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NuMA assembles into an extensive filamentous structure when expressed in the cell cytoplasm

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SUMMARY

NuMA is a 236 kDa protein that participates in the organization of the mitotic spindle despite its strict localization in the nucleus during interphase. To test how cells progress through mitosis when NuMA is localized in the cytoplasm instead of the nucleus, we have deleted the nuclear localization sequence of NuMA using site-directed mutagenesis and transiently expressed this mutant protein (NuMA- Δ NLS) in BHK-21 cells. During interphase, NuMA- Δ NLS accumulates in the cytoplasm as a large mass approximately the same size as the cell nucleus. When cells enter mitosis, NuMA- Δ NLS associates normally with the mitotic spindle without causing any apparent deleterious effects on the progression of mitosis. Examination of the cytoplasmic mass formed by NuMA- Δ NLS using transmission electron microscopy (TEM) revealed an extensive network of ~5 nm

filaments that are further organized by the presence of dynamic microtubules into a dense web of solid, ~23 nm cables. Using flow cytometry, we have isolated the intact filamentous mass formed by NuMA- Δ NLS from lysates of transiently transfected cells. These isolated structures are constructed of networks of interconnected 5 nm filaments and are composed exclusively of NuMA. These data demonstrate that NuMA is capable of assembling into an extensive filamentous structure supporting the possibility that NuMA serves a structural function either in the nucleus during interphase or at the polar ends of the mitotic spindle.

Key words: NuMA, Nucleus, Mitosis, Filament, Nuclear matrix

INTRODUCTION

The nucleus of a typical vertebrate cell undergoes extensive physical rearrangement during mitosis (Newport and Forbes, 1987; Gerace and Burke, 1988). These structural changes are necessary for the efficient segregation of the nuclear components into each of the daughter cells, and examples of these physical rearrangements include the condensation of the chromosomes, the dissolution of the nuclear lamina, and the vesicularization of the nuclear envelope. To date, there appear to be three segregation pathways for the components of the nucleus during mitosis. The chromosomes and chromatin-associated proteins are segregated equally into each daughter cell during anaphase through their association with the microtubules of the mitotic spindle (Mitchison, 1989; Rieder, 1991). The nuclear envelope vesicles, nuclear lamina, and many other nuclear proteins are distributed into each daughter cell by passive diffusion (McKeon, 1991; Nigg, 1992; Wiese and Wilson, 1993). Finally, the NuMA protein is distributed equally into each daughter cell through its association with the polar ends of the mitotic spindle (Lyderon and Pettijohn, 1980; reviewed by Compton and Cleveland, 1994; Cleveland, 1995).

NuMA is a 236 kDa protein that is distributed uniformly throughout the interior of the cell nucleus although it appears

to be excluded from the nucleoli (Price and Pettijohn, 1986, Compton et al., 1991; Kallajoki et al., 1991; Tousson et al., 1991; Yang et al., 1992; Maekawa et al., 1991). When the nuclear envelope disassembles during prometaphase, NuMA is released from the nuclear compartment and accumulates at the polar end of the mitotic spindle. The prevailing evidence suggests that once NuMA is released from the nucleus and accumulates at the mitotic spindle pole it plays an essential role in organizing the microtubules at the polar region of the mitotic spindle. Experimental support for this functional role include the perturbation of the organization of the mitotic spindle following the microinjection of NuMA-specific antibodies (Gaglio et al., 1995; Kallajoki et al., 1991, 1992, 1993; Yang and Snyder, 1992), the elimination of microtubule organization in a cell free mitotic extract following the specific depletion of NuMA (Gaglio et al., 1995), and the perturbation of the organization of the mitotic spindle following the expression of a mutant form of the NuMA protein that fails to associate with the mitotic spindle and exerts dominant negative phenotype (Compton and Luo, 1995).

The possible functional role(s) for NuMA in the nucleus, however, are much less certain due to the lack of defined functional assays for specific nuclear function. It has been proposed that NuMA is a structural component of the core fibers of the

nuclear matrix (Yang et al., 1992; Zeng et al., 1994a), that it participates in the re-assembly of the daughter cell nuclei at the end of mitosis (Compton and Cleveland, 1993), and that it is a component of splicing complexes (Zeng et al., 1994b). In addition to these proposed specific functions for NuMA within the cell nucleus, there have also been proposals that NuMA is only localized in the nucleus to sequester it away from the cytoplasmic microtubules during the interphase stages of mitosis (Compton et al., 1992). Indeed, we have recently proposed that the localization of NuMA in the nucleus establishes an inherent asymmetry to its distribution which is necessary for its role in directing the microtubules of the developing mitotic spindle toward the chromosomes during assembly of the mitotic spindle (Compton and Luo, 1995).

Given the strict localization of NuMA in the nucleus during interphase, and the proposals that NuMA may only be localized in the cell nucleus to sequester it away from the cytoplasmic microtubules we decided to examine how cells progress through mitosis if NuMA is present in the cell cytoplasm instead of the nucleus. The amino acid sequence necessary and sufficient for NuMA's localization in the nucleus has been mapped to a short segment in its carboxyl-terminal globular tail domain (Tang et al., 1994). To test the fate of NuMA in the cell cytoplasm we have used site-directed mutagenesis to delete the nuclear localization sequence from the NuMA protein and then transiently expressed this mutant form of NuMA (NuMA- Δ NLS) in BHK-21 cells. In this article we show that NuMA- Δ NLS accumulates in the cell cytoplasm in a filamentous mass approximately the same size as the cell nucleus, and that NuMA- Δ NLS associates normally with the microtubules of the mitotic spindle without exerting any dominant negative effects. The cytoplasmic structure formed by NuMA- Δ NLS is composed of an extensive network of ~5 nm filaments that become organized into solid ~23 nm cables in the presence of dynamic microtubules. These results demonstrate that NuMA is capable of assembling into an extensive filamentous array consistent with the proposed structural roles for NuMA in the interphase nucleus and at the polar ends 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.

Transfection and microinjection

Cells were transiently transfected using Lipofectamine reagent (Gibco BRL/Life Technologies, Gaithersburg, MD) as described by the manufacturer. Cells growing on 35 mm dishes with (for immunofluorescence and electron microscopy) or without (for immunoblot analysis) glass coverslips were washed twice, and equilibrated in 800 μ l Opti-MEM serum free medium. Plasmid DNA (1 μ g) diluted in 100 μ l of Opti-MEM serum free medium, and 10 μ g of the Lipofectamine reagent diluted in 100 μ l 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 μ l 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 in the nucleus with plasmid DNA at a concentration of 100 μ g/ml in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA. Injected cells were followed by phase contrast microscopy and processed for immunofluorescence and electron 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 extracted and fixed by immersing the coverslips in microtubule stabilizing buffer (MTSB: 4 M glycerol, 100 mM Pipes, pH 6.8, 1 mM EGTA, 5 mM MgCl₂) 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 (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 1% bovine serum albumin and all subsequent steps were performed in TBS containing 1% bovine serum albumin at room temperature. In some cases the cells were fixed without prior detergent extraction by immersion in phosphate-buffered saline containing 3.5% paraformaldehyde for 5 minutes at room temperature. The cells were then extracted with 0.5% Triton X-100 in TBS containing 1% bovine serum albumin for 5 minutes at room temperature. Following fixation, 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 μ g/ml; Sigma Chemical Co., St Louis, MO). Coverslips were mounted in FITC-Guard (Testog, Chicago, IL) and observed with a Nikon Optiphot microscope equipped for epifluorescence. No fewer than 12 cells were examined for each panel presented in Figs 2-4, and the panels shown depict a representative cell for each experiment.

