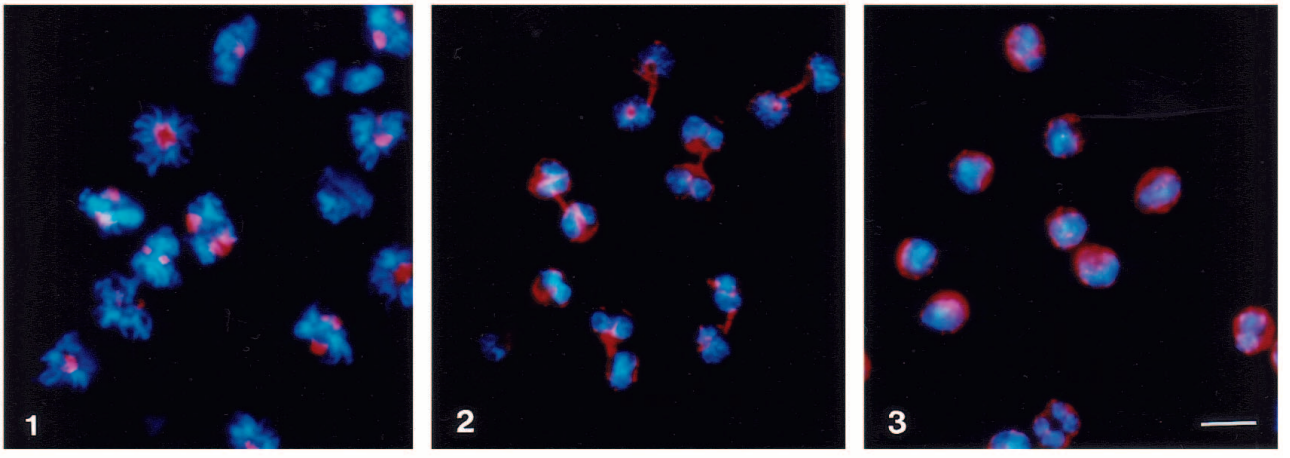
NuMA's phosphorylation state was initially determined in vivo by ^{32}P-orthophosphate labeling and immunoprecipitation. In interphase cells, a 250 kDa phosphoprotein was resolved on SDS-PAGE after autoradiography (Fig. 1, lane I). In addition, a phosphoprotein of approximately 180 kDa coprecipitated with NuMA (Fig. 1, asterisk), consistent with previous results (Price and Pettijohn, 1986). It is unlikely that the 180 kDa protein is a breakdown product of NuMA since this protein was not detected with several NuMA antibodies raised against different parts of the molecule (data not shown) nor when phosphatase inhibitors were omitted (data not shown). In mitotic cells labeled with ^{32}P-orthophosphate, NuMA appeared as a broader band of higher molecular mass than in interphase cells (lane M). Western blots confirmed that the interphase and mitotic phosphoproteins were NuMA (data not shown; Compton and Luo, 1995). Interestingly, the 180 kDa protein did not coprecipitate with NuMA in extracts prepared from mitotic cells.

To determine whether the mobility shift seen in mitosis was due to phosphorylation, immunoprecipitates from unlabeled cells were treated with alkaline phosphatase and western blotted (Fig. 2). Following optimization of the gel system for the resolution of the high molecular mass species (see Materials and Methods), alkaline phosphatase treatment demonstrated that the shifted mitotic protein was reduced to interphase mobility (lanes 2 and 4). Inclusion of phosphatase inhibitors in the preparation prevented the shift down to interphase mobility (lane 6) and controlled for contaminating proteolytic activity in the phosphatase preparation. The phosphatase sensitivity of NuMA demonstrated that the change in the electrophoretic mobility was the result of phosphorylation. The fact that the electrophoretic mobility of interphase NuMA was unaffected by phosphatase treatment (Fig. 2, lane 3) made it possible to study the mitosis-specific phosphorylation of NuMA in unlabeled cells.

