Formation of Spindle Poles by Dynein/Dynactin-dependent Transport of NuMA

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Abstract. NuMA is a large nuclear protein whose relocation to the spindle poles is required for bipolar mitotic spindle assembly. We show here that this process depends on directed NuMA transport toward microtubule minus ends powered by cytoplasmic dynein and its activator dynactin. Upon nuclear envelope breakdown, large cytoplasmic aggregates of green fluorescent protein (GFP)-tagged NuMA stream poleward along spindle fibers in association with the actin-related protein 1 (Arp1) protein of the dynactin complex and cytoplasmic dynein. Immunoprecipitations and gel filtration demonstrate the assembly of a reversible, mitosis-specific complex of NuMA with dynein and dynactin. NuMA transport is required for spindle pole assembly and maintenance, since disruption of the dynactin complex (by increasing the amount of the dynamitin subunit) or dynein function (with an antibody) strongly inhibits NuMA translocation and accumulation and disrupts spindle pole assembly.

Key words: mitosis • microtubules • motor proteins • centrosome • spindle pole matrix

Introduction

The formation of bipolar mitotic spindles is a prerequisite to ensure the symmetrical distribution of chromosomes to each daughter cell. Spindles consist of two arrays of microtubules, anchored with their minus ends at the poles and their plus ends extending towards the equator, where they partially overlap in an antiparallel fashion. The spindle microtubules can provide both force and guidance for chromosome movement. Further, by converging towards the spindle poles, they concentrate chromosomes in two defined areas, where these are packed into daughter nuclei.

A variety of proteins that are involved in spindle morphogenesis have been characterized during the last few years. Essential proteins for pole formation include centrosomal components, such as γ-tubulin (Joshi et al., 1992; Stearns and Kirschner, 1994) and pericentrin (Doxsey et al., 1994), as well as proteins that are not directly anchored to the pericentriolar material, such as NuMA (Lydersen and Pettijohn, 1980), dynactin (Gaglio et al., 1996, 1997), several microtubule-dependent motor proteins, including dynein (Hald et al., 1996, 1997) and XKLP2 (Boleti et al., 1996), and the small GTPase, Ran (reviewed by Kahana and Cleveland, 1999).

Prior efforts have shown that NuMA, a nuclear protein that relocates to the spindle poles during mitosis and meiosis, is necessary to focus microtubules into spindle poles and to control the size of mitotic spindles (Merdes et al., 1996). It is enriched in a crescent-shaped area at the spindle poles, and EM demonstrates that the majority of the protein is located between microtubules (Dionne et al., 1999). Immunoprecipitation of NuMA from metaphase-arrested frog egg extracts further revealed an association of NuMA with cytoplasmic dynein and its activator complex dynactin. The mechanisms by which this complex is formed, how NuMA becomes pole-associated, and the precise role of this complex in linking spindle microtubules to each other or the centrosome have not been identified.

Several studies have shown that the formation of spindle poles is inhibited in the presence of a mAb against the dynein intermediate chain (Heald et al., 1996, 1997; Gaglio et al., 1997). At the same time, however, dynein motor activity seems unaffected by this treatment (Heald et al., 1997). The situation is further complicated by the fact that bundling of microtubules into a convergent polar array needs one or more components with multiple microtubule binding sites. Although the dynein motor possesses one such binding site on each subunit of the heavy chain dimer, these two sites are both needed for processive movement and, therefore, unlikely to be involved in tethering microtubule bundles. Rather, the known dynein dependency for...
tethering spindle microtubules into poles must involve proteins with additional microtubule binding sites. Candidates for these include dynactin (Waterman-Storer et al., 1995) and NuMA (M. Erdes et al., 1996), both of which have been shown to bind to microtubules themselves.

From this background, we now use a combination of green fluorescent protein (GFP)1-tagging of NuMA in vivo, immunoprecipitation and gel filtration, and antibody disruption to identify the mechanism of formation of focused spindle poles.