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 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 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 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 (Compton and Luo, 1995). This plasmid was maintained in the DH1 strain of *Escherichia coli* and purified by CsCl centrifugation (Maniatis et al., 1982). Mutation of the NuMA cDNA was performed using the transformer site-directed mutagenesis kit (Clontech laboratories Inc., Palo Alto CA) precisely as described by the manufacturer. The sequence of the mutagenic primer (Operon

Technologies Inc., Alameda CA) was 5'-GTGGGGCTCTAGGGA-GACCCGGGTGGTGATGCCAGTGC-3'. This oligonucleotide creates an internal deletion of 15 nucleotides in the human NuMA cDNA that eliminates amino acid residues 1971-1975 (1985-1989 using the amino acid numbering system of Yang et al., 1992). This oligonucleotide also introduces a novel *Sma*I site into the cDNA that permits rapid identification of the mutant clones. The appropriate mutation was verified by DNA sequence analysis, and no multiple mutations occurred as judged by restriction analysis using the mutation-associated restriction enzyme site. 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.

Electron microscopy

Cells growing on photo etched alpha-numeric glass coverslips expressing NuMA- Δ NLS were identified by phase contrast microscopy. The coverslips were removed from the culture medium and the cells fixed with 2% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.4, for 1 hour at room temperature either with or without (as indicated in the text) prior extraction with MTSB containing 0.5% Triton X-100. Following fixation, the cells were rinsed in 0.1 M Na-cacodylate buffer, post-fixed with 1% OsO₄ in 0.1 M Na-cacodylate buffer for 30 minutes at room temperature, and en-bloc stained in 2% aqueous uranyl acetate. Cells were dehydrated through a graded series of ethanols and propylene oxide, and flat-embedded in epon(LX112)/araldite(502). The glass coverslip was removed by etching in cold concentrated hydrofluoric acid as described by Moore (1975), and Rieder and Bowser (1987). The area of interest was identified with the help of a dissecting microscope, cut out of the flat-embedded rectangle and remounted onto epoxy blanks. 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. No fewer than four cells were examined for each panel in Figs 5 and 6, and the panels shown depict a representative cell for each experiment.

Flow cytometry

BHK-21 cells were harvested 18 to 24 hours after transfection with the plasmid driving the expression of NuMA- Δ NLS. The cells were removed from the culture dish by trypsinization, washed twice with PBS, and lysed at a concentration of 1.5×10^6 cells/ml by agitation in lysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM MgCl₂, 0.25 M sucrose, 0.5% Triton X-100, 20 μ g/ml PMSF, 1 μ g/ml each of leupeptin, pepstatin, antipain, and DAPI) for 1 hour at 4°C. The crude lysate was then subjected to flow cytometry with phosphate-buffered saline as the vehicle using a FACStar Plus (Becton Dickinson). The data was collected and analyzed using the Lysys II software package.

RESULTS

Expression of human NuMA in the cell cytoplasm

To determine the functional properties of NuMA when it accumulates in the cell cytoplasm rather than in the cell nucleus we have used site-directed mutagenesis to delete five amino acids (15 nucleotides) from the globular tail of the NuMA protein. These five amino acids span the core of NuMA's nuclear localization sequence based on the previous results of Tang et al. (1994). To verify that the mutant NuMA cDNA (NuMA- Δ NLS) is 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 hamster BHK-21 cells (Fig. 1). In BHK-21 cells transfected with the expression vector alone (containing no cDNA; Fig. 1, Mock) there is no detectable protein when analyzed using a primate-specific anti-NuMA rabbit polyclonal antibody (Gaglio et al., 1995). In BHK-21 cells transfected with the expression plasmid driving the expression of either the wild-type human NuMA protein (Fig. 1, NuMA 1-2101) or NuMA- Δ NLS (Fig. 1, NuMA- Δ NLS) a single protein is detected that migrates with a molecular mass >200 kDa. These data are consistent with previous experiments involving the expression of human NuMA in the context of hamster BHK-21 cells (Compton and Cleveland, 1993; Compton and Luo, 1995), and indicate that NuMA- Δ NLS migrates in SDS-PAGE with the same relative molecular mass as the wild-type human NuMA protein.

To determine the intracellular localization of NuMA- Δ NLS we performed indirect immunofluorescence microscopy using the primate-specific anti-NuMA rabbit serum (Gaglio et al., 1995) and the tubulin-specific monoclonal antibody DM1 α (Fig. 2). In interphase cells that express NuMA- Δ NLS but have not yet passed through mitosis the human NuMA protein accumulates in a large mass adjacent to the nucleus in the cell cytoplasm. This cytoplasmic mass formed by NuMA- Δ NLS is frequently similar in size to the cell nucleus, and careful focusing of the microscope indicates that it is fibrous in nature (Fig. 2A). In addition to these qualities, the cytoplasmic structures formed by NuMA- Δ NLS consistently stain positive for tubulin although the microtubule array in other regions of the cytoplasm do not appear to be affected in any noticeable way. The morphology of the cytoplasmic mass formed by NuMA- Δ NLS is identical regardless of the fixation condition (methanol, paraformaldehyde, glutaraldehyde) or whether the

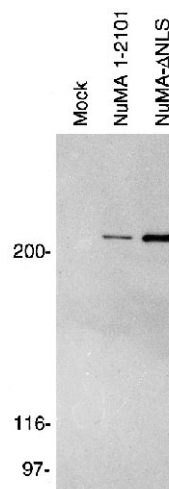


Fig. 1. Expression of human NuMA in hamster BHK-21 cells. The primate-specific anti-NuMA rabbit antibody was used to detect the exogenously expressed human NuMA proteins in ~ 40 μ g of total cell extract after electrophoresis on a 5% SDS-polyacrylamide gel. BHK-21 cells were transfected with the expression vector alone (Mock), or the expression vector driving the expression of either the full length human NuMA protein (NuMA 1-2,101) or the NuMA lacking the nuclear localization sequence (NuMA- Δ NLS). The migration positions of myosin (200 kDa), β -galactosidase (116 kDa), and phosphorylase B (97 kDa) are indicated at the left.

cells are fixed directly or subjected to extraction with non-ionic detergents prior to fixation under conditions that either preserve microtubules or conditions for the observation of the nuclear matrix. Moreover, we observed that NuMA- Δ NLS formed a filamentous structure in the cell cytoplasm whether the expression level is relatively high (Fig. 3A) or low (Fig. 3B), and in all cases these filamentous structures stained positively for tubulin if they were assembled in the presence of dynamic microtubules. Finally, while the localization of NuMA- Δ NLS to the cell cytoplasm is probably the direct result of the mutation we introduced into the NuMA cDNA, the assembly of NuMA into a filamentous mass in the cell cytoplasm is not an artifact of the expression of a mutant form of NuMA because we have observed similar structures in the cytoplasm of cells expressing extremely high levels (approximately 5% of transfected cells) of the wild-type human NuMA protein (data not shown). These results verify that amino acids 1971-1975 form the core of NuMA's nuclear localization sequence (Tang et al., 1994), and further show that the accumulation of NuMA in the cell cytoplasm leads to the formation of a mass that has fibrous qualities which are detectable by immunofluorescence microscopy.

When cells expressing NuMA- Δ NLS enter mitosis, the human NuMA- Δ NLS protein localizes normally at the polar ends of the mitotic spindle although the staining intensity at the spindle poles is reproducibly weaker than the staining

intensity of the cytoplasmic mass that forms prior to mitosis (Fig 2B). Following the completion of mitosis, the human NuMA- Δ NLS protein accumulates in the nuclei of the two daughter cells (Fig. 2C). The most likely explanation for the post-mitotic nuclear accumulation of NuMA- Δ NLS is that it interacts with the endogenous hamster NuMA protein during mitosis and the nuclear localization sequence on the endogenous protein acts in a dominant fashion to direct the nuclear accumulation of the human NuMA- Δ NLS following mitosis. Regardless of the mechanism of post-mitotic nuclear import of NuMA- Δ NLS, these data indicate that despite the accumulation of NuMA- Δ NLS in the cell cytoplasm prior to mitosis, this mutant form of NuMA localizes correctly during other phases of mitosis and does not exert any observable dominant negative effect on the assembly of the mitotic spindle or the progression of mitosis.