NuMA phosphorylation occurs in the absence of an intact spindle

The timing of NuMA's mitotic phosphorylation was consistent with its release from the nucleus as cells enter mitosis (Price and Pettijohn, 1986; Kallajoki et al., 1991; Tousson et al., 1991; Compton et al., 1992; Yang et al., 1992; Tang et al., 1993). In fact, it is strikingly similar to the phosphorylation-dependent disassembly of lamin B from the nuclear lamina (Gerace and Blobel, 1980). However, it is also possible that the phosphorylation of NuMA occurs when it redistributes to the mitotic spindle. To determine whether NuMA's mitotic phosphorylation was the result of its association with the spindle apparatus, the phosphorylation state of the protein was examined in nocodazole-treated cells in which microtubule spindle formation was prevented and cells were arrested in prometaphase (Zieve et al., 1980). NuMA was phosphorylated (Fig. 3, lane N) to the same level as in metaphase cells with intact spindles (lane M) indicated by the consistent presence of bands with equivalent electrophoretic mobilities. In addition, a previously undetected doublet could be resolved in both cases indicating that multiple phosphorylation events had occurred. Phosphatase treatment confirmed that both shifted bands were phosphorylated species of NuMA (data not shown). To ensure that the mitotic spindles were disrupted by nocodazole treatment and to determine where NuMA was located under these conditions, immunofluorescence microscopy was performed (data not shown). In non-drug treated mitotic cells, normal microtubule spindles were observed with NuMA at the spindle poles. In nocodazole-treated mitotic cells lacking

A



B

Phase	0 minutes	60 minutes
Metaphase	85.7%	1.0%
Anaphase	10.2%	0.1%
Telophase	2.7%	0.3%
G_1	1.4%	98.6%

Fig. 5. Preparation of highly synchronized mitotic cells. (A) Fluorescence micrographs of non-drug treated metaphase cells incubated in media for 0, 25, or 60 minutes (panels 1, 2, 3, respectively) at 37°C. Cells were stained with a tubulin monoclonal antibody (red), and DAPI (blue). Bar, 20 μm. (B) Quantitation of synchrony. More than 2,000 cells were counted in five separate experiments and results are summarized as the percentage of cell population in each phase.

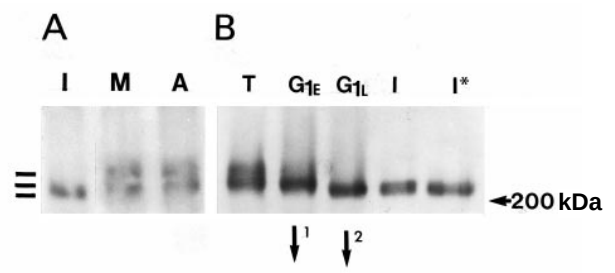


Fig. 6. Dephosphorylation of NuMA during the cell cycle. NuMA was immunoprecipitated from cells at various stages of the cell cycle and western blotted (see Fig. 2). Three forms of NuMA were detected (bars at left). (B) NuMA's downward shift in G_1 early (G_{1E}) and G_1 late (G_{1L}) is indicated by arrows 1 and 2. Cell cycle stages: (I) interphase cells in log phase; (*I) previously frozen interphase cells; (A) anaphase-t_{10}; (T) telophase-t_{25}; (G_{1E}) early G_1-t_{60}; and (G_{1L}) late G_1-t_{240}.

microtubule spindles, the majority of NuMA was dispersed throughout the cell. These data demonstrate that NuMA does not require spindle formation to undergo these mitotic phosphorylation events and indicate that the phosphorylation occurs prior to spindle assembly in metaphase, while NuMA is still in the nucleus. In addition, the presence of only the interphase form of NuMA in late G_2 cells (data not shown) indicates that the phosphorylation events occur at the G_2/M transition.

A fraction of NuMA associated with the centrosome in nocodazole-treated cells (data not shown) suggesting that it was an intrinsic centrosomal component. To examine this possibility, centrosomes were isolated from CHO cells and co-stained for NuMA and pericentrin. NuMA was detected at all centrosomes (Fig. 4B) stained with pericentrin (Fig. 4A). Centrosome localization of NuMA is more striking in preparations where microtubules are regrown from isolated centrosomes then fixed and co-stained with tubulin and NuMA antibodies (Fig. 4C,D). Under these conditions, NuMA is located at the center of microtubule asters. Other nuclear proteins such as lamin B did not localize to the centrosomes (data not shown). These results demonstrate that a small fraction of NuMA is present in centrosomes throughout the cell cycle. The phosphorylation state of this fraction of NuMA is not known.