Materials and Methods

Transfection Experiments and Microscopy

For the expression of GFP-tagged NuMA in tissue culture cells, a construct was assembled that contained full-length human NuMA (Compton et al., 1992). NuMA was modified at its 5′ end by PCR using a primer (GCAGGGCCGCGCATAGACTCCAC) that encoded a Not1 restriction site followed by the start codon. This site was used to join NuMA in frame to the 3′ end of GFP that was in turn modified by adding a Not1-containing hinge region of 42 bp (CCAGAGCGCCGGCGAAGTG-GCTGGAGACAGGTGGCAGGCGGCCG), eliminating the stop codon of GFP. The construct was inserted into the expression vector pcDNA3 (Invitrogen) using EcoRI and XbaI restriction sites. HeLa cells were grown on glass coverslips in DME containing 10% FBS (GIBCO BRL). HeLa cells were transfected with the DNA construct using calcium phosphate precipitation, as described in Sambrook et al. (1989). The transfected efficiency was tested by immunoblotting, using the mAb 1F1 against human NuMA (Compton et al., 1991), and by counting GFP-positive cells under the fluorescence microscope. To assess the degree of GFP-NuMA overexpression in individual cells, immunofluorescence staining with the 3F1 antibody, recognizing both GFP-NuMA and endogenous NuMA, and a secondary Texas red-coupled anti-mouse antibody was performed. The intensity of the Texas red fluorescence in interphase nuclei of 23 randomly selected GFP-NuMA–expressing cells and 30 control cells was quantified from confocal microscope sections as pixel intensity per nuclear section area, after subtraction of the background fluorescence calculated from five different representative areas.

For time-lapse observations of GFP-NuMA, coverslips containing cells were mounted in growth medium onto microscope slides using a vaseline-coated rubber O-ring as spacer. Recordings of 0.5-s exposure time were made at 19°C in 2-min intervals using a Photometrics Sensys cooled CCD camera, containing a KAF 1400-G chip. The camera was mounted on a Zeiss Axiophot, using a Plan-Apochromat 63×/1.40 lens, and a filter-wheel/shutter device (American Precision LEP/LUDL), controlled by Quips Imaging Software (Vysis) on a PowerMacintosh 8600/200 computer (Apple). Alternatively, recordings at 37°C were made with the same equipment, and a temperature-controlled fan heater mounted next to the microscope stage.

Spindle Formation Assays and Immunofluorescence

For the formation of spindles in vitro, Xenopus laevis egg extract and Xe-nopus sperm were prepared and centrifuged onto glass coverslips as described in M. Erdes et al. (1996). Direct formation of spindles from frog sperm in cytostatic factor (CSF)-arrested extracts was monitored for ~1 h. Dynein inhibition experiments were carried out by adding monomelic dynein intermediate chain antibody 70.1 (Sigma Chemical Co.) dialyzed against PBS to the extract at the beginning of the incubation with frog sperm, at a final concentration of 0.1 mg/ml immunoglobulin. Dynein was inhibited by the addition of dynactin at a final concentration of 0.75 μg/ml. For this experiment, a dynamitin clone was obtained by PCR from a Hela cell cDNA library (provided by S. Kandell-Lewis, University of Edinburgh, Scotland) using primers ATGGCGGACCCTAAATACGCC and TCTCACTTTCCCAGCTTCTTC. Sequencing revealed that a dynamitin isoform was cloned that lacked amino acids 36-40, but was otherwise identical to the previously published human dynamitin sequence (Cheverev et al., 1996). The dynamitin PCR product was cloned in the vector pCR-TM 2.1, excised using EcoRI, and cloned into the bacterial expression vector pET 28A (both from Invitrogen). Bacterial cells were isolated using 8M urea and dialyzed against PBS before the assay. NuMA was inhibited by adding antibodies against the distal Xenopus NuMA tail (M. Erdes et al., 1996) to preformed spindles for 10 min. Control spindles were assembled while adding equivalent volumes (up to 20% of the extract volume) of PBS.

Xenopus laevis sperm for 5 min in methanol at −20°C. After rehydration and rinsing in PBS, cells were labeled with antibodies against human NuMA (clone 3F1, see Compton et al., 1991), Xenopus NuMA/distal tail domain (M. Erdes et al., 1996), actin-related protein 1 (A rp1)/α27 (Clark and Moyer, 1999), dynein heavy chain (Heald et al., 1997), Eg5 (Sawin et al., 1992), α-tubulin, clone DMA1 (Sigma Chemical Co.). Secondary antibody coupled to FITC or Texas red, were from Vector. Chromosomes were stained using 4',6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co.), and coverslips were embedded in Vectashield (Vitro). For confocal fluorescence microscopy, equipment was used as described above. For confocal microscopy, a Leica DMRB microscope with a PL APO 100×/1.4 lens was used; the device was equipped with argon, krypton, and UV lasers for excitation (at 488, 566, and 360 nm, respectively), and a Leica TCS SP multi band spectrophotometer for detection, controlled by Leica TCS NT software. Default settings for FITC, Texas red and DAPI were used.

The dependence of NuMA transport on intact microtubules was studied in GFP-NuMA–expressing and control cells. GFP-NuMA aggregates were visualized in living cells as described above. Cells were then exposed to nocodazole at 10 μg/ml for 15 min. For control cells, they were either incubated with fresh culture medium for 45 min to remove the nocodazole or directly stained, and stained for tubulin immunofluorescence. Cells were relocated on the microscope using the stage coordinates, as well as reference pictures taken with phase-contrast microscopy.

