

Mutation of the predicted p34^{cdc2} phosphorylation sites in NuMA impair the assembly of the mitotic spindle and block mitosis

Duane A. Compton* and Chenghua Luo

Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755, USA

*Author for correspondence

SUMMARY

NuMA is a 236 kDa intranuclear protein that is distributed into each daughter cell during mitosis through association with the pericentrosomal region of the mitotic spindle. NuMA's interaction with the microtubules of the mitotic spindle is mediated through its 45 kDa carboxyl-terminal globular tail, and there is indirect evidence suggesting that NuMA's interaction with the mitotic spindle is controlled in a mitosis-specific manner. Consistent with this evidence is the fact that all four of the predicted p34^{cdc2} consensus phosphorylation sites in the NuMA protein are located in the carboxyl-terminal globular domain, and we demonstrate here that NuMA is phosphorylated in a mitosis-specific fashion *in vivo*. To test if the predicted p34^{cdc2} phosphorylation sites are necessary for NuMA's mitosis-specific interaction with the mitotic spindle, we have introduced mutations into the human NuMA cDNA that convert these predicted p34^{cdc2} phosphorylation sites from threonine or serine residues into alanine residues, and subsequently determined the cell cycle-dependent localization of these altered NuMA proteins following their expression in tissue culture cells. While none of these specific

mutations in the NuMA sequence alters the faithful targeting of the protein into the interphase nucleus, mutation of threonine residue 2040 alone or in combination with mutations in other potential p34^{cdc2} phosphorylation sites abolishes NuMA's ability to associate normally with the microtubules of the mitotic spindle. Instead of binding to the mitotic spindle these mutant forms of NuMA concentrate at the plasma membrane of the mitotic cell. Cells expressing these mutant forms of NuMA have disorganized mitotic spindles, fail to complete cytokinesis normally, and assemble micronuclei in the subsequent interphase. These data suggest that NuMA's interaction with the microtubules of the mitotic spindle is controlled by cell cycle-dependent phosphorylation in addition to differential subcellular compartmentalization, and the characteristics of the dominant negative phenotype induced by these mutant forms of NuMA support a role for NuMA in the organization of the mitotic spindle apparatus.

Key words: NuMA, mitosis, mitotic spindle, p34^{cdc2} phosphorylation site, microtubule

INTRODUCTION

At the onset of mitosis, the major components of the nucleus of a typical vertebrate cell must undergo complete physical restructuring to permit their efficient segregation into each daughter cell. This restructuring includes the condensation of the chromatin into chromosomes visible by light microscopy, the vesicularization of the nuclear envelope, and the disassembly of the nuclear lamina (Newport and Forbes, 1987; Gerace and Burke, 1988). To date, there are three distinct segregation pathways for the components of the nucleus during mitosis. First, there is the highly documented segregation pathway taken by the chromosomes following their attachment to the microtubules of the mitotic spindle (Mitchison, 1989; Rieder, 1991). The second distinct segregation pathway for nuclear components during mitosis is passive diffusion into each daughter cell following nuclear envelope dissolution. This mode of segregation is used by a multitude of nuclear proteins, and is typified by the nuclear lamin proteins (McKeon, 1991; Nigg, 1992). The third mitotic segregation pathway for nuclear

components is defined by the intranuclear protein NuMA (Nuclear protein that associates with the Mitotic Apparatus; Lydersen and Pettijohn, 1980; reviewed by Compton and Cleveland, 1994).

NuMA is a 236 kDa protein that was first identified in a hamster/human somatic cell hybrid as a human-specific high molecular mass component of the nuclear matrix (Lydersen and Pettijohn, 1980). During interphase, NuMA is distributed in a punctate fashion uniformly throughout the cell nucleus, except for its exclusion from the nucleolus (Price and Pettijohn, 1986; Compton et al., 1991; Kallajoki et al., 1991; Maekawa et al., 1991; Yang et al., 1992; Tang et al., 1993). At the onset of mitosis, when the chromosomes condense and the nuclear envelope disassembles, NuMA is released from the nuclear compartment and rapidly associates with the microtubules of the mitotic spindle. As cells progress from prometaphase to metaphase, NuMA progressively accumulates at the polar ends of the mitotic spindle independently of chromosome movements. NuMA remains associated with the microtubules at the polar ends of the mitotic spindle through

anaphase, and is ultimately released from the microtubules in telophase and incorporated into the nuclei of each daughter cell by nuclear pore-dependent import (Compton et al., 1992).

The amino acid sequence of the NuMA protein, as deduced from the DNA sequence of the full-length cDNA, is predicted to have three structural domains (Compton et al., 1992; Yang et al., 1992; Maekawa and Kuriyama, 1993; Tang et al., 1993). The amino-terminal head and carboxyl-terminal tail are composed of proline-rich globular domains of approximately 24 kDa and 45 kDa, respectively. These terminal globular head and tail domains are separated by a 1500 amino acid central α -helical domain that contains significant regions of hydrophobic amino acids arranged in a heptad repeat, indicating that NuMA has the potential to oligomerize through coiled-coil interactions similar to the intermediate filament family of proteins. Except for this central α -helical domain, the predicted NuMA protein does not contain any identifiable functional motifs, nor does it show any significant sequence homology with any protein in the Genbank or EMBL databases.

Despite NuMA's interesting structure and the detailed descriptions of its cell cycle distribution, the function of NuMA as well as the mechanism and control of NuMA's interaction with the microtubules of the mitotic spindle are not understood. The accumulation of NuMA at the polar ends of the mitotic spindle is dependent on the presence of intact microtubules, since disruption of the spindle microtubules with microtubule-destabilizing drugs results in the displacement of NuMA from its polar location in a mitotic cell (Price and Pettijohn, 1986; Tousson et al., 1991). Reversing these microtubule-destabilizing conditions by removal of the microtubule-destabilizing drug results in the polymerization of the microtubules and coordinate relocalization of NuMA to the polar ends of the newly assembled spindle (Tousson et al., 1991). Furthermore, when mitotic cells are treated with the microtubule-stabilizing drug (taxol), a complex series of microtubule asters assemble at the cell periphery independently of the microtubule organizing center (centrosomes), and NuMA is associated with the distal or minus ends of those microtubules (Maekawa et al., 1991; Kallajoki et al., 1992). These data, in conjunction with double-label immunofluorescence microscopy using centrosome-specific antibodies, have demonstrated that NuMA is not a component of the mitotic centrosome per se, but is associated with the microtubules that emanate from the centrosome (Compton et al., 1992; Yang et al., 1992; Kallajoki et al., 1992). Collectively, these data suggest that NuMA has a high affinity for microtubules during mitosis, a conclusion that has been supported by *in vitro* microtubule-dependent sedimentation of NuMA from mitotic cell extracts (Maekawa et al., 1991; Kallajoki et al., 1992).

The affinity of NuMA for microtubules, however, appears to be regulated in a mitosis-specific manner because NuMA fails to associate with microtubules in the context of the interphase cell cytoplasm (Compton et al., 1992). Previous experiments have demonstrated that NuMA is a phosphoprotein (Price and Pettijohn, 1986), and it is likely that NuMA's mitosis-specific interaction with microtubules is controlled through phosphorylation by p34^{cdc2} kinase. All four of the predicted p34^{cdc2} phosphorylation sites in the NuMA polypeptide reside in the carboxyl-terminal globular tail of the protein (Yang et al., 1992), and recent experiments have shown that NuMA's carboxyl-terminal globular tail domain is necessary

for NuMA's interaction with the mitotic spindle during mitosis (Compton and Cleveland, 1993).

In this article we demonstrate that NuMA is phosphorylated in a mitosis-specific manner, and we show that mutant forms of NuMA lacking one or more of the predicted p34^{cdc2} phosphorylation sites are incapable of targeting onto the microtubules of the mitotic spindle. In the absence of spindle binding these mutant forms of NuMA accumulate at the plasma membrane of the cell, and exert a dominant negative phenotype characterized by the failure to assemble a well organized mitotic spindle and the inability to complete mitosis. These results suggest that NuMA's interaction with the microtubules of the mitotic spindle is controlled by mitosis-specific phosphorylation as well as differential subcellular compartmentalization, and the dominant negative phenotype observed here is consistent with previous anti-NuMA antibody microinjection experiments (Yang and Snyder, 1992; Kallajoki et al., 1991) that support a functional role for NuMA in the organization of the mitotic spindle.

MATERIALS AND METHODS

Cell culture

The hamster BHK-21 cell line was maintained in DME containing 10% fetal calf serum, 2 mM glutamine, 100 i.u./ml penicillin, and 0.1 μ g/ml streptomycin. Cells were grown at 37°C in a humidified incubator with a 5% CO₂ atmosphere.

In vivo ³²P-labeling and immunoprecipitation

HeLa cells in T-75 cm² flasks were synchronized at the G₁/S boundary of the cell cycle by double thymidine block. Synchronized HeLa cells were released from the thymidine block into complete medium, allowed to resume the cell cycle for either two (for S/G₂ cells) or five (for M cells) hours, and washed twice for thirty minutes each in phosphate-free DMEM containing 10% dialyzed FCS (GibcoBRL/Life Technologies, Gaithersburg, MD). Following these washes the cells were incubated for three hours in phosphate-free DMEM/10% dialyzed FCS supplemented with 200 μ Ci/ml of [³²P]orthophosphoric acid (Amersham, Arlington Heights, IL). Mitotic cells were enriched by the inclusion of 1 μ g/ml nocodazole and collected by mitotic shake-off, and interphase cells (S/G₂) were collected by trypsinization. In both cases the cells were immediately chilled to 4°C and washed twice with cold Tris-buffered saline (TBS).