NuMA is dephosphorylated in two distinct steps after mitosis

To better understand the role of phosphorylation in NuMA localization and function, we examined changes in the phosphorylation state of the protein as cells exit mitosis. To precisely correlate morphological and biochemical events during mitosis, synchronized cell populations were used (Fig. 5A). Quantitative analysis demonstrated that a highly enriched population of

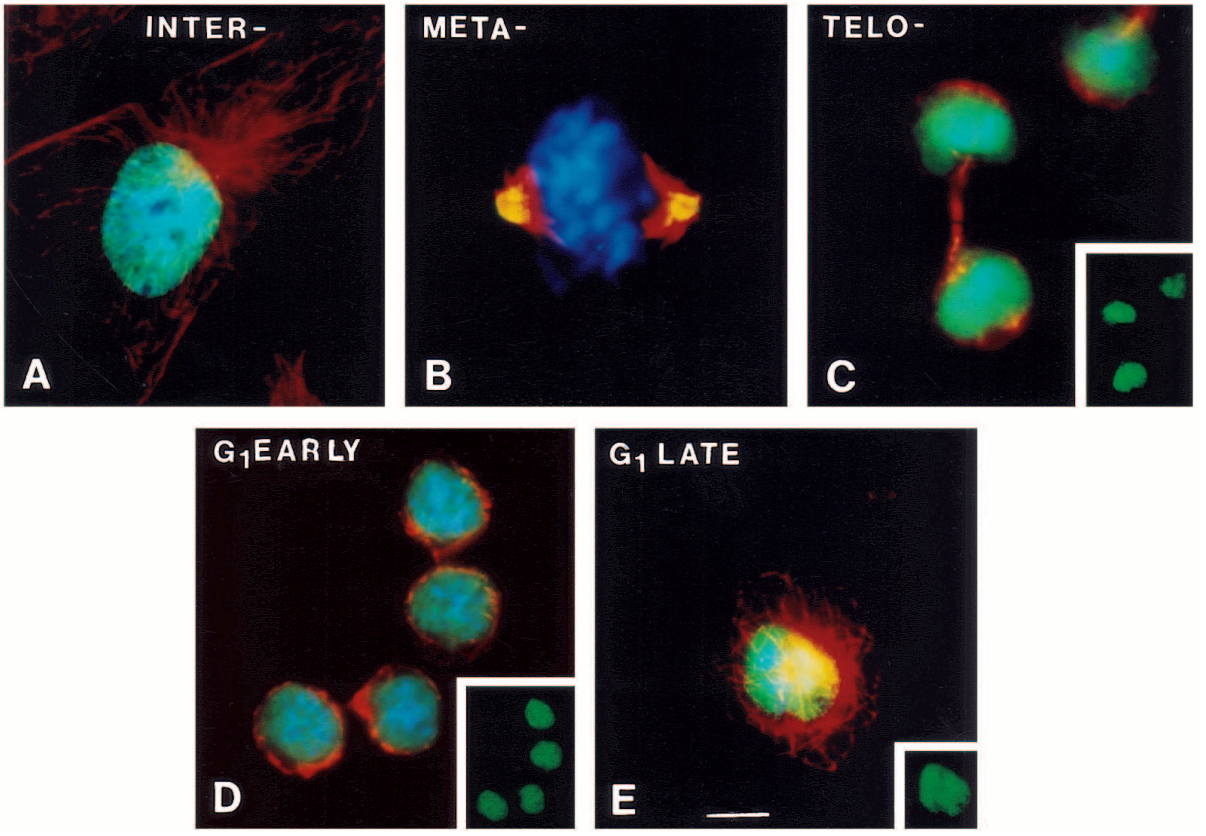


Fig. 7. NuMA distribution through the mitotic cell cycle and G_1. Immunofluorescence microscopy was used to localize NuMA (green), microtubules (red) and DNA (DAPI, blue) in synchronized cells as described in Fig. 5. NuMA is nuclear in interphase (A), relocates to the poles of the mitotic spindle at metaphase (B, yellow-green), and reassembles back into the reforming nucleus in telophase (C). Throughout G_1 (D,E), NuMA remains in the nucleus and does not undergo any detectable morphological changes. Inset (C,D,E) shows NuMA stain alone for each group of cells. Bar, 5 μm.