Immunoprecipitation of NuMA from Frog Egg Extracts

For the immunoprecipitation of NuMA, affinity beads were prepared by coupling peptide-treated goat anti-rabbit antibody (Jackson Immunoresearch Laboratories) to Affi-Prep-H2 hydrazide support (BioRad) according to the manufacturer’s instructions. These beads were then coated with antibody against the Xenopus NuMA distal tail region, and NuMA was removed from extracts as described (M. Erdes et al., 1996). After immunoprecipitation experiments, beads were washed five times with 60 mM KCl, 15 mM NaCl, 15 mM Tris/HCl, pH 7.4, once with buffer containing 0.2% Triton X-100, and finally boiled for 5 min in gel loading buffer, containing SDS and mercaptoethanol. Coimmunoprecipitation of other proteins was tested by gel electrophoresis and immunoblotting, using antibodies against dynactin heavy chain, dynactin intermediate chain, A rp1, and Eg5 as described above. After SDS-PAGE, the proteins were transferred to a nitrocellulose membrane (Q. Qiu et al., 1999), and antidynein p150/glued, mAb b150B (Q. Qiu et al., 1999). Inhibition of dynein or dynactin coprecipitation was performed using mAb b70.1 or dynamin, as described above. Experiments in interphase frog egg extract were carried out by converting metaphase arrested egg extract by addition of 2 mM calcium chloride and incubation at room temperature for 45 min. The cell cycle stage of the extract was monitored by testing 1-μl aliquots for histone H3 kinase activity exactly as described by Murray (1991). Subsequent NuMA immunoprecipitation was performed within 20 min, and aliquots of the immunoprecipitation supernatants of both interphase and metaphase extracts were tested again for kinase activity, to verify that these extracts had not proceeded in the cell cycle.

Gel Filtration Chromatography

A gel filtration column of 80-cm length was prepared using 200 ml Sepharose 4B (Sigma Chemical Co.) in 60 mM KCl, 15 mM NaCl, 15 mM Tris/HCl, pH 7.4, 1 mM β-mercaptoethanol, and 0.1 mM PMFS. The flow rate was 0.14 ml/min. The column was calibrated using blue dextran (2,000 kD), thyroglobulin (669 kD), ferritin (440 kD), catalase (232 kD), and aldolase (138 kD) as markers. CSF–arrested frog egg extract was cleared by centrifugation at 18,000 g for 15 min. 100 μl of extract was loaded onto the column. Fractions of 4-ml size were precipitated with 10% trichloroacetic acid, washed with acetone, and analyzed by gel electrophoresis and immunoblotting. The migration position of tubulin dimers (110 kD) was determined by immunoblotting and used as an internal size standard. A different gel filtration column of 45-cm length was prepared with 30 ml Sephacryl S-400 (Sigma Chemical Co.) in the same buffer as above. 50 μl
of extract was loaded, and fractions of 1-ml size were collected and treated as above.

Microtubule Seed Transport Assay

Spindles were assembled around DNA-coated magnetic beads in control and NuMA-depleted frog egg extracts, cycled from interphase into mitosis (Heald et al., 1996, 1997). NuMA depletion was performed as described (Merdes et al., 1996). Rhodamine-labeled seeds were prepared and video microscopy was performed as described (Heald et al., 1996, 1997).

Results

NuMA Colocalizes with Dynactin after Nuclear Envelope Breakdown

To determine how NuMA is transported after nuclear envelope disassembly, NuMA location was identified in cells just transitioning from prophase to prometaphase. As described previously (Compton et al., 1992; Yang et al., 1992), NuMA was found almost homogeneously distributed in the nucleoplasm during interphase (not shown). However, during prophase, while chromosome condensation took place, the localization of NuMA was restricted to the interchromatin space. Fig. 1 A shows a typical conventional immunofluorescence picture of a late prophase, in which NuMA was found in those areas of the nucleus that were not occupied by the condensed chromosomes (for comparison, see also Compton et al., 1992; Yang et al., 1992). At this stage, the actin-related protein A rp1, a subunit of the motor-cargo-mediating complex dynactin, decorated the duplicated centrosomes and astral microtubules radiating from them (Fig. 1 B). With the transition to prometaphase (identified by the absence of the round contour of the nuclear envelope), small aggregates of NuMA were grouped in radial arrays colocalizing with astral A rp1 (Fig. 1, C and D).