For immunoprecipitation of NuMA from ³²P-labeled cells, 2×10⁵ mitotic and 1×10⁶ interphase cells were resuspended in 150 μ l of extraction buffer (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) and heated to 100°C three times for three minutes each with vigorous agitation between each incubation. Following this extraction, the samples were cooled to room temperature and diluted eightfold with 20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 10 mM sodium pyrophosphate, 3.4% Triton X-100. The mitotic and interphase extracts were then divided in half and 20 μ g of rabbit preimmune IgG or anti-NuMA immune IgG were added to each and incubated at 4°C for 12 hours with gentle agitation. Following this incubation, 50 μ l of Protein A-conjugated agarose (50% slurry in TBS) was added and the extracts were incubated for 2 hours at 4°C with gentle agitation. The Protein A-conjugated agarose containing the IgG was then collected by centrifugation at 10,000 *g* for 15 seconds, washed three times with 20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 30 mM sodium pyrophosphate, 1.5% Triton X-100, washed twice in 20 mM Tris-HCl, pH 7.4, 250 mM NaCl, 30 mM sodium pyrophosphate, 1.5% Triton X-100, and finally solubilized in 50 μ l SDS-PAGE sample buffer. The immunoprecipitated material was

then subjected to size fractionation on 5% SDS-PAGE and processed for immunoblotting as described below using enhanced chemiluminescence (exposure times varied from 5 to 30 seconds at room temperature without an intensifying screen). The chemiluminescence signal was then allowed to be extinguished overnight and the blot subsequently exposed to film at -70°C with an intensifying screen for 2 to 5 hours.

Transfection and microinjection

Cells were transiently transfected using Lipofectamine reagent (GibcoBRL/Life Technologies, Gaithersburg, MD) as described by the manufacturer. Cells growing on 35 mm dishes with (for immunofluorescence microscopy) or without (for immunoblot analysis) glass coverslips were washed twice, and equilibrated in 800 μl Opti-MEM serum-free medium. A 1 μg sample of plasmid DNA diluted in 100 μl of Opti-MEM serum-free medium, and 10 μg of the Lipofectamine reagent diluted in 100 μl of Opti-MEM serum-free medium were mixed and incubated at room temperature for 15 to 20 minutes. The DNA/lipid complex contained in 200 μl was then added to the cells dropwise while mixing. The cells were then incubated at 37°C for 6 to 8 hours, and then washed twice and incubated overnight with complete medium.

Cells growing on photo-etched alpha-numeric glass coverslips (Bellco Glass Co., Vineland, NJ) were microinjected following the procedures of Compton and Cleveland (1993) and Capecchi (1980). Interphase cells were microinjected into the nucleus with plasmid DNA at a concentration of 100 $\mu\text{g}/\text{ml}$ in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA. Injected cells were followed by phase-contrast microscopy until they reached the desired stage of the cell cycle, and then processed for immunofluorescence microscopy.

Immunological techniques

Intracellular localization of expressed human NuMA in the hamster cell line BHK-21 was determined following procedures outlined by Compton et al. (1991). Cells growing on glass coverslips were fixed by immersion in PBS containing 3.5% paraformaldehyde for 5 minutes at room temperature. The cells were then extracted with 0.5% Triton X-100 in TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 1% bovine serum albumin for 5 minutes at room temperature. The cells were then rinsed and maintained through all subsequent steps in TBS containing 1% bovine serum albumin at room temperature. Primary antibodies were added to the cells and incubated for 30 minutes at room temperature in a humidified chamber. Coverslips were washed in TBS containing 1% bovine serum albumin and the bound antibodies were detected with the appropriate fluorescein-conjugated or Texas Red-conjugated secondary antibodies (Vector Labs, Burlingame, CA). DNA was detected with 4',6-diamidino-2-phenylindole (DAPI; 0.4 $\mu\text{g}/\text{ml}$; Sigma Chemical Co., St Louis, MO). Coverslips were mounted in Gel/mount (Biomed, Foster City, CA) or FITC-Guard (Testog, Chicago, IL) and observed with a Nikon Optiphot microscope equipped for epifluorescence.

In some cases (as indicated in the text) cells were extracted with Triton X-100 prior to fixation. This was performed by immersing the coverslips in microtubule stabilizing buffer (MTSB: 4 M glycerol, 100 mM PIPES, pH 6.8, 1 mM EGTA, 5 mM MgCl_2) for 1 minute at room temperature followed by extraction with MTSB containing 0.5% Triton X-100 at room temperature for 2 minutes, rinsing in MTSB for 2 minutes at room temperature, and finally fixing in -20°C methanol for 10 minutes. Cells were rehydrated using TBS containing 1% bovine serum albumin and all subsequent steps were performed as stated above.

Proteins were analyzed from transiently transfected cells by immunoblot analysis following SDS-PAGE (Laemmli, 1970). Cells were washed three times in ice-cold PBS and harvested directly in SDS-PAGE sample buffer. Proteins were separated by size using 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 6 hours at room temperature in TBS containing 1% nonfat milk. Non-bound primary antibody was removed by washing five times for 3 minutes each in TBS containing 0.25% Tween-20 and the bound antibody was detected using horseradish peroxidase-conjugated Protein A (Bio-Rad Co., Hercules CA). Non-bound Protein A was removed by washing five times for 3 minutes each in TBS and the bound protein A was detected using enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

Site-directed mutagenesis

The full-length 7.2 kb human NuMA cDNA was transferred from the Bluescript plasmid (Compton and Cleveland, 1993) into the multiple cloning site of the pUC19 plasmid vector in the same 5' to 3' orientation as the β -galactosidase gene using the unique *Bam*HI site and the *Sal*I sites. This plasmid was maintained in the DH1 strain of *Escherichia coli* and purified by CsCl centrifugation (Maniatis et al., 1982). Mutations in the NuMA cDNA were introduced using the transformer site-directed mutagenesis kit (Clontech Laboratories Inc., Palo Alto CA) precisely as described by the manufacturer. Each mutagenic primer (Operon Technologies Inc., Alameda CA) altered the DNA sequence of the NuMA cDNA so that the reading frame was maintained with the alteration of only one amino acid as well as introducing a new restriction site into the NuMA cDNA to aid in rapid identification of the mutant plasmids. The sites of mutation and the sequence of the mutagenic primers were as follows (the associated restriction enzyme site introduced by each primer is given in parenthesis): 2000TA, 5'-CGGTCTCGGGGAGCCATGGGGCGTGG-3' (*Nco*I); 2040TA, 5'-GCTTCTTGGGCGCGTTGAGGATGC-3' (*Bst*UI); 2072SA, 5'-GCAATGCGCGGTGCACGGCGGGTTC-3' (*Apa*LI); 2091TA, 5'-GGCTCGAGGGGCGCCACCAATGGC-3' (*Nar*I). The appropriate mutations in each plasmid were verified by DNA sequence analysis, and no multiple mutations occurred as judged by restriction analysis using the mutation-associated restriction enzyme site. The amino acids threonine 2000, threonine 2040, serine 2072, and threonine 2091 were each altered to alanine residues independently or in combinations by repeated mutagenesis reactions. (These amino acids are the same as threonine 2015, threonine 2055, serine 2087, and threonine 2106, using the sequence numbering of Yang et al. (1992).) The nomenclature used in this article to denote the conversion of a threonine or serine residue to an alanine residue is TA or SA, respectively, and is immediately preceded by the amino acid residue number. Following mutagenesis, the 7.2 kb *Eco*RI fragment containing the full-length NuMA cDNA was transferred into the unique *Eco*RI site of the multiple cloning site of the mammalian expression vector GW1CMV. These plasmids were maintained in the DH1 strain of *E. coli* and purified by CsCl centrifugation.