mitotic cells (85%, Fig. 5B) progressed synchronously through telophase and into G_1 (98%). These synchronous populations were used to determine the phosphorylation state of NuMA (Fig. 6) in relationship to its intracellular distribution (Fig. 7).

NuMA's metaphase phosphorylation state (Fig. 6A, lane M) persisted through telophase (Fig. 6B, lane T) even though the protein redistributed from the spindle poles (metaphase, Fig. 7B) to the reforming nucleus at this time (Fig. 7C). With NuMA still present in reforming nuclei at early G_1 (Fig. 7D) the higher of the shifted bands became undetectable (Fig. 6B, lane G_{1E}) apparently shifting down to the intermediate mobility as a result of dephosphorylation. In fact, results from several experiments suggest that the intermediate band increases in intensity as the upper band is lost. The disappearance of the intermediate form occurred three hours later (Fig. 6B, lane G_{1L}) and did not correlate with any changes in NuMA distribution or morphology (Fig. 7E). The timing of NuMA phosphorylation and disassembly from the nucleus were similar to that of lamin B, another protein of the interphase nucleus (data not shown; Gerace and Bobel, 1980). However, lamin B dephosphorylation correlated with its reassembly in the anaphase to telophase transition, while NuMA dephosphorylation occurred later, in G_1 (data not shown; Gerace and Bobel, 1980).

DISCUSSION

NuMA phosphorylation

We have demonstrated that NuMA is a phosphoprotein in interphase that undergoes additional phosphorylation events at the G_2/M transition and is dephosphorylated postmitotically in several steps. We believe that these phosphorylation/dephosphorylation events demarcate specific points in the 'life cycle' of NuMA that are important for its localization and/or function. Importantly, we show that the mitosis-specific phosphorylation is not a consequence of the protein's redistribution to the mitotic spindle and that dephosphorylation is not required for its release from the spindle. In fact, since both phosphorylation and dephosphorylation of NuMA seem to occur while the protein is in the nucleus, we propose that these post-translational modifications may regulate nuclear events.

The mitosis-specific phosphorylation events described here have previously gone unnoticed (Maekawa et al., 1991; Kempf et al., 1994) probably due to technical difficulties associated with detection of the phosphorylation-induced shift in electrophoretic mobility. Such shifts in electrophoretic mobility occur in many (Buchkovich et al., 1989; Cooper, 1991), but not all (Gerace and Blobel, 1980), phosphoproteins. Although it has been hypothesized that the addition of phosphate affects charge or tertiary structure, even under conditions of SDS-PAGE, the cause of the shifts remains unclear (Cooper, 1991). Changes in the mobility of mitotic NuMA have been recently observed (Compton and Luo, 1995). However, multiple phosphorylated species (Figs 3,6) were not revealed, the 180 kDa phosphoprotein was not observed (most likely due to omission of appropriate phosphatase inhibitors, Fig. 1, and data not shown) and dephosphorylation was not examined.

NuMA phosphorylation and spindle pole association

NuMA interacts with both microtubules and centrosomes at the mitotic spindle. For example, drug-induced depolymerization of spindle microtubules results in the redistribution of NuMA throughout the cytoplasm and into heterogeneously-sized cytoplasmic foci (not shown; Lydersen and Pettijohn, 1980; Kallajoki et al., 1991; Tousson et al., 1991). In addition, NuMA is found at centrosomes in vitro (Fig. 4) and in vivo (Tousson et al., 1991; Tang et al., 1994), at the center of taxol-induced microtubule asters (Maekawa et al., 1991; Kallajoki et al., 1992), and in purified microtubule fractions (Maekawa et al., 1991; Kallajoki et al., 1992). Many NuMA antibody microinjection and overexpression studies have resulted in aberrant spindle formation and function (reviewed by Compton and Cleveland, 1994). These data suggest that NuMA plays a role in the formation and stability of the mitotic spindle. A natural assumption from these observations is that the mitotic phosphorylation events that we describe here affect NuMA's ability to redistribute to the mitotic spindle.