In many prometaphase cells, besides the material that had already accumulated at the poles, larger aggregates of NuMA (Fig. 1 E) in association with A rp1 (Fig. 1 F) were found approximately in the midzone of the spindle. The occurrence of nonpolar NuMA-containing aggregates was found in cells from various species, including HeLa cells, Xenopus A6 cells (Fig. 1, I and J) and chicken DU 249 cells (not shown). The staining of these aggregates was detected with three different antibodies: mAb 1F1 against human...
NuMA (Compton et al., 1991), polyclonal anti-X enus
NuMA tail (Merdes et al., 1996), and a newly generated
mAb against chicken NuMA (Merdes, A., unpublished
observation). The shape and size of these aggregates var-
ed from cell to cell, but nonpolar NuMA aggregates were
found in 64% of prometaphases (n = 244) of HeLa cell
cultures. It appeared that these aggregates were mainly
found in early stages of prometaphase, but were absent af-
after a bipolar spindle apparatus had fully formed, and after
the majority of the chromosomes had been bi-oriented.
Many NuMA aggregates were found in focal planes differ-
ent from the spindle poles, which may explain why they
have not been documented in previous reports. As shown
in Fig. 1, G and H, this aggregated NuMA frequently
stretched along spindle fibers towards the spindle poles,
suggesting that NuMA was under a microtubule-depen-
dent pulling force.

Because the kinesin-related microtubule motor Eg5 has
been shown to participate in spindle pole organization
(Gaglio et al., 1996) and because Eg5 was also reported to
bind to the dynactin complex (Blangy et al., 1997), we
wanted to test for potential localization of Eg5 on NuMA
aggregates. Although Eg5 was enriched at developing
spindle poles in prometaphase cells, it was not enriched on
the NuMA aggregates outside the pole regions (Fig. 1, Q
and R). Cytoplasmic dynein, on the other hand, partially
colocalized in prometaphase cells with NuMA aggregates
(Fig. 1, K and L, arrowhead). This colocalization was con-
firmed using the three-dimensional resolution of a confocal
microscope, equipped with a spectrophotometer for
detection to exclude bleed-through of NuMA fluorescence
onto the dynein signal (Fig. 1, M–P). In addition to dynein
associated with NuMA, the bulk of dynein was found
more diffusely within the cytoplasm (Fig. 1, L, N, and P) or
at kinetochores (Fig. 1 P, arrow), as also seen for the dy-
nactin subunit, A rp1 (Fig. 1 F). This suggests that only a
proportion of the pool of dynein and dynactin attaches to
NuMA aggregates, to pull these towards the poles.

Poleward Transport of GFP-tagged NuMA in
Living Cells

To test the idea of dynein/dynactin-dependent NuMA
transport to the spindle poles in living HeLa cells, we
expressed full-length NuMA tagged at its extreme NH2
terminus with GFP. This GFP-NuMA showed a cell cycle-
dependent relocation from the interphase nucleus to the
spindle poles that was indistinguishable from endoge-
nous NuMA (Fig. 2, A–D). Cells expressing GFP-NuMA
were observed at all stages of the cycle with no apparent
consequences arising from accumulation of the chimeric
NuMA. To determine the levels of GFP-NuMA overex-
pression, we performed immunoblotting of transiently
transfected HeLa cell cultures using an antibody that rec-
ognizes both GFP-NuMA and endogenous NuMA. Be-
sides the 240-kD band of endogenous human NuMA, the
transfected cells revealed an additional band representing
GFP-tagged NuMA (Fig. 2 E, right lane). By fluoresc-
ce microscopy we counted that ~12% of the cells (252 in
2,035) were overexpressing GFP-NuMA in a typical
experiment. The level of overexpression in individual cells,
as measured by quantitative immunofluorescence micro-
copy (see Materials and Methods), ranged from 1.2- to 3.8-
fold, with an average level of 2.2-fold of the endogenous
NuMA level (n = 23 for GFP-NuMA expressors, n = 30
for controls). GFP-tagged NuMA accumulated in nonpo-
lar aggregates during early stages of prometaphase (Fig. 2
F) in the same way as endogenous NuMA staining with antii-
obodies (Fig. 1). In combination, these observations ruled
out the possibility of an artifact due to overexpression, or
vice versa, a staining artifact of our antibodies.