RESULTS

Mitosis-specific phosphorylation of NuMA

To test if NuMA is phosphorylated in a mitosis-specific manner, we synchronized HeLa cells at the G_1/S boundary by double thymidine block and incubated the cells in phosphate-free medium containing [^{32}P]orthophosphate for three hours. Interphase cells (S/G_2) were labeled two hours after release from the thymidine block and collected by trypsinization. Mitotic cells (M) were labeled six hours after release from the thymidine block, accumulated by the addition of nocodazole, and collected by mitotic shake-off. NuMA was isolated from each ^{32}P -labeled cell population by immunoprecipitation, and the relative phosphate content of NuMA derived from interphase and mitotic cells was determined by autoradiography following size fractionation by SDS-PAGE. Immunoblot

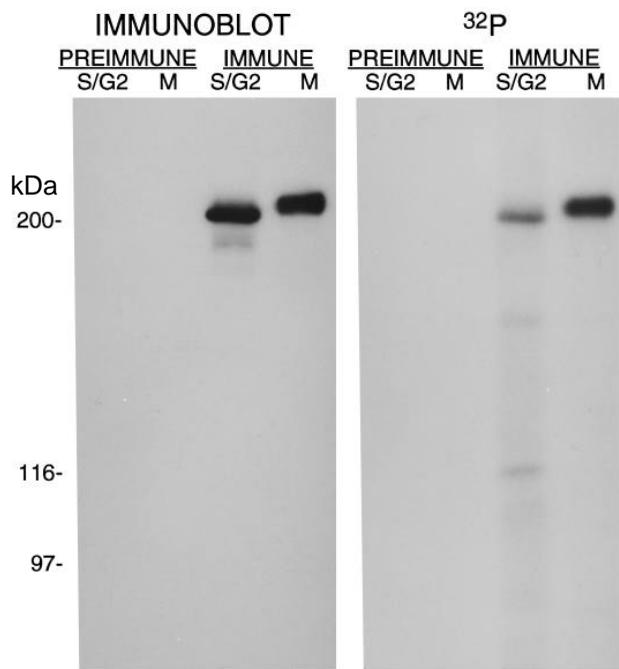


Fig. 1. Mitosis-specific phosphorylation of NuMA in vivo. Synchronized HeLa cells were incubated with [^{32}P]orthophosphate for three hours, harvested in either interphase (S/G₂) or mitosis (M), and subjected to immunoprecipitation using a preimmune serum (PREIMMUNE) or an anti-NuMA specific serum (IMMUNE). The resulting immunoprecipitates were then subjected to size fractionation on a 5% SDS-PAGE and the proteins identified by immunoblot using an anti-NuMA antibody (IMMUNOBLOT) followed by autoradiographic exposure (^{32}P).

analysis of the resulting immunoprecipitates with a polyclonal anti-NuMA antibody demonstrates that no proteins are precipitated using IgG purified from the preimmune serum, and approximately equal quantities of NuMA are precipitated from both interphase and mitotic cells using IgG purified from the anti-NuMA immune serum (Fig. 1, IMMUNOBLOT). Interestingly, the mobility of NuMA derived from mitotic cells in the SDS-PAGE appears to be slightly retarded relative to NuMA derived from interphase cells. Autoradiographic exposure of this filter for the detection of the incorporated ^{32}P demonstrates that NuMA derived from mitotic cells contains approximately 3 \times (determined by scanning densitometry using multiple film exposures) more phosphate relative to NuMA derived from interphase cells (Fig. 1, ^{32}P). These data corroborate the original findings of Price and Pettijohn (1986) demonstrating that NuMA is a phosphoprotein, and extend those findings to demonstrate that NuMA is hyperphosphorylated during mitosis.

Mutation of potential p34^{cdc2} phosphorylation sites abolishes NuMA's association with the mitotic spindle

To determine the functional properties of NuMA proteins lacking one or several of the potential p34^{cdc2} phosphorylation sites we have systematically altered each potential p34^{cdc2} phosphorylation site by site-directed mutagenesis, and assayed the properties of each altered NuMA protein following

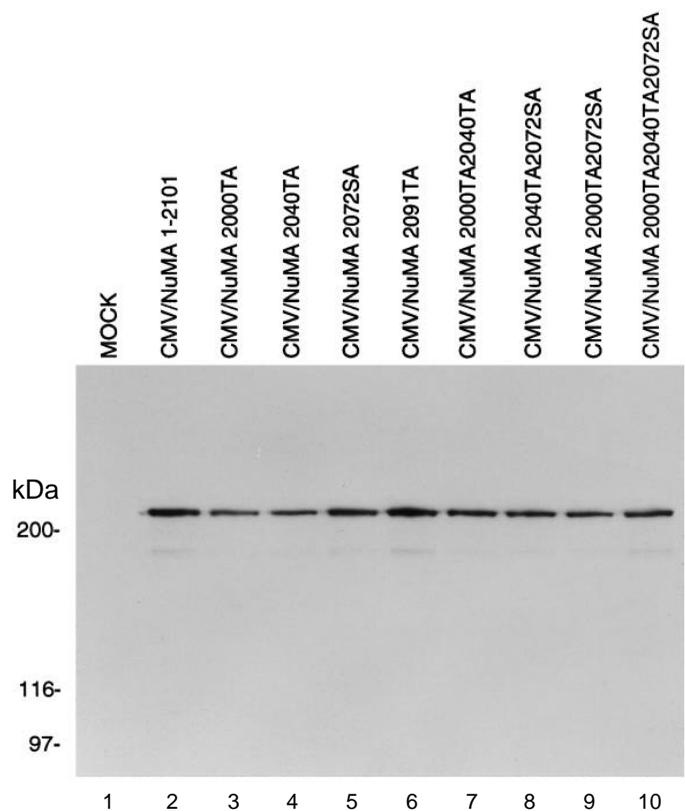


Fig. 2. Immunoblot detection of the expressed human NuMA proteins following transient transfection of hamster BHK-21 cells. The human-specific anti-NuMA monoclonal antibody was used to detect the exogenously expressed human NuMA proteins in $\sim 40 \mu\text{g}$ of cell extract after electrophoresis on a 5% SDS-polyacrylamide gel. Lane 1, mock-transfected BHK-21 cells; lane 2, BHK-21 cells expressing the wild-type human NuMA protein; lanes 3-10, BHK-21 cells expressing human NuMA proteins with the indicated amino acid substitutions. The migration positions of myosin (200 kDa), β -galactosidase (116 kDa), and phosphorylase B (98 kDa) are indicated at the left.

transient transfection of hamster BHK-21 cells. To insure that each mutant NuMA cDNA was competent to drive the expression of a protein with the appropriate molecular mass, we determined the size of the expressed human NuMA protein by immunoblot analysis following transient transfection of the hamster cells (Fig. 2). In hamster BHK-21 cells transfected with the expression vector alone (containing no cDNA; Fig. 2, MOCK) there is no detectable protein when analyzed with the human-specific anti-NuMA monoclonal antibody mAb1F1 (Compton et al., 1991). In BHK-21 cells transfected with the expression plasmid driving the expression of the wild-type human NuMA protein (Fig. 2, CMV/NuMA1-2101), one protein species is detected migrating at a molecular mass of 210 kDa. These data are consistent with previous experiments demonstrating that exogenous expression of the human NuMA protein in BHK-21 cells resulted in a protein that migrated in SDS-PAGE with a molecular mass indistinguishable from the endogenous human protein (Compton and Cleveland, 1993). Transfection of BHK-21 cells with plasmids that drive the expression of NuMA proteins containing mutations in one, two or three of the p34^{cdc2} phosphorylation sites resulted in the

accumulation of a human-specific NuMA protein with a relative molecular mass indistinguishable from that of the wild-type human NuMA protein (Fig. 2). These data demonstrate that the human NuMA proteins containing the various amino acid alterations are of the same size and accumulate to similar levels as the wild-type human NuMA protein following transient transfection of hamster BHK-21 cells.

The intracellular localization of human NuMA proteins carrying mutations in each of the four potential p34^{cdc2} phosphorylation sites was determined by indirect immunofluorescence microscopy using the human-specific anti-NuMA monoclonal antibody following transient transfection of the BHK-21 cells (Fig. 3). In no case did the alteration of any of these specific amino acids affect the faithful localization of the human NuMA protein into the nucleus of an interphase cell (Fig. 3A-D). Similarly, there was no detectable effect on the localization of the NuMA proteins onto the microtubules of the mitotic spindle when threonine 2000, serine 2072 or threonine 2091 was substituted by an alanine residue (Fig. 3F,H,I). Mutation of threonine 2040 into an alanine residue, however, resulted in an altered localization pattern of the expressed human NuMA protein during mitosis (Fig. 3G). Although some of this mutant NuMA protein localized normally to the pericentrosomal region of the mitotic spindle (only one pole is visible in this panel) this mutant form of NuMA also consistently localized to the periphery of the cell cytoplasm. Careful focusing of the microscope through different focal planes indicated that NuMA2040TA was localized not only at the mitotic spindle pole, but also at the plasma membrane of the cell rather than diffusely localized throughout the cell cytoplasm (data not shown). These data suggest that threonine 2040 plays a critical role in efficiently targeting the NuMA protein onto the microtubules of the mitotic spindle.

Since mutation of threonine 2040 leads to a partial defect in the targeting of the NuMA protein onto the mitotic spindle, we tested the possibility that mutation of multiple p34^{cdc2} phosphorylation sites in the NuMA polypeptide would show a more dramatic mis-localization phenotype (Fig. 3). As with the mutant NuMA proteins altered at a single amino acid residue, mutation of multiple p34^{cdc2} phosphorylation sites had no observable effect on the accumulation of the expressed human NuMA in the interphase nucleus (Fig. 3E). Expression of NuMA with alanine residues substituted for both threonine 2000 and serine 2072 displayed a normal localization pattern of the protein on the mitotic spindle, and expression of NuMA with alanine residues substituted for both threonine 2040 and serine 2072 resulted in a localization pattern similar to the mutant form of NuMA carrying a single amino acid substitution at threonine 2040 (data not shown). Finally, expression of NuMA carrying mutations in both threonine 2000 and threonine 2040 or mutations in three of the potential p34^{cdc2} phosphorylation sites (threonine 2000, threonine 2040, and serine 2072) displayed no detectable localization to the pericentrosomal region of the mitotic spindle, and accumulated at the plasma membrane of the mitotic cell (Fig. 3J). These data demonstrate that mutation of as few as two amino acids in the carboxyl-terminal globular tail of the NuMA protein (threonine 2040 and threonine 2000) are sufficient to abolish NuMA's ability to associate with the microtubules of the mitotic spindle. In the absence of localization of the mutant NuMA proteins

onto the mitotic spindle the proteins accumulate at the plasma membrane of the cell.