Our results do not support a role for these mitotic phosphorylation events in spindle association for several reasons. First, they occur at the time of nuclear disassembly, between G_2 and prometaphase, prior to spindle assembly. Second, in mitotic cells lacking spindles, phosphorylation still occurs. Third, if phosphorylation is required for spindle association then one would expect that dephosphorylation would be required for dissociation of NuMA in telophase. However, dephosphorylation of NuMA occurs much later, in G_1. Thus, it is unlikely that the phosphorylation and dephosphorylation events observed in this study are involved in NuMA's spindle localization. However, it is formally possible that other phosphorylation events, not detected by our analyses, play a role in spindle association, assembly, or stabilization.

NuMA phosphorylation and nuclear function

NuMA's phosphorylation occurs at roughly the same time that it disassembles from the nucleus. These events are mechanistically and temporally analogous to the phosphorylation-driven disassembly of lamin B from the nuclear lamina (Gerace and Blobel, 1980). Thus, it is possible that NuMA's disassembly from the nucleus at mitosis is also driven by phosphorylation. Assembly of lamin B into the reforming nuclear lamina occurs concomitantly with its dephosphorylation (Gerace and Blobel, 1980). In contrast, reassembly of NuMA into the nucleus does not correlate with its dephosphorylation. In fact, the first dephosphorylation event occurs after NuMA is already nuclear, partially associated with the nuclear matrix (data not shown). The dephosphorylation is complete within 10 minutes (data not shown). This rapid dephosphorylation does not correlate with NuMA's localization, its detergent-insolubility (data not shown) or with changes in nuclear morphology.

The second dephosphorylation event takes place near the G_1/S boundary as shown by the rapid (not shown) and quantitative shift in electrophoretic mobility (Fig. 6). It is possible that dephosphorylation of NuMA plays a role in the activation of DNA replication which occurs at this time. In support of this idea are recent data demonstrating that NuMA binds to matrix attachment regions (MARs) (Luderus et al., 1994) which are DNA sequences believed to be responsible for organizing chromatin into constrained loops. MARs have been implicated in the regulation of DNA replication and transcription (Phi-van et al., 1990; Razin et al., 1991).

Based on these results, we propose that the dephosphorylation events described in this study may play a role in the

assembly of NuMA into higher order structures in the nucleus or in other nuclear events not detectable in this analysis. Dephosphorylation drives the assembly of other coiled-coil proteins such as lamin B (Heald and McKeon, 1990), vimentin (Evans, 1988), and others (Inagaki et al., 1988; Pasternak et al., 1989). There is some debate over whether NuMA will form filamentous structures via the coiled coil domain since the sequence lacks periodicity in acidic and basic residues (Parry, 1994). However, the complex phosphorylation pattern of NuMA in both mitosis and interphase may contribute to changes in charge distribution and tertiary structure of the molecule which would enable the protein to form filaments.

Regulation of NuMA phosphorylation and dephosphorylation

The kinases and phosphatases that act on NuMA at different stages of the cell cycle have not been identified. The mitosis-specific cdc2 kinase may regulate the disassembly of NuMA from the nucleus as it does the disassembly of lamin B from the nuclear lamina (Peter et al., 1990). Although site-directed mutagenesis of the consensus sites for cdc2 kinase in NuMA affects it's subcellular distribution (Compton and Luo, 1995), there has been no direct demonstration that NuMA is a substrate for cdc2 kinase. This will require identification of the specific amino acids that are phosphorylated at mitosis.

Identification of the phosphatases that act on NuMA in late G_1 may provide insights into potential mechanisms for the activation of DNA replication. Future investigations designed to elucidate the role of phosphorylation and dephosphorylation in NuMA distribution and function will focus on the identification and role of specific phosphoamino acids, kinases and phosphatases.

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