Time-lapse recordings of GFP-NuMA revealed that the
midzone aggregates were stretched towards both spindle
poles (Fig. 2 F, 5–13 min) and then moved towards the
poles along tracks of spindle fibers (Fig. 2 F, 13–37 min).
Whereas some of the initial movements seemed to occur
along few defined tracks (between 13 and 21 min), later
stages showed NuMA transport across the entire half spin-
dles (for example, see diffuse GFP-NuMA signal in the
upper half spindle at 37 min in Fig. 2 F). This might simply
reflect the fact that the density of spindle microtubules is
lower early in prometaphase (Roos, 1973). Between 37
and 63 min, nearly all GFP-NuMA had accumulated at
the spindle poles. Small cytoplasmic aggregates of GFP-
NuMA were also visible in some fields (Fig. 2 G, arrow),
being pulled towards the spindle poles at an average speed
of 1 μm/min (± 0.3, n = 3) when these experiments were
conducted at 19°C. When the microscopy was performed
at 37°C instead, movements ranging from 1.7–4.5 μm/min
were measured, yielding an average speed of 2.6 μm/min
(± 1.0, n = 8). These transport velocities are in good
agreement with rates for dynein-dependent movement of
microtubule seeds measured in spindles, which yielded a
median speed of ~2.5 μm/min, with several very rapid
movements producing an average of 6 μm/min (Heald et
al., 1997).

To examine the dependence of poleward NuMA trans-
port on the integrity of spindle microtubules, we subjected
prometaphase cells to treatment with nocodazole (Fig. 2
H). After incubation with nocodazole, all microtubules
had been dissolved (Fig. 2 H, right). At the same time, all
pole-accumulated NuMA, as well as nonpolar NuMA ag-
gregates that were visible initially in the untreated cell
(Fig. 2 H, left) were completely solubilized (Fig. 2 H, mid-
dle). When nocodazole-treated cells were allowed to re-
cover in fresh culture medium, the spindle apparatus re-
formed (Fig. 2 I, left), and NuMA accumulated at the
poles again (Fig. 2 I, right). Besides, new aggregates of
NuMA formed in the cytoplasm and along the spindle ap-
paratus (Fig. 2 I, right).
components. This revealed that dynein heavy and intermediate chains, as well as dynactin p150 and the actin-related protein Arp1, were complexed with NuMA (Fig. 3A). The pole-enriched microtubule-severing factor, katanin (McNally et al., 1996), or the kinesin-related motor, Eg5, did not coimmunoprecipitate with NuMA (Fig. 3A), underlining the specificity of these coimmunoprecipitation results. Further, the association of NuMA with dynein and dynactin is cell cycle dependent: when NuMA was immunoprecipitated from frog egg extracts released from metaphase arrest, the level of both coimmunoprecipitated dynactin and dynein was significantly reduced (Fig. 3B).

To dissect the associations within this complex, the effects of inhibitors disrupting dynein and dynactin were examined. Coprecipitation was significantly reduced by each of two different inhibitors. First, the 50-kD dynactin subunit dynamin, a well-characterized inhibitor known to disrupt the integrity of the dynactin complex (Echeverri et al., 1996; Quintyne et al., 1999), abolished coimmunoprecipitation of dynactin subunits with NuMA. Further, probably as a consequence of dynactin disassembly, this also inhibited coprecipitation of dynein. Thus, dynein binding to NuMA apparently requires an intact dynactin complex as an adaptor. Second, coimmunoprecipitation of both dynein
and dynactin components was strongly inhibited by mAb 70.1 against dynein intermediate chain. As discussed by Gaglio et al. (1997), this antibody does not only affect the function of dynein itself in HeLa cell extracts. It does, however, apparently solubilize dynactin subunits from larger microtubule aster assemblies, thereby implicating a cooperative interaction between the dynein intermediate chain and its dynactin binding partner, p150/glued, as essential to the stabilization of a larger microtubule-binding complex that is disrupted by the mAb (Gaglio et al., 1997). Release of dynactin from NuMA by mAb 70.1 (Fig. 3A) thus indicates that NuMA must also be a part of this larger complex.

To determine the size and stability properties of the cytoplasmic dynein and dynactin associated with NuMA, mitotic frog egg extracts were fractionated over a Sepharose 4B gel filtration column. This revealed a small amount of both dynactin p150 and NuMA coeluting with the size...
marker dextran, consistent with a complex of 2,000 kD or higher (Fig. 3 C). The dynactin p150 always appeared as an unfocused band in these fractions, suggestive of an as yet unidentified covalent modification. The majority of NuMA and dynactin, however, was detected in fractions of an intermediate size, at \( \sim 1,000 \) kD, whereas the bulk of the dynein intermediate chain eluted at a much later position (corresponding to \( \sim 290 \) kD). Dynein heavy chains were found at \( \sim 800 \) kD and did not copurify with the intermediate chains (data not shown). This indicated that most components of the basic dynein motor complex (consisting of at least two heavy chains of 400–500 kD, two intermediate chains of 70 kD, and several light chains and a native molecular weight expected to exceed 1,000 kD) had disassembled during chromatography. This reinforces the immunoprecipitation findings that NuMA, dynactin, and dynein form a low-affinity complex that is easily disassembled by dilution. Moreover, they also demonstrate that the weakest or most transient interaction is of NuMA with the dynein motor component itself.