In the analysis of these mutant forms of NuMA, no fewer than 20 mitotic cells and 50 interphase cells were scored following transfection with each individual plasmid. The localization patterns presented in Fig. 3 for each mutant form of NuMA are representative of the population of cells obtained during these transfection experiments, and in all cases the observed localization pattern was found in >95% of the cells. In addition, despite variable expression levels within the cell populations that arise as an unavoidable consequence of transient transfection, the subcellular localization of these mutant NuMA polypeptides did not change as a function of expression level.

NuMA is insoluble in the absence of spindle association

In previous experiments NuMA has been found to be an insoluble component of the mitotic spindle and, therefore, not sensitive to extraction by Triton X-100 (Compton et al., 1991; Tousson et al., 1991). To directly test if the solubility of NuMA is dependent on its association with the microtubules of the mitotic spindle, BHK-21 cells were transiently transfected with the plasmids driving the expression of the wild-type NuMA protein (CMV/NuMA 1-2101) or the mutant NuMA protein that fails to associate with the mitotic spindle (CMV/NuMA2000TA2040TA2072SA). These cells were then processed for indirect immunofluorescence microscopy to determine the distribution of the expressed human NuMA protein either with or without Triton X-100 extraction prior to fixation (Fig. 4). Extraction with Triton X-100 was performed for 2 minutes at room temperature using microtubule-stabilizing buffer (see Materials and Methods for composition), and the cells were fixed and processed identically as described above. These extraction conditions have been demonstrated to efficiently remove soluble proteins (Gill et al., 1991), and immunoblot analysis of parallel samples for tubulin confirmed the efficiency of extraction in this case (data not shown). As expected, expression of the wild-type NuMA protein localizes correctly to the polar ends of the mitotic spindle (the mitotic spindle axis is perpendicular to the plane of focus in this case), and is not appreciably affected by extraction with Triton X-100 (Fig. 4A and C). As described above, expression of NuMA carrying mutations in three potential p34^{cdc2} phosphorylation sites fails to localize at the polar ends of the mitotic spindle, and accumulates at the plasma membrane of the cell if the cells are fixed prior to Triton X-100 extraction (Fig. 4B). Extraction of mitotic cells that are expressing this mutant form of NuMA with Triton X-100 prior to fixation, however, results in a clear reorganization of the expressed mutant NuMA protein away from the region that was occupied by the plasma membrane (Fig. 4D). While the staining pattern for this mutant NuMA protein appears more intracellular and displays a pronounced punctate staining pattern, it is clearly retained as an insoluble component of the cell cytoplasm.

NuMA mutants that fail to bind to the mitotic spindle inhibit mitosis and perturb mitotic spindle organization

To test if cells are capable of completing mitosis normally in the presence of NuMA proteins that fail to target correctly onto

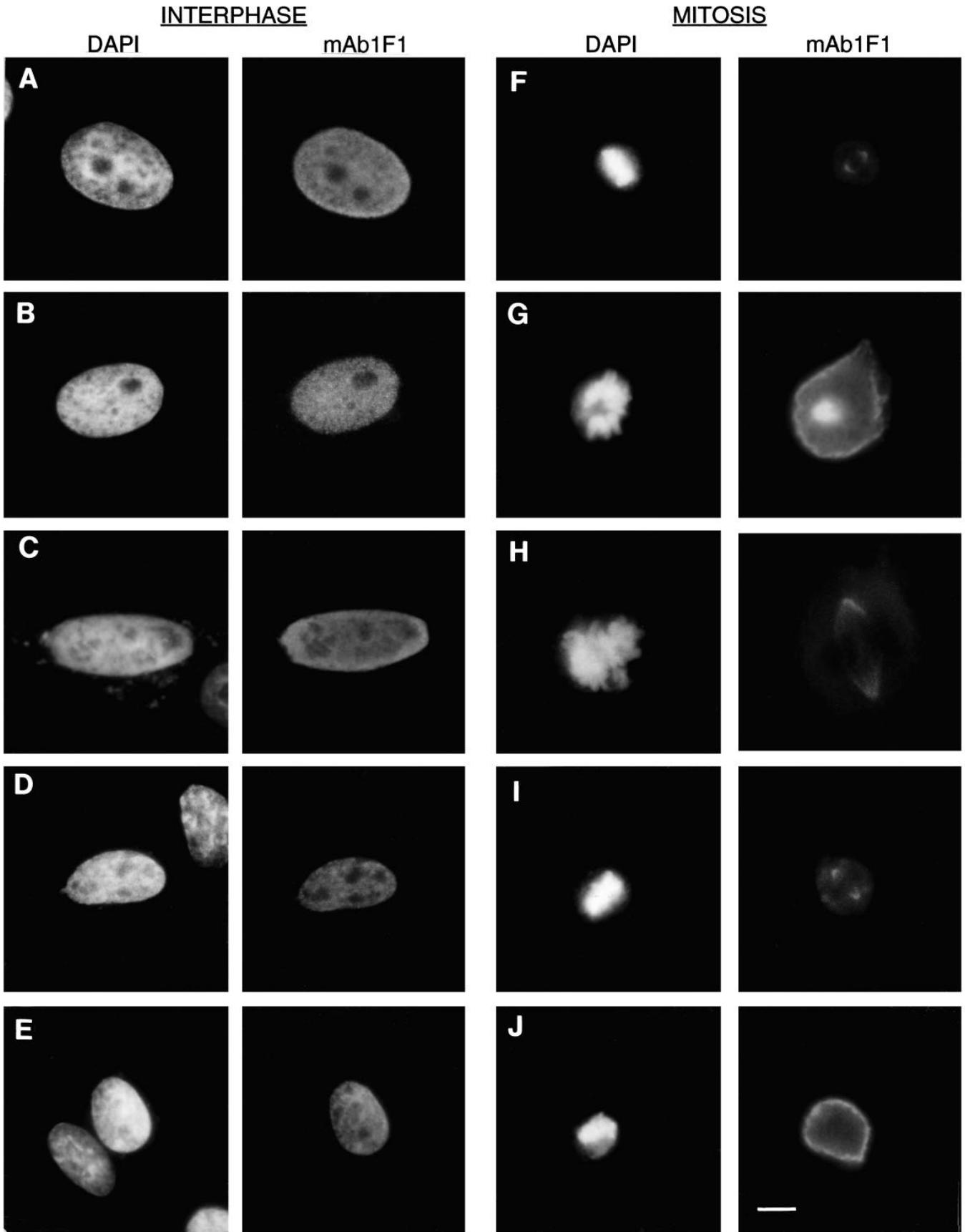


Fig. 3. Cellular localization of NuMA proteins carrying mutations in the predicted p34^{cdc2} phosphorylation sites. Hamster BHK-21 cells were transiently transfected with plasmids driving the expression of NuMA carrying an alanine residue substituted for either threonine 2000 (NuMA2000TA; A,F), threonine 2040 (NuMA2040TA; B,G), serine 2072 (NuMA2072SA; C,H), threonine 2091 (NuMA2091TA; D,I), or threonine 2000, threonine 2040 and serine 2072 (NuMA2000TA2040TA2072SA; E,J). Cells were fixed and processed for indirect immunofluorescence microscopy with a DNA-specific dye (DAPI) and a human-specific anti-NuMA monoclonal antibody (mAb1F1). Bar, 10 μ m.

the mitotic spindle during mitosis we introduced the expression plasmids into the BHK-21 cells by microinjection, and carefully monitored the progression of each injected cell through mitosis (Fig. 5). Previous studies had determined that expression of NuMA proteins introduced into BHK-21 cells in this fashion resulted in detectable protein levels (by immunofluorescence microscopy) within one hour post-microinjection, and that the wild-type NuMA protein targeted correctly in the interphase nucleus and onto the mitotic spindle and had no deleterious effects on the completion of mitosis (Compton and Cleveland, 1993). When BHK-21 cells were microinjected with the plasmid driving the expression of the NuMA2000TA, the mutant protein accumulated and targeted correctly as anticipated, and had no deleterious affect on the completion of mitosis (Fig. 5A). Out of 18 microinjected cells that completed mitosis in the presence of NuMA2000TA, all 18 completed

cell division normally yielding two daughter cells, in which each nucleus stains positively for the expressed human NuMA protein (Fig. 5A). Similarly, expression of NuMA2072SA and NuMA2091TA had no obvious affect on the ability of cells to progress through mitosis normally, as normal daughter cells were observed following mitosis in 12 and 11 microinjected cells, respectively (data not shown). Interestingly, despite the compromised ability of NuMA2040TA to target onto the mitotic spindle, the expression of NuMA2040TA had little affect on the normal completion of mitosis (Fig. 5B). Normal daughter cells were observed following the completion of mitosis in 18 out of 22 injected cells. The remaining 4 cells either failed to undergo cytokinesis and became binucleate (3 cells) or completed cytokinesis and assembled micronuclei in the subsequent interphase (data not shown).