Because the cytoplasmic structures formed by NuMA- Δ NLS appear to have a filamentous nature and they stain positively for tubulin we tested if the assembly of these structures required microtubules. For this experiment we transiently expressed the human NuMA- Δ NLS protein in the hamster cells and then perturbed the cytoplasmic array of microtubules using either taxol or nocodazole. To test if microtubules were necessary to maintain these cytoplasmic structures we allowed NuMA- Δ NLS to accumulate and assemble into the cytoplasmic mass prior to the addition of either taxol or nocodazole.

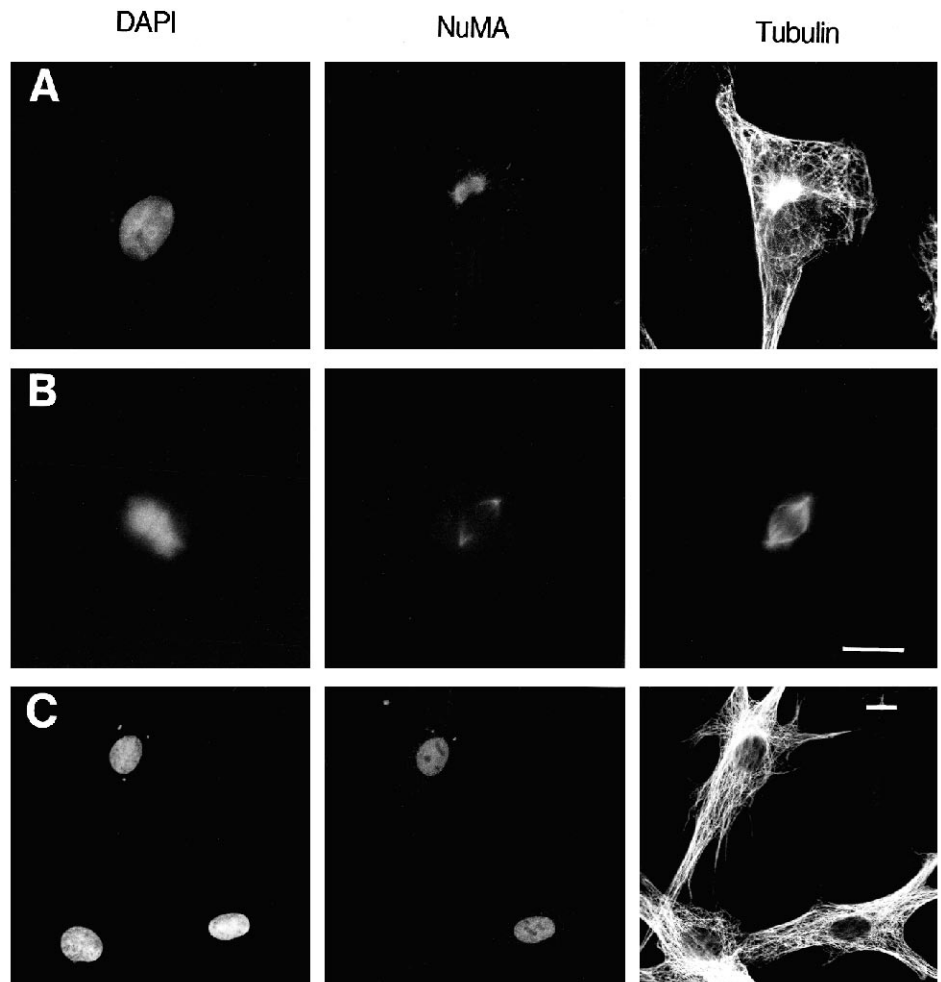


Fig. 2. Localization of human NuMA- Δ NLS in BHK-21 cells. Cells were transiently transfected with the plasmid driving the expression of NuMA lacking the nuclear localization sequence. Cells in interphase prior to mitosis (A), during mitosis (B) or in interphase following the completion of mitosis (C) were fixed and processed for immunofluorescence microscopy with the DNA-specific dye (DAPI), the primate-specific anti-NuMA rabbit serum (NuMA), and the tubulin-specific mouse monoclonal antibody DM1 α (Tubulin). Note that the magnification in A and B is different from C. Bars, 20 μ m.

Under these conditions, the addition of the microtubule perturbing drugs has little effect on the organization of the structure formed by NuMA- Δ NLS in the cell cytoplasm (data not shown). To test if microtubules are necessary for the assembly of the cytoplasmic mass of NuMA- Δ NLS we added either taxol or nocodazole to the culture medium during the expression of the NuMA- Δ NLS protein in the cell cytoplasm. For this experiment we microinjected the plasmid DNA directly into the cell nucleus to induce protein expression, allowed the cells to recover from the microinjection at 37°C for 15 minutes, and then added either taxol or nocodazole to the culture medium (each at 10 μ M for 8 hours). In the presence of taxol, NuMA- Δ NLS still accumulates in the perinuclear region of the cytoplasm as a solid mass, but it no longer has a filamentous quality nor does it stain positively for tubulin (Fig. 3C). In the presence of nocodazole, NuMA- Δ NLS accumulates in a ring-like aggregate around the nucleus in

addition to a number of foci dispersed throughout the cytoplasm. These structures lack any obvious filamentous quality observable by immunofluorescence microscopy, but contrary to the results using taxol, these NuMA- Δ NLS foci do stain positively for tubulin (Fig. 3D). These results indicate that NuMA- Δ NLS accumulates in discrete foci in the cell cytoplasm regardless of the presence of microtubules, but that dynamic microtubules are necessary during the accumulation of NuMA- Δ NLS for the cytoplasmic mass to attain the fibrous quality that is detectable by immunofluorescence microscopy.

Next, we examined whether other cytoskeletal components localize in the NuMA- Δ NLS structure (Fig. 4). Using Texas red-conjugated phalloidin to identify actin and an antibody specific for vimentin (the only intermediate filament protein expressed in BHK-21 cells) we failed to detect either vimentin or actin associated with the NuMA- Δ NLS structure (Fig. 4A,B). Indeed, the structure formed by NuMA- Δ NLS, like the nucleus, appears