To test whether smaller dilutions and/or shorter chromatography times would preserve higher amounts of co-fractionating NuMA and dynactin, similar extracts were fractionated on a sephadex S-400 column of only 45-cm length. Under these conditions, dynein again eluted in fractions distinct from NuMA and dynactin. However, almost all NuMA chromatographed with a proportion of dynactin p150, which again displayed a diffuse electrophoretic mobility selectively in the NuMA-containing fractions (Fig. 3 D). Further evidence for the transient and reversible nature of the NuMA complexes with dynactin and dynein was obtained by sedimentation analysis on 10-40% sucrose gradients (not shown). This revealed that associations between NuMA and dynactin or dynein were completely lost during the long times required for sedimentation, with NuMA sedimenting in two peaks of 8S and 35S. The bulk of dynein heavy chain and dynactin components sedimented at 20S, as reported previously (Schroer and Sheetz, 1991).

**Disruption of Dynactin or Dynein Inhibits NuMA Transport and the Formation of Spindle Poles**

To directly test whether NuMA is transported towards the spindle poles by association with dynein and dynactin, spindles assembled in metaphase-arrested frog egg extracts were monitored for the effects of dynein and dynactin inhibitors. Control extracts yielded regularly shaped spindles \( \sim 60 \) min after addition of frog sperm DNA (Fig. 4 A).

Even preformed spindles were sensitive to treatment with antibodies against NuMA, which provoked the release of the centrosomes from the spindles and the splaying of microtubule ends previously focused to form each pole (Fig. 4 B). Serial sectioning and EM confirmed that the disconnected small microtubule asters (with anti-NuMA staining in their core) indeed contained centrosomes (not shown). A dilution of high levels of the dynactin inhibitor dynamitin during the process of spindle formation produced a very similar phenotype of unfocused poles (Fig. 4 C). In this case, NuMA was no longer restricted to the microtubule minus ends, but seen along the entire length of the spindle fibers (Fig. 4 C, bottom). Consistent with earlier observations (Heald et al., 1997; Gaglio et al., 1997), an almost identical effect was seen by addition of the dynein intermediate chain antibody 70.1 (Fig. 4 D). These data indicate that there is a direct dependence of NuMA accumulation at the poles on dynein/dynactin-mediated transport. It should be noted that a certain amount of NuMA is nevertheless seen at microtubule minus ends, de-
NuMA antibody (top) or antidynein heavy chain (bottom). Bar, 20 

Despite dynein or dynactin inhibition. This partly could be due to NuMA's direct binding affinity to microtubules (Merdes et al., 1996), and an inherent microtubule translocation mechanism of the spindle, termed poleward flux (Mitchison, 1989). The disruption of dynactin and dynein in our experiments did, however, result in a dramatic disruption of the spindle poles, adding further weight to the model in which cohesion of microtubules at poles depends on the interaction between NuMA, dynein, and dynactin.

NuMA-dependent Tethering and Focusing of Polar Microtubules

Experiments in Xenopus egg extracts using rhodamine-labeled microtubule seeds previously have been used to show that focusing of poles may involve cytoplasmic dynein-mediated, minus end-directed sliding of microtubules along each other (Heald et al., 1997). In this model, stationary microtubules would provide the tracks, along which motile microtubules would be transported by dynein as a cargo. To analyze whether NuMA played any role in this microtubule movement, spindles in frog egg extracts were assembled around DNA-coated magnetic beads. These spindles were incubated with small microtubule seeds that were brightly labeled at their minus ends with rhodamine-tubulin. In control spindles, these microtubule seeds accumulated at the poles, on the minus ends of spindle fibers (Fig. 5A). When we attempted to form spindles in extracts from which NuMA had been depleted with immobilized NuMA antibodies, only aberrant structures, lacking microtubules focused into poles were assembled around the DNA beads (Fig. 5B). These structures were almost identical to spindles assembled in NuMA-free extracts around sperm chromat (Merdes et al., 1996), or those formed in the presence of inhibitors, as shown in Fig. 4. Thus, NuMA is necessary for pole formation in spindle assembly either in the presence or absence of centrosomes. Moreover, virtually no microtubule seeds attached to these structures (Fig. 5B). Thus, in these mitotic extracts NuMA is also necessary either for transport of microtubules relative to each other or for stabilizing the tethering of those microtubules after transit to the microtubule minus ends, or both. These effects are due to loss of NuMA, rather than depletion of dynein, since immunoblotting revealed that dynein was still present in abundance in these samples after nearly complete NuMA removal (Fig. 5C), consistent with our earlier report that dynein is ~30 times more abundant than NuMA in these extracts (Merdes et al., 1996).