We next tested the ability of cells to complete mitosis in the presence of NuMA proteins lacking multiple potential p34^{cdc2} phosphorylation sites. Expression of NuMA2000TA2072SA or NuMA2040TA2072SA had no obvious effects on the completion of mitosis in 10 or 12 cells that completed mitosis following microinjection, respectively (data not shown). Expression of NuMA2000TA2040TA and NuMA2000TA2040TA2072SA, however, consistently resulted in the appearance of cells with abnormal morphology following the completion of mitosis. In 11 out of 16 cells expressing NuMA2000TA2040TA, and 24 out of 28 cells expressing NuMA2000TA2040TA2072SA, the cells failed to

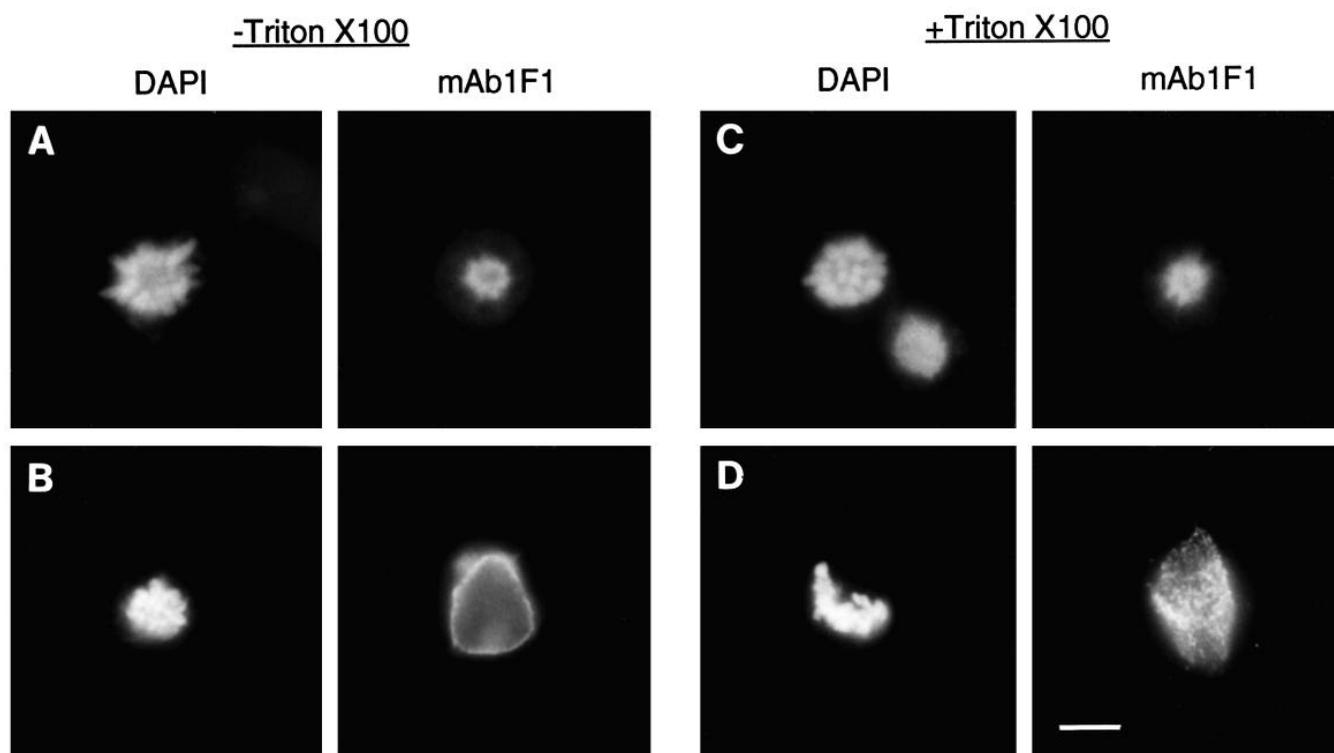


Fig. 4. Solubility of NuMA proteins carrying mutations in three potential p34^{cdc2} phosphorylation sites. Hamster BHK-21 cells were transiently transfected with either the plasmid driving the expression of the wild-type NuMA protein (NuMA 1-2101; A,C), or the plasmid driving the expression of the NuMA protein carrying alanine substitutions in three of the potential p34^{cdc2} phosphorylation sites (NuMA2000TA2040TA2072SA; B,D). The cells were processed for indirect immunofluorescence microscopy using Triton X-100 extraction prior to (+Triton X-100) or following (-Triton X-100) fixation, and stained with a DNA-specific dye (DAPI) and a human-specific anti-NuMA monoclonal antibody (mAb1F1). Bar, 10 μ m.

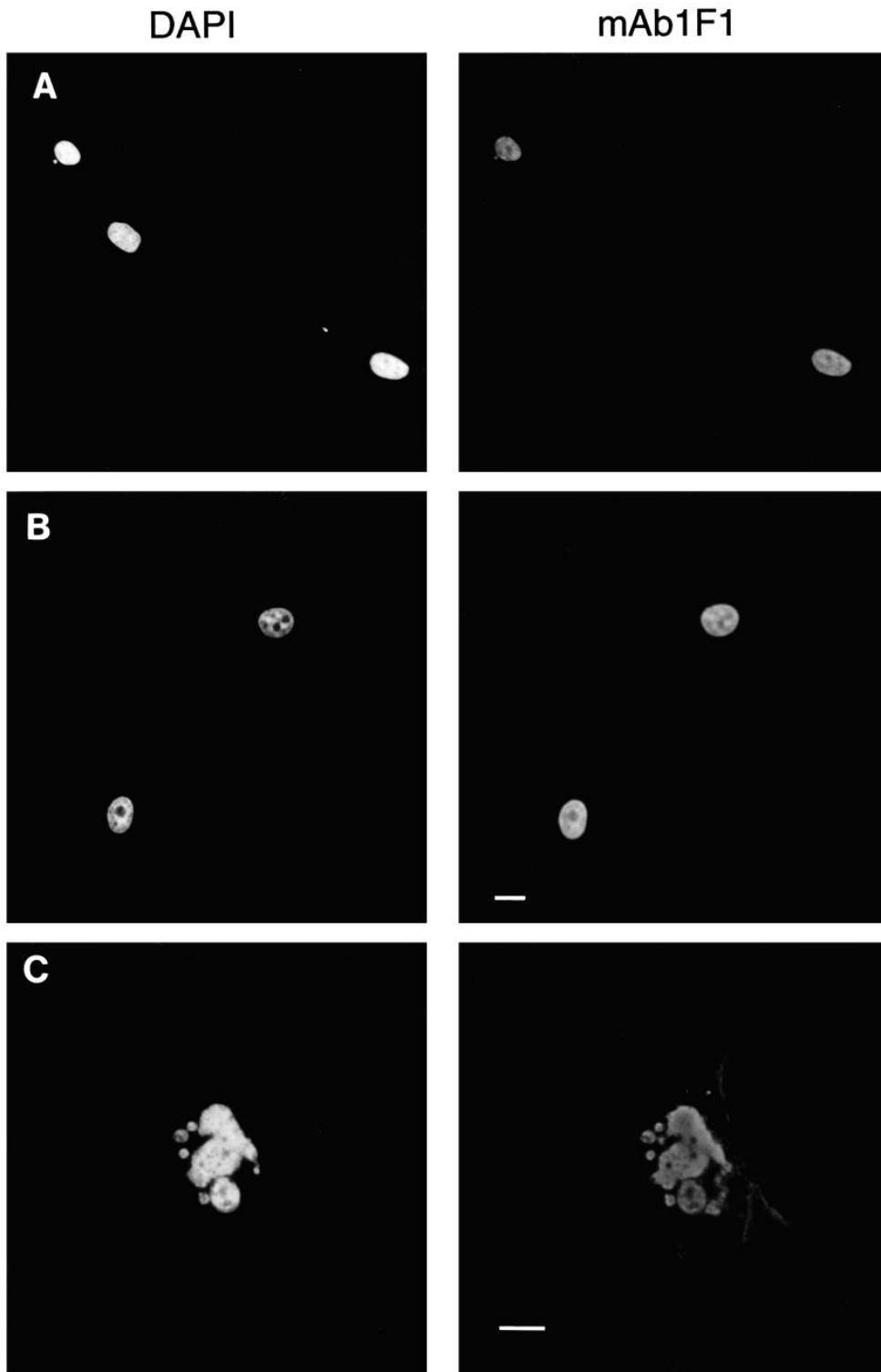


Fig. 5. Completion of mitosis in hamster cells expressing various mutant forms of the human NuMA protein. Hamster cells were microinjected with the plasmid driving the expression of NuMA carrying mutations in either threonine 2000 (NuMA2000TA; A), threonine 2040 (NuMA2040TA; B), or threonine 2000, threonine 2040 and serine 2072 (NuMA2000TA2040TA-2072SA; C). Cells were allowed to complete mitosis following microinjection, and processed for indirect immunofluorescence microscopy using a DNA-specific dye (DAPI) and a human-specific anti-NuMA monoclonal antibody (mAb1F1). Please note that the magnification in A and B is different from C. Bar, 10 μ m.

complete cytokinesis (as judged by formation of only a single interphase daughter cell) and assembled multiple small nuclei (micronuclei) in the subsequent interphase (Fig. 5C). These data demonstrate that expression of mutant forms of NuMA that are incapable of associating normally with the mitotic spindle induce a dominant negative phenotype characterized by the inability of cells to complete mitosis and the assembly of micronuclei in the ensuing interphase.