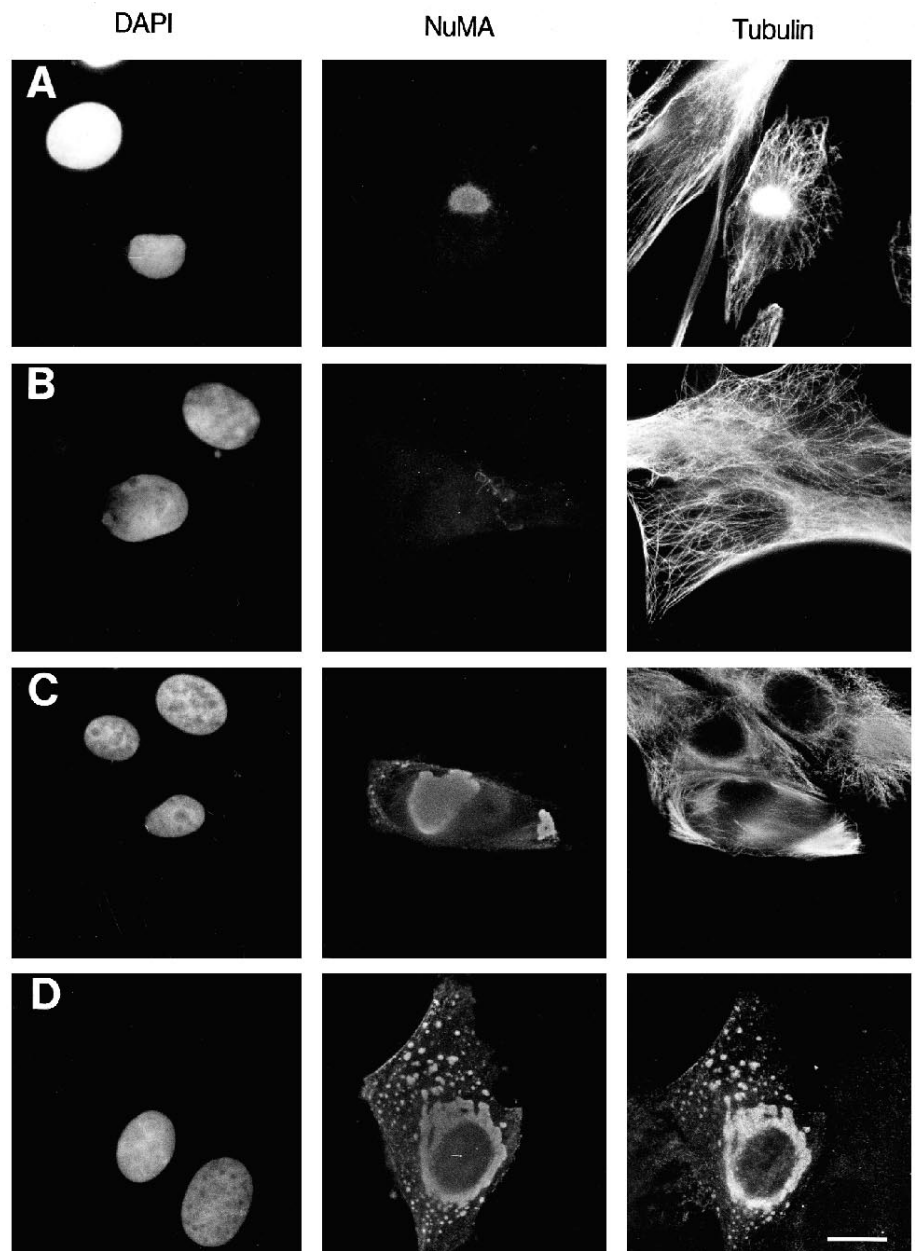


Fig. 3. Localization of various quantities of human NuMA- Δ NLS in BHK-21 cells and localization of NuMA- Δ NLS in BHK-21 cells in the presence of either taxol or nocodazole. Cells were either transiently transfected (A,B) or microinjected (C,D) with the plasmid driving the expression of NuMA lacking the nuclear localization sequence. Cells were fixed directly (A,B) or treated with either 10 μ M taxol (C) or 10 μ M nocodazole (D) for 8 hours and then fixed and processed for immunofluorescence microscopy with the DNA-specific dye (DAPI), the primate-specific anti-NuMA rabbit serum (NuMA), and the tubulin-specific mouse monoclonal antibody DM1 α (Tubulin). Bar, 20 μ m.

to exclude a majority of the cytoplasmic intermediate filaments (Fig. 4B). Next, because of the shape of the NuMA- Δ NLS structure we tested if the nuclear lamins associated with NuMA- Δ NLS. Using an antibody that recognizes both lamin A and C (courtesy of Dr L. Gerace) we failed to detect any association of the nuclear lamin proteins A or C with the NuMA- Δ NLS structure in the cytoplasm (Fig. 4C). Finally, we tested for the presence of the centrosome using antibodies specific for pericentrin (courtesy of Dr S. Doxsey). Despite the accumulation of NuMA- Δ NLS in the perinuclear region of the cell there is no consistent association of this structure with the centrosome (Fig. 4D, arrow). Taken together, these results indicate that no other cytoskeletal proteins appear to be associated with the cytoplasmic structure formed by NuMA- Δ NLS, and suggest that the fibrous quality of the cytoplasmic mass formed by NuMA- Δ NLS that is observable by immunofluorescence microscopy is derived from an interaction between NuMA and dynamic microtubules.

Ultrastructure of NuMA in the cell cytoplasm

The apparent filamentous organization of the structures formed by NuMA in the cell cytoplasm prompted us to examine these structures at high magnification using transmission electron microscopy (TEM). For these experiments we grew the BHK-21 cells on photo-etched gridded coverslips, transfected them with the plasmid that drives the expression of NuMA- Δ NLS, and then used phase contrast microscopy to select the cells to examine by TEM. When viewed by TEM the filamentous nature of the cytoplasmic mass created by the expression of NuMA- Δ NLS is clearly obvious (Fig. 5A). A large region of the cytoplasm adjacent to the nucleus is occupied by a dense web of solid, unbranching cables that extend up to 2 μ m in length (the actual length of each cable may be longer, but thin section prohibits their observation), and appear to exclude adjacent components of the cytoplasm (i.e. mitochondria). At high magnification (Fig. 5B, large arrow), the solid core of

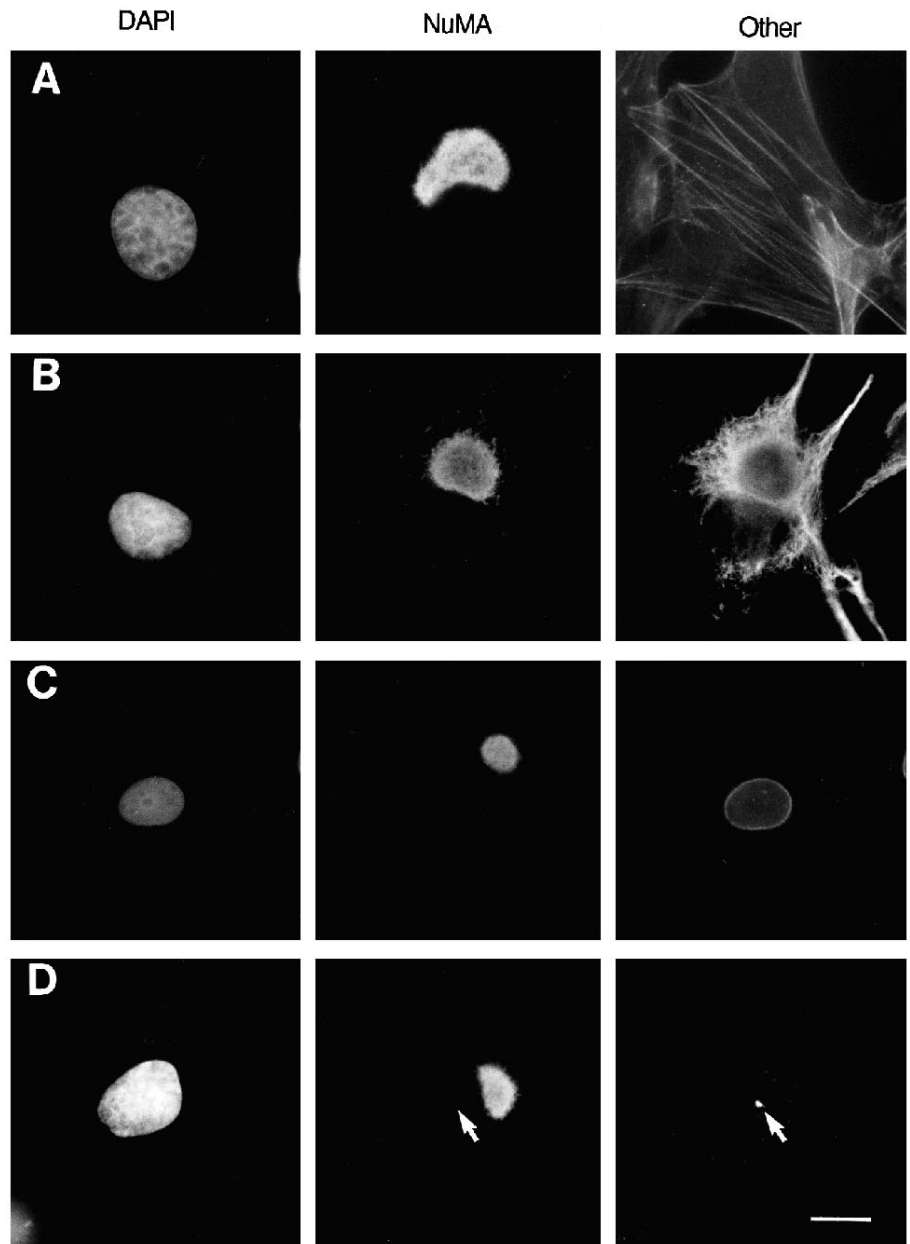


Fig. 4. Localization of human NuMA- Δ NLS relative to other cytoskeletal systems in BHK-21 cells. Cells were transiently transfected with the plasmid driving the expression of NuMA lacking the nuclear localization sequence. Cells were fixed and processed for immunofluorescence microscopy with the DNA-specific dye (DAPI), the NuMA-specific monoclonal antibody 1F1 (NuMA), and either Texas red-conjugated phalloidin (A), a rabbit antibody recognizing vimentin (B), a rabbit antibody recognizing nuclear lamins A and C (C), or a rabbit antibody recognizing the centrosome-associated protein pericentrin (D). Bar, 20 μ m.