Discussion

The Role of NuMA, Dynein, and Dynactin at the Spindle Poles

We have for the first time observed the centripetal trafficking of NuMA to the poles during prometaphase. We have demonstrated that NuMA is transported along the mitotic spindle by the minus end-directed motor cytoplasmic dynein, in a complex with the activator dynactin. Further, we show that NuMA transport to the poles is necessary to form and stabilize the spindle and the spindle poles. Previously it was reported that dynein can drive the movement of microtubules within the spindle, with their minus ends leading towards the poles (Heald et al., 1996, 1997). From these studies and from work by others, a role of dynein and dynactin in the focusing of polar microtubule arrays was inferred (Gaglio et al., 1996, 1997; Echeverri et al., 1996). We now extend these observations by demonstrating that only a fraction of cytoplasmic dynein that associates with both dynactin and NuMA contributes to the formation of the spindle poles. In fact, the dynein-dependent accumulation of microtubule ends at the poles is only possible in the presence of NuMA, suggesting that NuMA is a critical factor involved in binding microtubules to each other.

We have shown previously that a region in the NuMA tail domain can bind microtubules and induce the formation of parallel microtubule bundles in vitro (Merdes et al., 1996). Therefore, in association with dynein and dynactin, NuMA can provide the necessary link that is required to attach a motile microtubule to the surface of a stationary microtubule, along which the dynein motor can glide (see model in Fig. 6, bottom). Alternatively, NuMA could reside on the surface of a stationary microtubule (Fig. 6, top) and the force production of the attached dynein motor could then be used to counterbalance outward-oriented forces of spindle-associated plus end-directed motors (Gaglio et al., 1996), thereby controlling the size of the spindle. This model is supported by our earlier finding that aberrant spindles assembled in NuMA-depleted frog egg

Figure 5. Microtubule seeds are transported towards the poles in a NuMA-dependent mechanism. A and B, Spindles assembled around DNA-coated magnetic beads in frog egg extract cycled from interphase into mitosis. Green, FITC-labeled tubulin; red, rhodamine-labeled microtubule seeds. (In addition, the magnetic beads used in this assay show a substantial amount of red autofluorescence.) A, Control spindle. B, A aberrant spindle assembled in NuMA-depleted extract. C, Immunoblots of untreated control extract, mock-depleted extract with a control antibody, and extract after NuMA depletion probed with anti-NuMA antibody (top) or antidynein heavy chain (bottom). Bar, 20 μm.
extracts are on average 1.5 times longer than control spindles. During the process of spindle formation, complexes of NuMA and dynein/dynactin could act as molecular ratchets that control the position of microtubules. In particular, this mechanism could help to stabilize parallel arrays of kinetochore fibers by preventing their minus ends from splaying apart, and thereby ensuring that linear tracks for anaphase chromosome separation are formed.

NuMA can assemble into oligomeric structures that are part of an insoluble, fibrous matrix. Indeed, recent EM work revealed the existence of NuMA in small electron-dense material located between spindle pole microtubules (Dionne et al., 1999). This, and the recent documentation of 12-arm NuMA oligomers formed in vitro or by overexpression of NuMA (Harborth et al., 1999), supports the idea that NuMA is a multivalent tethering factor that stabilizes the spindle poles independent of the centrosomes. Other than NuMA, spindle pole factors with similar functions are found in nonvertebrate organisms. For example, the recently characterized protein Asp in Drosophila melanogaster (Avides and Glover, 1999) localizes to the poles and possesses microtubule-binding capacity, and Asp mutants in flies display an aberrant spindle morphology similar to that observed after inhibition of NuMA, dynein, or dynactin.