One possible explanation for the observed disruption in the cells' ability to transit normally through mitosis in the presence of these mutant forms of NuMA is that the assembly or organization of the mitotic spindle has been perturbed. To directly test the integrity of the mitotic spindles assembled in the presence of these various mutant forms of NuMA we performed indirect immunofluorescence microscopy on cells expressing various mutant forms of NuMA using an anti-

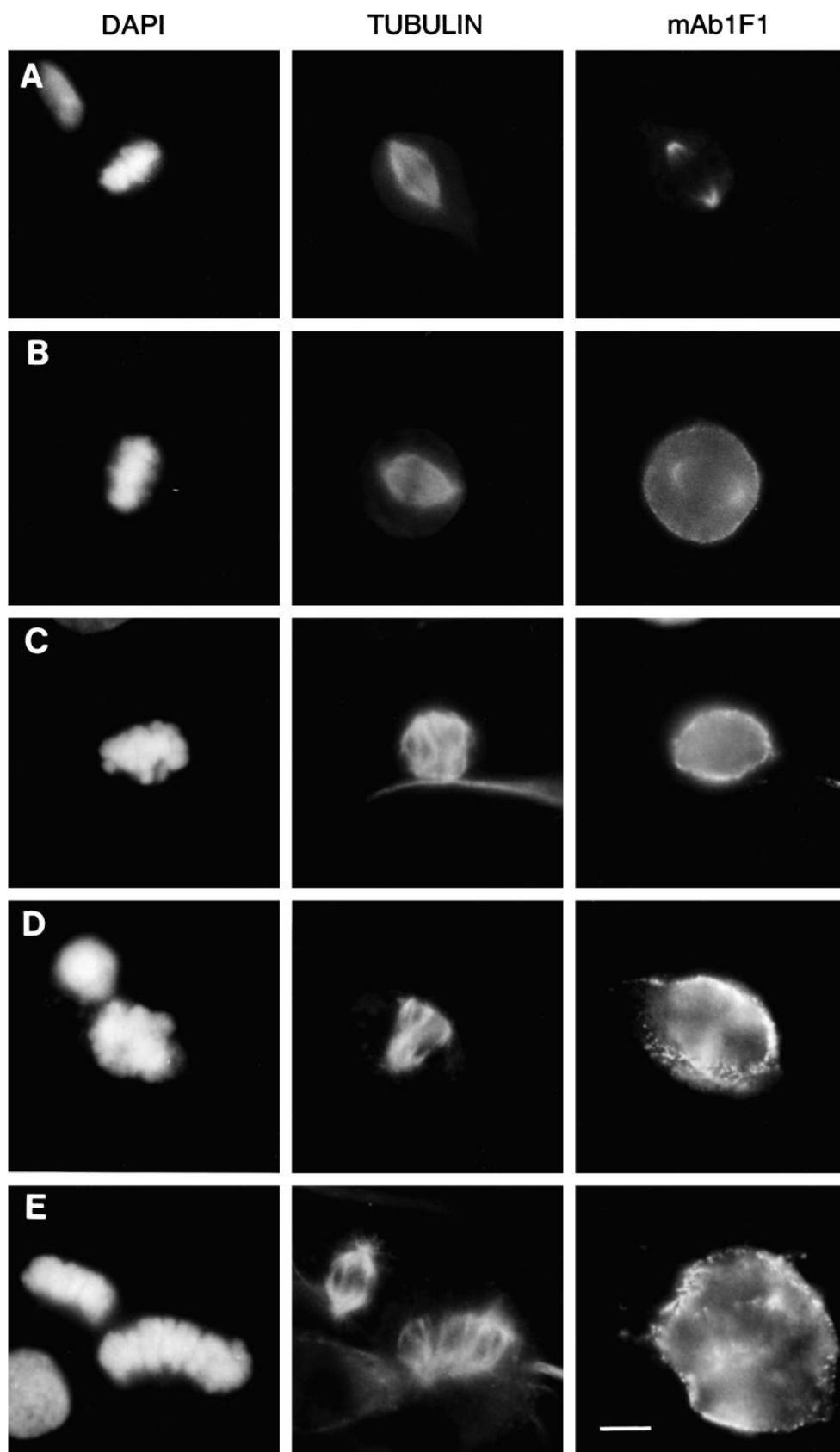


Fig. 6. Morphology of the mitotic spindle in the presence of various mutant forms of the human NuMA protein. Hamster BHK-21 cells were transiently transfected with plasmids driving the expression of NuMA carrying mutations in either threonine 2000 (NuMA2000TA; A), threonine 2040 (NuMA2040TA; B), or threonine 2000, threonine 2040 and serine 2072 (NuMA2000TA2040TA-2072SA; C-E). Cells were fixed and processed for indirect immunofluorescence microscopy using a DNA-specific dye (DAPI), an anti-tubulin antibody (TUBULIN), and a human-specific anti-NuMA monoclonal antibody (mAb1F1). Bar, 10 μ m.

tubulin antibody (Fig. 6). In cells expressing mutant forms of NuMA that target correctly or possess a mixed localization

phenotype (NuMA2000TA or NuMA2040TA), the mitotic spindles displayed a typical 'fusiform' morphology (Fig. 6A

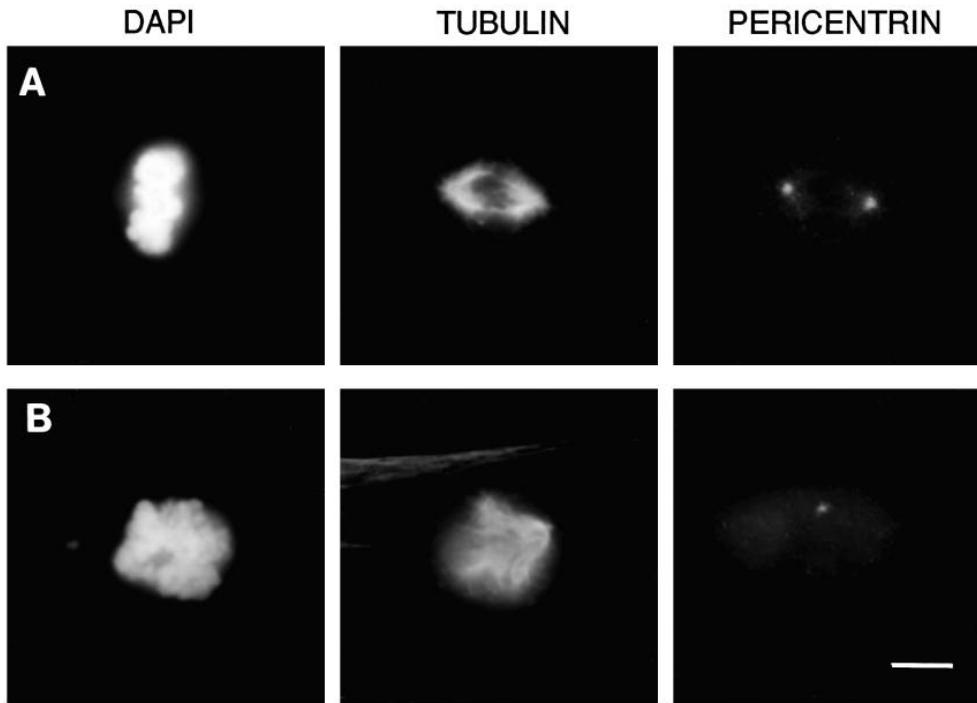


Fig. 7. Localization of the centrosome in cells expressing a mutant form of NuMA that fails to localize to the mitotic spindle. Untreated hamster BHK-21 cells (A) or cells microinjected with the plasmid driving the expression of NuMA carrying mutations of three of the predicted p34^{cdc2} phosphorylation sites (NuMA2000TA2040TA2072SA;B) were processed for indirect immunofluorescence microscopy using the DNA-specific dye (DAPI), anti-tubulin antibody (TUBULIN), and an anti-pericentrin antibody (PERICENTRIN). Bar, 10 μ m.

and B). In contrast, in mitotic cells expressing the NuMA protein, which is incapable of binding to the mitotic spindle, the organization of the mitotic spindle is abnormal. In the presence of NuMA2000TA2040TA2072SA, the mitotic spindle fails to obtain a symmetrical shape, and appears to lack clearly defined poles (Fig. 6C-D). Microtubules are present and appear to interact with the chromosomes leading, in some cases, to the formation of a metaphase chromosome array (Fig. 6E), but the microtubules do not appear to be organized from a single 'point source'. A clear example of this dominant affect on the organization of the mitotic spindle is shown in Fig. 6E, where the disorganized array of microtubules in a mitotic cell expressing NuMA2000TA2040TA2072SA can be directly compared with the well organized microtubule array of an adjacent non-transfected cell. These data suggest that the dominant negative phenotype of perturbation of mitosis that is induced by NuMA proteins that are unable to associate normally with the mitotic spindle is due to the disorganization of the mitotic spindle.