each cable is clearly visible when viewed in cross section or viewed laterally and is uniformly ~ 23 nm in diameter (23.01 ± 1.03 nm). Unlike typical cytoskeletal filaments,

however, these cables are laterally asymmetric. Both the lateral and the cross sectional views reveal that each cable has a smooth side and an electron dense 'fuzzy' side (Fig. 5B, small

Fig. 5. Ultrastructure on NuMA- Δ NLS expressed in the cytoplasm of BHK-21 cells. Cells were transiently transfected with the plasmid driving the expression of NuMA lacking the nuclear localization sequence and either fixed directly (A-C) or extracted with a microtubule stabilizing buffer containing 0.5% Triton X-100 prior to fixation (D,E) in 2% glutaraldehyde. Cells were then dehydrated, embedded, sectioned, and viewed by transmission electron microscopy. Large arrows indicate cables observed in cross section, and small arrows indicate regions of lateral asymmetry. Bars: (A,D), 1 μ m; (B,C,E), 200 nm.

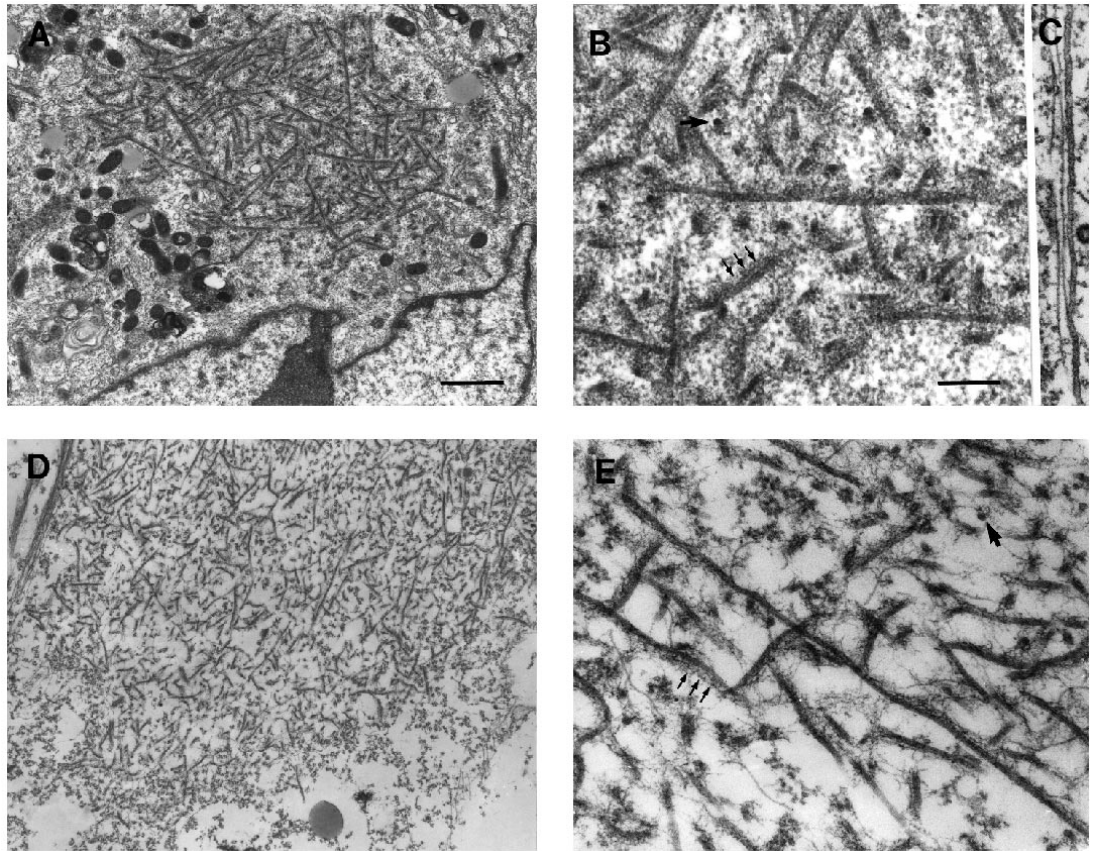
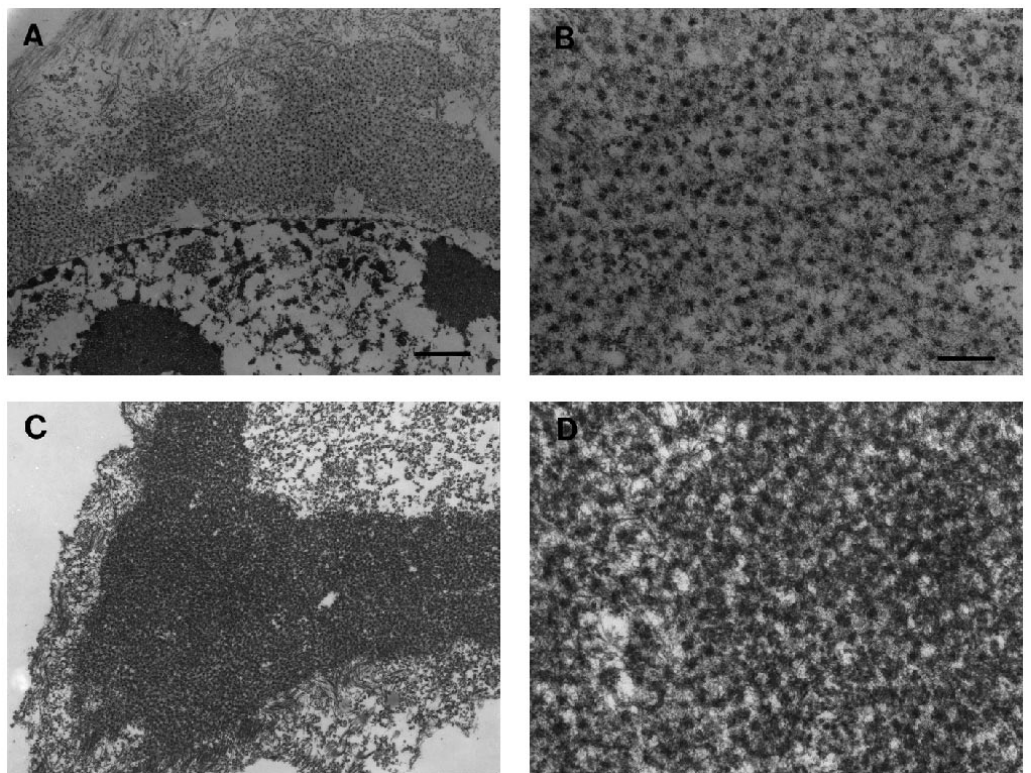


Fig. 6. Ultrastructure on NuMA- Δ NLS expressed in the cytoplasm of BHK-21 cells in the presence of microtubule perturbing drugs. Cells were microinjected with the plasmid driving the expression of NuMA lacking the nuclear localization sequence and treated with either 10 μ M nocodazole (A,B) or 10 μ M taxol (C,D) for 8 hours. Cells were then extracted with a microtubule stabilizing buffer containing 0.5% Triton X-100, fixed in 2% glutaraldehyde, processed, and viewed by transmission electron microscopy. Bars: (A,C), 1 μ m; (B,D), 200 nm.



arrows). Consistent with the results obtained with immunofluorescence microscopy, the filamentous assembly of NuMA- Δ NLS in the cell cytoplasm is not an artifact arising from the expression of a mutant form of NuMA, because we have observed similar filamentous structures by TEM in the cytoplasm of a minority of transfected cells that are expressing extremely high levels of the wild-type NuMA protein (data not shown). Finally, we failed to find any microtubules within this mass of NuMA-derived cables despite the fact that immunofluorescence analysis indicates that tubulin is present within this mass and that microtubules were visible in the adjacent regions of the cell cytoplasm (Fig. 5C).