**The Formation and Molecular Composition of the NuMA/Dynein/Dynactin Complex**

In somatic cells, the interaction between NuMA and dynein/dynactin is restricted to mitosis, because during interphase NuMA is segregated to the nucleus, and spatially separated from cytoplasmic dynein and dynactin by the nuclear membrane. This compartmentalization does not exist in frog egg cytoplasm, where the majority of NuMA, dynein, and dynactin remains soluble during mitosis as well as during interphase. Nevertheless, the binding of NuMA to dynein and dynactin is mitosis-specific even in this system, as demonstrated in our coprecipitation experiments from metaphase and interphase frog egg extracts. This indicates that the interaction between NuMA, dynein, and dynactin must be specifically regulated. Our experiments both in frog egg extract and in mitotic HeLa cells demonstrate that the transport and pole accumulation of NuMA is a gradual process. Aggregates of NuMA are found in as many as 64% of HeLa cells in prometaphase, of which the majority is attached to the mitotic spindle. The formation of these aggregates could be explained in two different ways: one possibility is that they represent remnants of a NuMA meshwork that has formed as part of an insoluble nuclear matrix during interphase and that has not yet completely dissolved at the time of nuclear envelope breakdown, when the mitotic spindle is formed. In support of this would be the observation of NuMA meshworks that persist as late as prophase, filling the intranuclear space between the condensed chromosomes (Fig. 1 A; as well as Compton et al., 1992; Yang et al., 1992). A nother possibility would be that all NuMA solubilizes at the prophase/prometaphase transition, but that the presence of spindle microtubules leads to condensation of soluble NuMA on their surface, if it cannot be transported to the spindle poles fast enough. This latter model would explain why NuMA aggregates are reversibly solubilized by the depolymerization of microtubules with nocodazole, but reform when microtubules are allowed to repolymerize. NuMA could thereby either bind directly to tubulin, as suggested before (Merdes et al., 1996), or bind to a different matrix formed in part by dynactin components. In any case, it appears that the poleward transport along spindle fibers is a rate-limiting step in dissolving these aggregates under physiological conditions.

Our microscopy data leave open the question whether NuMA is completely solubilized into homodimers (Harborth et al., 1995), or whether 12-arm oligomers, as recently described (Harborth et al., 1999), are transported along the spindle. What mechanisms promote disassembly of the interphase NuMA lattice or its mitosis-specific association with dynactin and dynein remains unknown, although it seems likely to involve posttranslational modification of NuMA: NuMA is phosphorylated at the G2/M transition (Sparks et al., 1995), and this phosphorylation seems to solubilize, at least in part, the fibrous meshwork residing in the nucleus during interphase (Saredi et al., 1997; Gueth-Hallonet et al., 1998; Harborth et al., 1999).

Our biochemical evidence makes it most likely that several NuMA dimers bind to dynactin to form a metastable complex, and recruit the dynein motor in a low-affinity interaction. This would explain why we can cofractionate NuMA and dynactin, but not dynein in our gel filtration experiments of metaphase-arrested frog egg extracts. The molecular size of our NuMA/dynactin fractions at ~2,000 kD would be consistent with the association of two dimers of NuMA (4 × 240 kD = 960 kD) with a dynactin complex.
of \( \approx 1,200 \text{kD} \), comprising ten subunits of Arp1, five subunits of p50/dynamitin, two subunits of p150/glued, and one subunit each of p62, p37, p32, p27, and p24 (Schafer et al., 1994). Consistently, after gel filtration, much of this complex is found disassembled, leaving both NuMA and dynactin at peaks of \( \approx 1,000 \text{kD} \). After initial dynactin binding to NuMA, dynine could then attach transiently to this complex by an interaction between the dynine intermediate chain and the p150/glued subunit (Kariki and Holzbaur, 1995; V. Vaughan and V. Allee, 1995). A notion by Schr"{o}er and Sheetz (1991), this interaction is not stable, a fact that is also reflected in the present study. The interaction of dynine with dynactin and NuMA, although detected by coimmunoprecipitation, is lost during the process of gel filtration. This might be due to the dilution in buffer and the long running times of our gel filtration columns (several hours), as compared with the immunoprecipitation protocol, in which washing is completed within a few minutes only. Further reflecting the transient nature of this interaction is the finding that dynine colocalizes only partially in some NuMA/dynactin midzone aggregates. Evidence for binding of NuMA to dynactin has also emerged from the findings of Clark and Meyers (1999), who showed that NuMA colocalizes to overexpressed wild-type Arp1, but not mutant forms of Arp1, even though these could still recruit other dynactin and dynine subunits. The binding of NuMA to dynactin was only seen in the cytoplasm of prometaphase cells in their experiments, but no longer during anaphase, indicating that the mitosis-specific binding between NuMA, dynactin, and dynine could be released as early as metaphase. Our in vivo observations add further support to this, revealing that all NuMA has been transported towards the poles at this stage, and might have been deposited to form an insoluble spindle pole matrix. A interaction between NuMA and the Arp1 filament of the dynactin complex thus seems likely, analogous to the suggested binding between Arp1 and Golgi apparatus-specific spectrin (Hollerman et al., 1996).

A further factor that may be involved in the formation of this complex is an isoform of protein 4.1 (Mattagajasingh et al., 1999). Protein 4.1 binds directly to a region of the NuMA tail, both during interphase and mitosis. A suggested by Clark and Meyers (1999), 4.1 could mediate the binding of NuMA to Arp1, reflecting a similar binding hierarchy as on the erythrocyte membrane skeleton, where 4.1 interacts with spectrin and actin. Further work will be needed to define the specific role of protein 4.1 at the spindle poles.

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