Since the presence of these mutant forms of NuMA induce a dominant negative phenotype characterized by a poorly organized mitotic spindle that lacks clearly defined poles, we tested the integrity of the centrosomes in these cells. Interphase BHK-21 cells were microinjected with the plasmid that expresses NuMA2000TA2040TA2072SA, monitored by phase-contrast microscopy until they entered mitosis, and then fixed and processed for indirect immunofluorescence microscopy using an anti-pericentrin antibody to localize the centrosomes (Fig. 7). In an uninjected cell the mitotic spindle attains a normal bipolar array with clearly defined poles that are decorated by the anti-pericentrin antibody (Fig. 7A). In mitotic cells expressing the mutant NuMA protein that is incapable of binding to the mitotic spindle the centrosomes are clearly detectable with the anti-pericentrin antibody and appear as discrete foci (Fig. 7B; only one pole is visible in this panel).

These data demonstrate that despite the disorganization of the mitotic spindle induced by the expression of a mutant form of NuMA that can not bind to the mitotic spindle the integrity of the centrosomes is not detectably altered.

DISCUSSION

In this article we demonstrate that NuMA is phosphorylated in a mitosis-specific fashion, and we show that mutation of the predicted p34^{cdc2} phosphorylation sites in the carboxyl-terminal tail of the human NuMA protein abolishes its capacity to associate with the pericentrosomal region of the mitotic spindle without affecting its capacity to target normally into the interphase nucleus. One amino acid (threonine 2040) appears to play a central role in directing NuMA onto the mitotic spindle; however, mutations of one or more potential p34^{cdc2} phosphorylation sites in addition to mutation of threonine 2040 are required to completely abolish NuMA's capacity to target onto the mitotic spindle. The two amino acids that are critical for NuMA to bind efficiently to the mitotic spindle, threonine 2000 and threonine 2040, each reside in clusters of at least 12 consecutive amino acids that are perfectly conserved between the human NuMA protein and the NuMA protein from *Xenopus laevis* (K. Ramyar, D. Compton and D. Cleveland, unpublished data), including the consensus sequence for phosphorylation by p34^{cdc2} (S/T-P-X-Z; where X is a polar amino acid and Z is generally basic; Monero and Nurse, 1990). The sequences surrounding serine 2072 and threonine 2091, on the other hand, are not highly conserved between the human and *Xenopus laevis* NuMA protein. While the demonstration that these amino acids are critical for NuMA's interaction with the mitotic spindle and that NuMA is phosphorylated in a mitosis-specific fashion in vivo is consistent with our hypothesis that NuMA's interaction with the

mitotic spindle is controlled by phosphorylation, future work will be needed to determine whether these amino acids in the NuMA protein are substrates for p34^{cdc2}. Despite the fact that the substitution of alanine residues would not be predicted to have a large impact on the overall three-dimensional structure of the protein, there remains the possibility that the mutations we have introduced into the globular tail of NuMA have altered the binding site or conformation of the protein, rendering it unable to bind to the mitotic spindle. In either case, these data clearly demonstrate that these select amino acids in NuMA's globular tail play an essential role in NuMA's localization to the pericentrosomal domain of the mitotic spindle.

In the absence of association with the mitotic spindle, it appears that the mutant NuMA protein accumulates at the plasma membrane of the cell and induces a dominant negative phenotype characterized by the assembly of a poorly organized mitotic spindle with ill-defined polar ends. The exact physical nature of this dominant negative phenotype is not obvious. It is possible that the mutant protein associates with the endogenous wild-type hamster NuMA protein leading to the mislocalization of the endogenous protein; the mutant protein could be titrating some other critical cellular component that normally binds to NuMA at the mitotic spindle pole; or the mutant NuMA polypeptide could aggregate into an insoluble complex that is unable to associate with the mitotic spindle. Unfortunately, antibodies that would allow us to track the endogenous hamster NuMA protein independently of the exogenously expressed human NuMA protein do not exist, so we are unable to address these possibilities. In any event, the result of this dominant negative phenotype, as with most treatments that perturb the normal assembly of the mitotic spindle, is that the cells fail to complete mitosis normally and assemble micronuclei in the subsequent interphase (for examples, see Rieder and Palazzo, 1992; Bernat et al., 1990; Nishimoto et al., 1978).

The localization of these mutant NuMA proteins to the plasma membrane of the mitotic cell is very similar to NuMA's distribution in mitotic cells that have been treated with the microtubule-stabilizing drug taxol. In taxol-treated mitotic cells the microtubules fail to assemble into a well organized mitotic spindle. Instead, the microtubules form a collection of aster-like bundles that extend from the plasma membrane of the cell into the cell cytoplasm. These bundles are arranged in a parallel array with their minus ends at the plasma membrane and their plus ends in the cytoplasm, and a bulk of the NuMA protein is localized at the minus ends of these microtubule bundles at the plasma membrane (Maekawa et al., 1991; Kallajoki et al., 1992). Similarly, in mitotic cells expressing mutant forms of NuMA that fail to associate normally with the pericentrosomal region of the mitotic spindle, the mutant NuMA protein concentrates at the plasma membrane of the cell and, in some cases, the microtubules appear to emanate from these regions of the cell (Fig. 6C and D). The mechanism behind NuMA's localization at the plasma membrane in the experiments described here or in taxol-treated mitotic cells is not understood at this time; however, it has been demonstrated that microinjection of an anti-NuMA antibody into taxol-treated mitotic cells will cause a redistribution of the microtubules into an interphase-like array, suggesting that NuMA plays a primary role in the organization of the taxol-induced microtubule aster at the plasma membrane (Kallajoki et al.,

1992). These data, in conjunction with the observed dominant negative phenotype described here and the perturbation of the mitotic spindle following microinjection of anti-NuMA antibodies (Kallajoki et al., 1991; Yang and Snyder, 1992) suggest that NuMA is playing a key role in the organization and/or stabilization of parallel arrays of microtubules during mitosis.

Why is NuMA in the interphase nucleus?

The data presented here indicate that NuMA's interaction with the microtubules of the mitotic spindle is controlled by both mitosis-specific phosphorylation and differential sub-cellular compartmentalization, and that NuMA plays a key role in organizing the mitotic spindle. If NuMA's interaction with microtubules is controlled in a mitosis-specific fashion through phosphorylation, then why is NuMA specifically localized in the nucleus of the interphase cell? One solution to this question is that NuMA has two distinct, and separable functions in the cell. In addition to organizing the microtubules of the mitotic spindle, NuMA could perform a second function within the interphase nucleus. Previously, we and others have proposed that NuMA plays a structural role within the nucleus (Compton and Cleveland, 1993; Price and Pettijohn, 1986; Yang et al., 1992) and, more recently, it has been suggested that NuMA

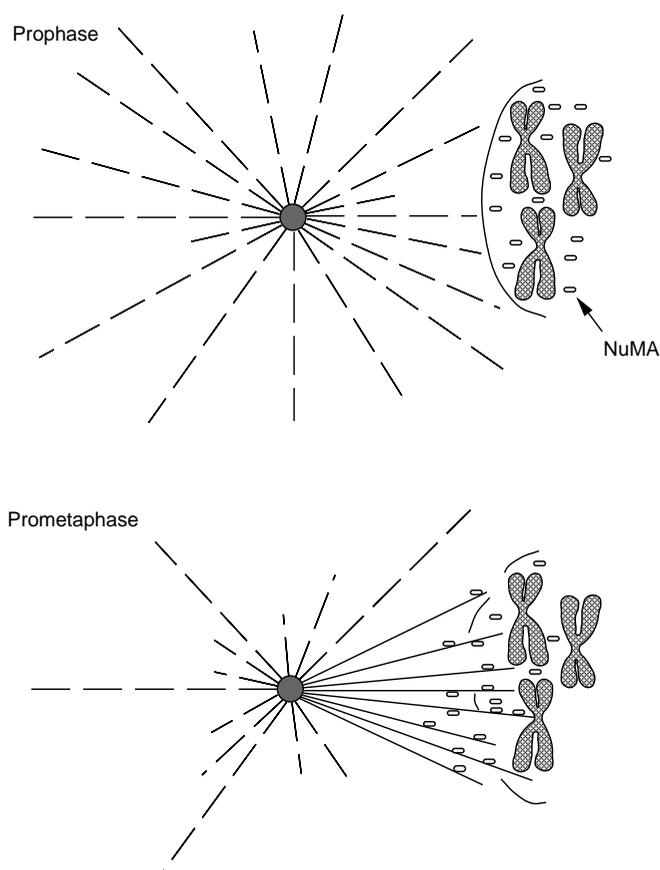


Fig. 8. Model for NuMA function during mitosis. Unstabilized aster microtubules (broken lines) nucleated from the centrosome in prophase are subject to dynamic shifts in their polymerization/depolymerization state. At prometaphase, when the nuclear envelope disassembles, the microtubules invade the nuclear compartment and become stabilized (continuous lines) by the interaction with NuMA prior to their interaction with the kinetochores of the chromosomes.

provides a link between the nuclear matrix and other nuclear components such as the spliceosomes (Zeng et al., 1994).