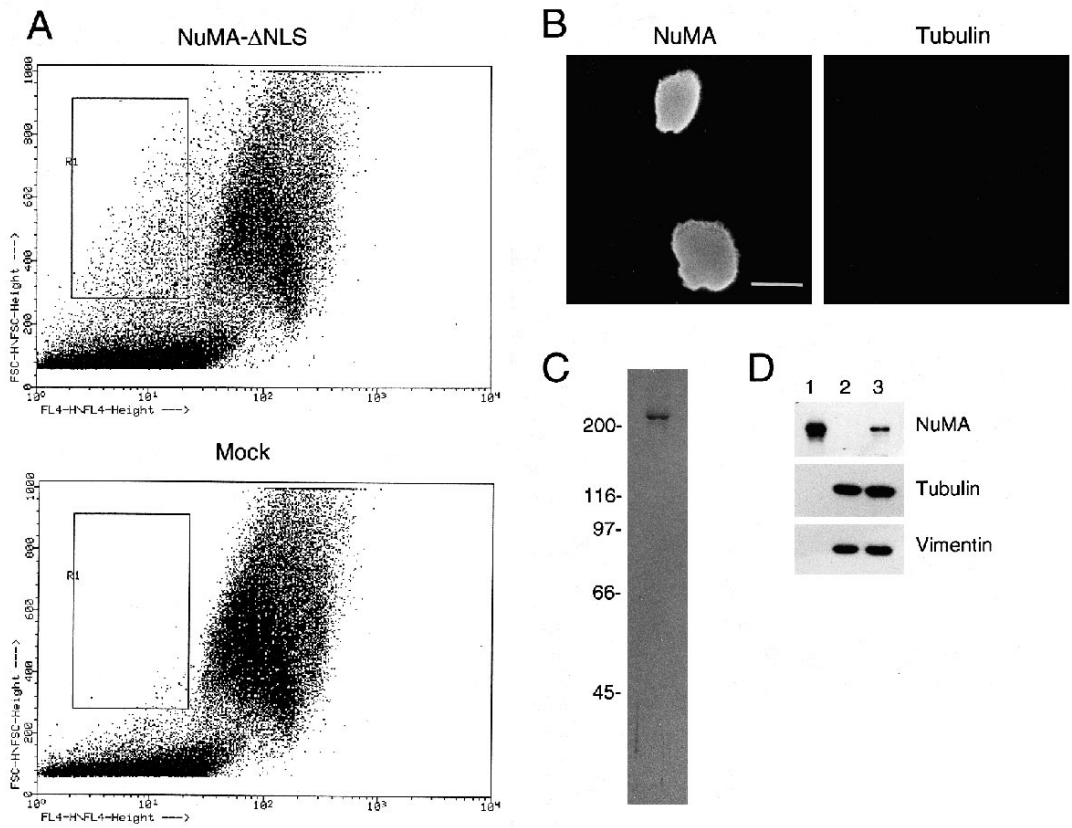
Extraction of the cells with Triton X-100 in a microtubule stabilizing buffer prior to fixation and processing for electron microscopy provides an enhanced view of the filamentous nature of the cytoplasmic mass formed by NuMA- Δ NLS (Fig. 5D). Under these conditions, the cytoplasmic mass formed by NuMA is primarily composed of two types of filamentous structures (Fig. 5E), and despite the exclusion of a majority of the cytoplasmic intermediate filaments from this filamentous mass (e.g. Fig. 4B) a few intermediate filaments are observable scattered throughout the NuMA- Δ NLS mass. One type of filament has the same morphology as the cables observed when cells are fixed directly without any prior detergent extraction.

These cables are up to 2 μ m long, solid, unbranching, \sim 23 nm in diameter, and laterally asymmetric (Fig. 5E, large arrow). The other type of filament has a diameter of \sim 5 nm (4.80 ± 0.67 nm), is highly branching, and appears to form a dense network that interconnects the larger cables. Indeed, the electron dense 'fuzzy' side of the 23 nm cables that was observed in cells prepared for TEM without prior detergent extraction appear to be composed of these 5 nm filaments (Fig. 5E, large and small arrows). These structures are resistant to extraction with high salt, because the same complex organization of \sim 23 nm cables interconnected by \sim 5 nm filaments is observed if the cells expressing NuMA- Δ NLS were extracted under conditions suitable for the observation of the nuclear matrix prior to fixation and examination by TEM (extraction conditions of He et al., 1990; data not shown). Finally, as in the cells fixed directly in glutaraldehyde without prior detergent extraction, no microtubules are visible within this cytoplasmic mass despite the presence of microtubules in adjacent regions of the cytoplasm (data not shown).

Since immunofluorescence analysis suggests that the filamentous nature of the mass formed by NuMA- Δ NLS is dependent on the presence of dynamic microtubules, we examined the ultrastructure of the products formed by expression of NuMA- Δ NLS in the presence of either taxol or

Fig. 7. Isolation of NuMA- Δ NLS-containing structures.

(A) BHK-21 cells were transiently transfected with either the expression plasmid driving the expression of NuMA lacking the nuclear localization sequence (NuMA- Δ NLS) or the empty expression vector (Mock), a lysate prepared from each cell population with the addition of 1 μ g/ml DAPI, and subjected to flow cytometry using the parameters of UV intensity (x axis) and light scattering intensity (y axis). The indicated window was used to sort the light scattering particles with low UV intensity. (B) Following flow cytometry the selected particles were spotted onto poly-L-lysine coated coverslips, fixed, and processed for immunofluorescence microscopy using the primate-specific anti-NuMA rabbit serum (NuMA) and the tubulin-specific mouse monoclonal antibody DM1 α (Tubulin). Bar, 20 μ m. (C) Approximately 100,000 particles selected by flow cytometry were solubilized in SDS sample buffer and fractionated by size on a 7.5% SDS-polyacrylamide gel. The gel was stained with Coomassie blue and the migration position of myosin (200), β -galactosidase (116), phosphorylase B (97), bovine serum albumin (66), and ovalbumin (45) are indicated on the left in kDa. (D) Immunoblot analysis with the primate-specific anti-NuMA rabbit serum (NuMA), the tubulin-specific mouse monoclonal antibody DM1 α (Tubulin), or the rabbit antibody recognizing vimentin (Vimentin) of approximately 5,000 flow cytometry-selected particles (lane 1), or total cell protein from approximately 15,000 BHK-21 cells that have been transiently transfected with either the empty expression plasmid (lane 2) or the plasmid driving the expression of NuMA- Δ NLS (lane 3).



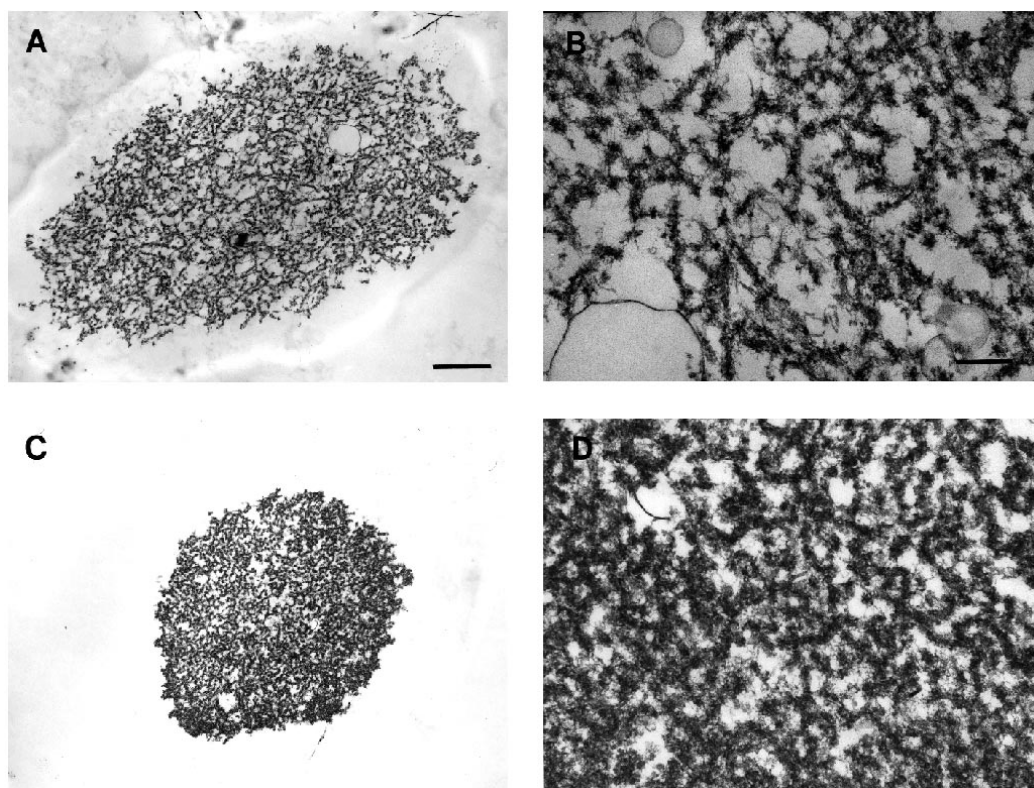


Fig. 8. Ultrastructure of the purified structures formed by expression of NuMA- Δ NLS in the cytoplasm of BHK-21. The structures formed by NuMA- Δ NLS were purified by flow cytometry, spotted onto poly-L-lysine coated coverslips, fixed in 2% glutaraldehyde, processed, and viewed by transmission electron microscopy. Bars: (A,C), 1 μ m; (B,D), 200 nm.

nocodazole (Fig. 6). In the presence of nocodazole (Fig. 6A,B) NuMA- Δ NLS accumulates in a ring-like aggregate surrounding the nucleus in addition to multiple foci dispersed throughout the cytoplasm. This cytoplasmic mass is composed of a collection of electron dense foci that are interconnected by a complex network of \sim 5 nm filaments whose morphology is similar to the \sim 5 nm filaments observed in cells expressing NuMA- Δ NLS in the absence of any microtubule perturbing drugs. There are no detectable cables resembling the \sim 23 nm cables that are observed when the protein is assembled in the presence of dynamic microtubules and no microtubules are observed in these cells. In the presence of taxol (Fig. 6C,D) NuMA- Δ NLS formed a mass in the cytoplasm adjacent to the nucleus that is substantially denser and less extended compared to when it assembles in the presence of dynamic microtubules or in the presence of nocodazole. Similar to the structure formed by NuMA- Δ NLS in the presence of nocodazole, the mass formed by NuMA- Δ NLS in the presence of taxol is composed of a regular array of electron dense foci interconnected by a network of 5 nm filaments that lack both the 23 nm cables and microtubules although bundles of microtubules were clearly visible at the periphery of the structure. These results suggest that the \sim 23 nm cables are derived from an interaction between dynamic microtubules and the expressed human NuMA- Δ NLS protein, and that the fibrous quality of the cytoplasmic mass formed by NuMA- Δ NLS that is observable by immunofluorescence microscopy is due to the formation of these \sim 23 nm cables.

To determine if any other cellular proteins participate with NuMA- Δ NLS in the assembly of these cytoplasmic structures we have devised a strategy using flow cytometry to purify the intact structures as if they were individual organelles. BHK-21 cells are transiently transfected with the plasmid to express

NuMA- Δ NLS and the transfected cells are harvested and lysed with Triton X-100. Under these conditions, the cytoplasmic structures formed by NuMA- Δ NLS remain intact as judged by both phase contrast and immunofluorescence microscopy (data not shown). We adapted the technique of flow cytometry to the isolation of these structures because the intact structures are large enough to be identified as particles by light scattering and they consistently co-purified with intact nuclei during conventional sedimentation-based protocols. Thus, we used flow cytometry to sort the intact structures formed by NuMA- Δ NLS away from the nuclei using the parameters of UV intensity (for DNA stained with DAPI) and light scattering intensity. In a typical flow cytometry analysis \sim 2.5% of the total number of light scattering particles appear in the selection window indicated in the plot following expression of NuMA- Δ NLS (Fig. 7A, NuMA- Δ NLS) whereas only 0.07% of the total light scattering particles are found in the analogous selection window following transfection with the expression plasmid alone (Fig. 7A, mock).

The structures isolated by this flow cytometry technique retain the morphology of the mass formed by NuMA- Δ NLS that is observed by immunofluorescence microscopy and TEM *in vivo* and are \sim 5-10 μ m in diameter. The isolated structures stain positively for NuMA but do not stain positively for tubulin (Fig. 7B) suggesting that any tubulin that was associated with the structures *in vivo* is lost during the isolation procedure. To determine what proteins are contained in these isolated structures we solubilized them with SDS and separated the proteins by size using SDS-PAGE. A single polypeptide is observed on both 7.5% (Fig. 7C) and 12.5% (data not shown) gels that migrates with a mass $>$ 200 kDa consistent with the mass of NuMA. Both immunofluorescence of the purified

structures (Fig. 7B) and immunoblot using a NuMA-specific antibody (Fig. 7D) confirm that this >200 kDa protein is NuMA. In addition, immunoblot analysis shows that there is no detectable tubulin or vimentin associated with these purified structures (Fig. 7D). Using various quantities of bovine serum albumin to estimate the staining sensitivities of these gels, we have calculated that each isolated particle contains approximately 10 pg of NuMA, and that if any protein with ~66 kDa molecular mass is associated with these structures then it is present in no greater than a 1:10 molar ratio with NuMA (data not shown). Thus, these data strongly suggest that these isolated structures are composed exclusively of NuMA.

Following the isolation of these cytoplasmic structures, we examined their morphology by TEM (Fig. 8). The purified structures are composed of a dense network of ~5 nm filaments that appear to intersect at small electron dense foci. The morphology of these purified structures is similar to the structures that are formed *in vivo* in the absence of dynamic microtubules (i.e. in the presence of either taxol or nocodazole), however, they have a somewhat more extended filamentous appearance which may be due to the initial assembly of these structures in the presence of dynamic microtubules prior to their isolation by flow cytometry. We do not observe either microtubules or the prominent ~23 nm cables that are observed by TEM when NuMA- Δ NLS is expressed in the presence of dynamic microtubules. The failure to observe any of the ~23 nm cables in these purified structures is consistent with the previous finding that the tubulin associated with the structures *in vivo* is lost during the isolation procedure (Fig. 7B,D), and that the ~23 nm cables form through an interaction between NuMA- Δ NLS and dynamic microtubules (Figs 3 and 6). Collectively, these results demonstrate that NuMA is capable of assembling into a complex structure in the cell cytoplasm that is composed of a network of ~5 nm filaments that are induced to form ~23 nm cables if it is assembled in the presence of dynamic microtubules.