A second possible explanation for NuMA's residence in the nucleus during interphase is that its role in organizing the microtubules of the mitotic spindle is coupled to its localization within the nucleus. A schematic diagram depicting this model for NuMA function is shown in Fig. 8. In this model, the primary function of NuMA is in organizing or stabilizing the parallel arrays of microtubules that emanate from the centrosome. During prophase, NuMA as well as the chromosomes are retained within the nuclear compartment as the mitotic aster microtubules assemble at each centrosome. At the onset of prometaphase, when the nuclear envelope disassembles, the microtubules begin to invade the nuclear compartment. In the context of this model for NuMA function, the NuMA protein would contact and stabilize those microtubules that invade the nuclear compartment. The aster microtubules that extend in the direction away from the nucleus would not encounter such a stabilizing interaction, and continue to rapidly fluctuate between growing and shrinking states. This NuMA-dependent stabilization of the microtubules that enter the nuclear compartment would favor the formation of a 'fusiform' spindle array and, thus, rely on the spatial restriction of the NuMA protein in the nuclear compartment. Therefore, during this critical stage of mitosis, this proposed NuMA-dependent stabilization of the subset of microtubules that extend into the nuclear compartment could enhance the probability that a microtubule would make contact with the kinetochore domain of a chromosome. Ultimately, all of the NuMA protein would become attached to the microtubules of the mitotic spindle with the eventual accumulation of NuMA at the polar ends of the mitotic spindle. Once at the pericentrosomal region of the mitotic spindle, NuMA could continue to provide a stabilizing influence for the microtubules of the mitotic spindle, allowing the microtubules to exchange tubulin subunits at each end.

Support for this model comes from *in vitro* spindle assembly experiments that suggest that the mitotic spindle is not organized strictly through interactions between the microtubules and the chromosomes, but rather, is assembled through a 'hierarchy of selective microtubule stabilization' (Sawin and Mitchison, 1991). This proposal is also consistent with the localization of NuMA at the minus ends of the taxol-induced microtubule asters (Maekawa et al., 1991; Kallajoki et al., 1992), as well as the localization of NuMA at the origin of the sites of microtubule assembly following the reversal of nocodazole-induced microtubule disassembly (Tousson et al., 1991). Moreover, this model is supported by the phenotypes observed following perturbation of NuMA function through anti-NuMA antibody microinjection (Kallajoki et al., 1991, 1992, 1993; Yang and Snyder, 1993), and expression of mutant forms of the NuMA polypeptide (Compton and Cleveland, 1993). Finally, this model emphasizes the role of NuMA during the early events in mitosis and not beyond the metaphase/anaphase transition, an aspect of the model that is directly supported by the anti-NuMA antibody microinjection experiments performed by Yang and Snyder (1993).

An essential question to be addressed with regard to the mechanism of how NuMA might act to selectively stabilize microtubules in the mitotic spindle is whether NuMA is contacting the microtubules directly or indirectly through an inter-

mediate protein such as a microtubule-dependent motor protein (Sawin et al., 1992; Yang and Snyder, 1993). As a final note, this proposed model for NuMA function could provide an evolutionary link between vertebrate cells that undergo an open mitosis (nuclear envelope disassembly) and the unicellular organisms that undergo a closed mitosis (assemble their mitotic spindle directly inside the nucleus).

This work was supported, in part, by a grant to D.A.C. from the Hitchcock Foundation. We thank Gus Lienhard for his critical review of this manuscript and Steve Doxsey for generously providing the anti-pericentrin antibody.

REFERENCES

- Bernat, R. L., Borisy, G. G., Rothfield, N. F. and Earnshaw, W. C. (1990). Injection of antacentromere antibodies in interphase disrupts events required for chromosome movements at mitosis. *J. Cell Biol.* **111**, 1519-1533.
- Capecchi, M. R. (1980). High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell* **22**, 479-488.
- Compton, D. A., Yen, T. J. and Cleveland, D. W. (1991). Identification of novel centromere/kinetochore-associated proteins using monoclonal antibodies generated against human mitotic chromosome scaffolds. *J. Cell Biol.* **112**, 1083-1097.
- Compton, D. A., Szilak, I. and Cleveland, D. W. (1992). Primary structure of NuMA, an intranuclear protein that defines a novel pathway for segregation of proteins at mitosis. *J. Cell Biol.* **116**, 1395-1408.
- Compton, D. A. and Cleveland, D. W. (1993). NuMA is required for the proper completion of mitosis. *J. Cell Biol.* **120**, 947-957.
- Compton, D. A. and Cleveland, D. W. (1994). NuMA, a nuclear protein involved in mitosis and nuclear reformation. *Curr. Opin. Cell Biol.* **6**, 343-346.
- Gerace, L. and Burke, B. (1988). Functional organization of the nuclear envelope. *Annu. Rev. Cell Biol.* **4**, 335-374.
- Gill, S. R., Schroer, T. A., Szilak, I., Steuer, E. R., Sheetz, M. P. and Cleveland, D. W. (1991). Dynactin, a conserved, ubiquitously expressed component of an activator of vesicle motility by cytoplasmic dynein. *J. Cell Biol.* **115**, 1639-1650.
- Kallajoki, M., Weber, K. and Osborn, M. (1991). A 210 kD nuclear matrix protein is a functional part of the mitotic spindle; a microinjection study using SPN monoclonal antibodies. *EMBO J.* **10**, 3351-3362.
- Kallajoki, M., Weber, K. and Osborn, M. (1992). Ability to organize microtubules in taxol-treated mitotic PtK2 cells goes with the SPN antigen and not with the centrosome. *J. Cell Sci.* **102**, 91-102.
- Kallajoki, M., Harborth, J., Weber, K. and Osborn, M. (1993). Microinjection of a monoclonal antibody against SPN antigen, now identified by peptide sequences as the NuMA protein, induces micronuclei in PtK2 cells. *J. Cell Sci.* **104**, 139-150.
- Laemmli, U. K. (1970). Cleavage of structural proteins during assembly at the head of the bacteriophage T4. *Nature* **227**, 680-682.
- Lydersen, B. K. and Pettijohn, D. E. (1980). Human specific nuclear protein that associates with the polar region of the mitotic apparatus: distribution in a human/hamster hybrid cell. *Cell* **22**, 489-499.
- Maekawa, T., Leslie, R. and Kuriyama, R. (1991). Identification of a minus-end specific microtubule-associated protein located at the mitotic spindle poles in cultured mammalian cells. *Eur. J. Cell Biol.* **54**, 255-267.
- Maekawa, T. and Kuriyama, R. (1993). Primary structure and microtubule-interacting domain of the SP-H antigen: a mitotic map located at the spindle pole and characterized as a homologous protein to NuMA. *J. Cell Sci.* **105**, 589-600.
- Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- McKeon, F. (1991). Nuclear lamin proteins: domains required for nuclear targeting, assembly, and cell-cycle-regulated dynamics. *Curr. Opin. Cell Biol.* **3**, 82-86.
- Mitchison, T. J. (1989). Mitosis: basic concepts. *Curr. Opin. Cell Biol.* **1**, 67-74.
- Monero, S. and Nurse, P. (1990). Substrates for p34^{cdc2}: *in vitro* veritas? *Cell* **61**, 549-551.
- Newport, J. W. and Forbes, D. J. (1987). The nucleus: structure, function and dynamics. *Annu. Rev. Biochem.* **56**, 535-565.

- Nigg, E. A.** (1992). Assembly-disassembly of the nuclear lamina. *Curr. Opin. Cell Biol.* **4**, 105-109.
- Nishimoto, T., Eilen, E. and Basilico, C.** (1978). Premature chromosome condensation in a tsDNA⁻ mutant of BHK cells. *Cell* **15**, 475-483.
- Price, C. M. and Pettijohn, D. E.** (1986). Redistribution of the nuclear mitotic apparatus protein (NuMA) during mitosis and nuclear assembly. *Exp. Cell Res.* **166**, 295-311.
- Rieder, C. L.** (1991). Mitosis: towards an understanding of chromosome behavior. *Curr. Opin. Cell Biol.* **3**, 59-66.
- Rieder, C. L. and Palazzo, R. E.** (1992). Colcemid and the cell cycle. *J. Cell Sci.* **102**, 387-392.
- Sawin, K. E. and Mitchison, T. J.** (1991). Mitotic spindle assembly by two different pathways in vitro. *J. Cell Biol.* **112**, 925-940.
- Sawin, K. E., LeGuilic, K., Philippe, M. and Mitchison, T. J.** (1992). Mitotic spindle organization by a plus-end-directed microtubule motor. *Nature* **359**, 540-543.
- Tang, T. K., Tang, C. C., Chen, Y.-L. and Wu, C.-W.** (1993). Nuclear proteins of the bovine epithelium. II. The NuMA gene gives rise to multiple mRNAs and gene products reactive with monoclonal antibody W1. *J. Cell Sci.* **104**, 249-260.
- Tousson, A., Zeng, C., Brinkley, B. R. and Valdivia, M. M.** (1991). Centrophilin, a novel mitotic spindle protein involved in microtubule nucleation. *J. Cell Biol.* **112**, 427-440.
- Yang, C. H., Lambie, E. J. and Snyder, M.** (1992). NuMA: an unusually long coiled-coil related protein in the mammalian cell nucleus. *J. Cell Biol.* **116**, 1303-1317.
- Yang, C. H. and Snyder, M.** (1992). The nuclear-mitotic apparatus protein (NuMA) is important in the establishment and maintenance of the bipolar mitotic spindle apparatus. *Mol. Biol. Cell* **3**, 1259-1267.
- Zeng, C., He, D., Berget, S. M. and Brinkley, B. R.** (1994). Nuclear-mitotic apparatus protein: A structural protein interface between the nucleoskeleton and RNA splicing. *Proc. Nat. Acad. Sci. USA* **91**, 1505-1509.

(Received 3 June 1994 - Accepted 3 October 1994)