DISCUSSION

In this article we demonstrate that the intranuclear protein NuMA assembles into an extensive filamentous network when expressed in the cell cytoplasm. This network is composed of two types of filamentous structures. One type of filament appears to result from the inherent assembly properties of the NuMA protein which leads to a highly branched, interlocking network of filaments that are uniformly ~5 nm in diameter. The second type of filament observed in these structures formed by NuMA- Δ NLS are solid, ~23 nm diameter, laterally asymmetric, unbranching cables that result from the interaction between the network of NuMA filaments and dynamic microtubules. The structures formed by NuMA- Δ NLS in the cell cytoplasm are stable to extraction with non-ionic detergents and high salt concentrations and behave as individual organelles when the cells are homogenized. To our knowledge, this type of filamentous organization has not been previously documented for any cyto- or nucleoskeletal proteins of the cell, and is the first observation of the extensive filamentous assembly of an intranuclear protein.

The finding that NuMA assembles into an extensive filamentous array in the cytoplasm is consistent with the prediction that NuMA has a coiled-coil secondary structure, but contrary to the structural predictions made using theoretical arguments (Parry,

1994) and a recent report examining the assembly properties of the purified recombinant human NuMA protein *in vitro* (Harborth et al., 1995). Harborth et al. (1995) reported that the recombinant human NuMA protein purified from bacteria following solubilization in 8 M urea assembled into ~200 nm long parallel, in register, coiled-coil dimers that did not further assemble into a filamentous array. The discrepancy between the assembly products observed *in vivo* (this report) and those obtained *in vitro* (Harborth et al., 1995) may be explained through a number of technical considerations as explained by Harborth et al. (1995) including the sub optimal buffer conditions used *in vitro*, the impaired renaturation of the protein following denaturation in 8 M urea, impaired filament formation due to the use of a bacterially expressed fusion protein, and/or the potential need for post-translational modification. These technical considerations are not applicable to the structures described in this article because we are expressing the native NuMA protein in the context of the normal cellular environment (cytoplasmic localization notwithstanding), although it is possible that *in vivo* conditions promote the higher-order assembly of the simple dimeric NuMA molecules described by Harborth et al. (1995) into the more complex arrangements of NuMA described in this article. In any event, the products of the higher-order assembly of NuMA in the cell cytoplasm are structurally unique relative to other cytoskeletal structures suggesting that in addition to the coiled-coil interactions that probably lead to the formation of the ~5 nm filaments, NuMA assembles into higher-order structures by a different mechanism compared to conventional intermediate filament proteins (end-to-end associations rather than lateral associations as suggested by Parry (1994), for example). It is also highly probable as we point out below and as Harborth et al. (1995) concede that NuMA will form a different structure depending on its interaction with additional cellular proteins. The interaction with additional cellular proteins and/or the cell cycle-dependent nature of these filaments may explain why we did not observe the formation of NuMA-containing filaments in cells where post-mitotic nuclear protein import was prevented by the microinjection of wheat germ agglutinin (Compton et al., 1992).

What is the physiological role for the assembly of NuMA into an extensive filamentous structure? We envision two potential structural roles depending on the phase of the cell cycle. First, NuMA may be serving a structural role inside the cell nucleus during interphase. NuMA is a component of the nuclear matrix (Kallajoki et al., 1991; Tang et al., 1993; Zeng et al., 1994a) that has recently been localized to a subset of the core filaments of the nuclear matrix (Zeng et al., 1994a). The core filaments of the nuclear matrix have been observed by electron microscopy following the extraction of chromatin-depleted nuclei with either high salt (He et al., 1990) or electroelution (Jackson and Cook, 1988), and are a complex network of filaments with variable diameters ranging from 9 to 13 nm that resemble the cytoplasmic intermediate filaments except that they are highly branching. The highly branching organization of the filaments formed by NuMA- Δ NLS in the cell cytoplasm, the stability of these structures to high salt extraction, and the localization of NuMA to the core filaments of the nuclear matrix (Zeng et al., 1994a) combine to support the possibility that NuMA may be a structural component of the nucleus and possibly a component of the core filaments of the nuclear matrix. On the other hand, the diameter of the filaments that are attributable to the assembly of NuMA

in the cell cytoplasm is only half the diameter of the core filaments of the nuclear matrix. This discrepancy could be explained if the core filaments of the nuclear matrix were heteropolymers composed of NuMA and some additional nuclear protein(s) leading to the formation of filaments with different dimensions compared to the filaments formed by NuMA alone in the cell cytoplasm. Alternatively, NuMA may participate in the formation of a filamentous structure within the nucleus that is distinct from the core filaments of the nuclear matrix that have been previously described. This alternative implies that there may be multiple, potentially redundant filamentous networks within the nucleus. The possibility of a redundant system of intranuclear filaments has been previously suggested by Yang and Snyder (1992) based on their findings that nuclei assemble normally following mitosis despite the antibody-induced sequestration of NuMA in the cytoplasm.

The second potential physiological function for the extensive filamentous assembly of NuMA is in organizing the microtubules at the polar ends of the mitotic spindle. We and others have generated substantial experimental evidence indicating that NuMA is required for the organization of the microtubules at the polar ends of the mitotic spindle (Compton and Luo, 1995; Gaglio et al., 1995; Kallajoki et al., 1991, 1992; Yang and Snyder, 1992), and the unique interaction between NuMA and dynamic microtubules that we describe here may be related to NuMA's ability to organize the microtubules during mitosis. However, the interaction between NuMA and microtubules is distinct from the interactions between microtubules and more conventional microtubule associated proteins (Olmsted, 1986; Lee, 1993; Hirokawa, 1994) for several reasons. First, the 23 nm cables appear to be co-polymers containing both NuMA and tubulin and are formed only when NuMA is expressed in the cytoplasm in the presence of dynamic microtubules. These 23 nm cables do not form if microtubules are depolymerized by nocodazole treatment or stabilized by taxol treatment. Second, the cytoplasmic network of microtubules appears to be consumed during the formation of these NuMA-tubulin complexes. In some instances NuMA- Δ NLS reaches expression levels high enough that all of the tubulin is complexed in the cytoplasmic mass formed by NuMA- Δ NLS and the cytoplasmic network of microtubules is absent from the adjacent regions of the cytoplasm (data not shown). This aspect of the formation of the 23 nm cables is clearly different from the microtubule binding properties of other microtubule binding proteins such as MAP-2 and tau which crosslink microtubules and MAP-4 which coats the microtubule surface. Third, NuMA appears to interact with the minus ends of the microtubules rather than the microtubule surface or the plus ends of the microtubules located at the cell periphery. Finally, the 23 nm cables formed by NuMA and dynamic microtubules are solid and laterally asymmetric with the 5 nm NuMA filaments extending outward along one surface. While a number of physical arrangements can be proposed to account for all of these features, one possibility is that NuMA interacts with (and perhaps destabilizes) the minus end of the dynamic microtubule by inducing the microtubule to open laterally into a curved sheet (Chretien et al., 1995) which forms a trough to support the ordered packing of the NuMA filaments. This highly speculative arrangement accounts for the observations that the cables are ~23 nm in diameter, solid, and laterally asymmetric. Regardless of the specific physical arrangement of NuMA and microtubules within these 23 nm

cables, however, the unique organization of these cables may indicate that NuMA's function as a component of the putative mitotic spindle matrix is mechanistically involved in the organization of the microtubules of the mitotic spindle and/or in the dynamic behavior of the microtubules in the mitotic spindle (i.e. poleward microtubule flux). In conclusion, these results demonstrate that the compartmentalization of NuMA within the nucleus may be necessary not only for a putative structural function for NuMA, but also to prevent any untimely interaction between NuMA and microtubules which could deleteriously effect the dynamics of the cytoplasmic microtubules.

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