Opposing Motor Activities Are Required for the Organization of the Mammalian Mitotic Spindle Pole

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Abstract. We use both in vitro and in vivo approaches to examine the roles of Eg5 (kinesin-related protein), cytoplasmic dynein, and dynactin in the organization of the microtubules and the localization of NuMA (Nuclear protein that associates with the Mitotic Apparatus) at the polar ends of the mammalian mitotic spindle. Perturbation of the function of Eg5 through either immunodepletion from a cell free system for assembly of mitotic asters or antibody microinjection into cultured cells leads to organized astral microtubule arrays with expanded polar regions in which the minus ends of the microtubules emanate from a ring-like structure that contains NuMA. Conversely, perturbation of the function of cytoplasmic dynein or dynactin through either specific immunodepletion from the cell free system or expression of a dominant negative subunit of dynactin in cultured cells results in the complete lack of organization of microtubules and the failure to efficiently concentrate the NuMA protein despite its association with the microtubules. Simultaneous immunodepletion of these proteins from the cell free system for mitotic aster assembly indicates that the plus end–directed activity of Eg5 antagonizes the minus end–directed activity of cytoplasmic dynein and a minus end–directed activity associated with NuMA during the organization of the microtubules into a morphologic pole. Taken together, these results demonstrate that the unique organization of the minus ends of microtubules and the localization of NuMA at the polar ends of the mammalian mitotic spindle can be accomplished in a centrosome-independent manner by the opposing activities of plus end– and minus end–directed motors.

THE mitotic spindle is a complex microtubule-based structure that is responsible for chromosome segregation during cell division (McIntosh and Koonce, 1989; Mitchison, 1989a; Rieder, 1991; Hyman and Karsenti, 1996). It is assembled in a spatially and temporally regulated manner during the cell cycle, and the organization of the microtubules and the movement and positioning of the chromosomes within the mitotic spindle are achieved through a complex set of forces exerted through the microtubule lattice. These forces derive from both the dynamics of microtubule polymerization (Inoue and Salmon, 1995) and the actions of microtubule-dependent motor proteins (Fuller and Wilson, 1992; Barton and Goldstein, 1996). Changes in microtubule polymerization occur primarily at the plus ends of the microtubules, and these changes have been postulated to exert force on the chromosomes in a number of ways, including chromosome movement mediated by the kinetochore (Koshland et al., 1988; Coue et al., 1991), the polar wind (Rieder and Salmon, 1994), and chromosome capture (Hayden et al.,

1990; Holy and Leibler, 1994). In addition to microtubule dynamics, forces exerted by both plus end– and minus end–directed microtubule-dependent motors are required for normal assembly of the mitotic spindle. The antagonistic nature of these oppositely acting motors has been elegantly explored in *Saccharomyces cerevisiae* (Saunders and Hoyt, 1992; Hoyt, 1994; Saunders et al., 1995). Prevailing evidence indicates that the counteractive forces generated by both microtubule dynamics and microtubule motors are placing the entire mitotic spindle under tension, and the tension created by these counteracting forces appears to be necessary for the inactivation of a spindle-dependent cell cycle checkpoint (Rieder et al., 1994; Li and Nicklas, 1995; Murray, 1995; Nicklas et al., 1995).

The mitotic spindle must be organized in such a way as to support dynamic changes at the microtubule ends while simultaneously integrating the forces exerted by microtubule-dependent motor proteins. Indeed, the observation of poleward microtubule flux (Mitchison, 1989b; Sawin and Mitchison, 1991) demonstrates that the microtubules within the mitotic spindle are arranged in a configuration that is sufficiently stable to perform chromosome movements while both the plus and minus ends are available to exchange tubulin subunits. To accomplish this organiza-

tional task, cells have devised two distinct structural complexes that coordinate the dynamics at microtubule ends with motor-dependent force production (Desai and Mitchison, 1995). The best characterized of these is the kinetochore (Earnshaw and Tomkiel, 1992; Pluta et al., 1995). Kinetochores are located at the chromosome centromeres and are responsible for both the attachment of microtubule plus ends to chromosomes and force production during chromosome movements (Koshland et al., 1988; Nicklas, 1989; Rieder et al., 1990; Coue et al., 1991; Hyman and Mitchison, 1991). Thus, kinetochores must not only serve as the sites for the localization (and function) of the motors that power chromosome movement, but they must also serve to maintain the attachment of microtubule plus ends with the chromosomes as the microtubules convert between growing and shrinking states.

The minus ends of microtubules focused at the spindle pole must also be organized by a unique structural complex. This putative complex would maintain the organization of microtubule minus ends in a morphologic pole while permitting them to release from the centrosome and allow for tubulin subunit exchange during poleward microtubule flux. The identity of the proteins contained within this hypothetical mitotic spindle pole organizing complex are currently unknown, although there is substantial experimental evidence indicating that a key component is the NuMA protein (Nuclear protein that associates with the Mitotic Apparatus)[1] (for reviews see Compton and Cleveland, 1994; Cleveland, 1995). NuMA is localized in the nucleus during interphase but concentrates at the polar ends of the mitotic spindle during mitosis (Lyderson and Pettijohn, 1980; Kallajoki et al., 1991; Maekawa et al., 1991; Tousson et al., 1991; Compton et al., 1992; Yang et al., 1992). Both in vitro and in vivo experiments indicate that NuMA is required for the organization of the mitotic spindle (Kallajoki et al., 1991, 1992, 1993; Yang and Snyder, 1992; Compton and Cleveland, 1993; Compton and Luo, 1995; Gaglio et al., 1995); however, it is unclear if NuMA interacts with any of the microtubule-dependent motors involved in the organization of the microtubules at the mitotic spindle poles or how it becomes concentrated at the polar ends of the mitotic spindle. To address these questions, we have used both in vitro and in vivo approaches to analyze the roles of cytoplasmic dynein (Holzbaur and Vallee, 1994; Schroer, 1994a), dynactin (Schroer and Sheetz, 1991; Schroer, 1994a,b; Schroer et al., 1996; Vallee and Sheetz, 1996), and Eg5 (Sawin et al., 1992a; Blangy et al., 1995). The results of these experiments demonstrate that oppositely directed motors antagonize each other during the organization of the microtubules into a morphologic pole and further show that these motors are necessary for the accurate positioning of NuMA at the minus ends of microtubules.

Materials and Methods

Cell Culture

The human HeLa cell line and the monkey CV-1 cell line were both main-

tained in DME containing 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, and 0.1 μg/ml streptomycin. Cells were grown at 37°C in a humidified incubator with a 5% CO_2 atmosphere.

Antibodies

NuMA was detected with either a human-specific mouse mAb (mAb1F1; Compton et al., 1991), a human autoimmune serum (courtesy of Dr. D. Pettijohn, University of Colorado, Boulder, CO), or the rabbit polyclonal antibody (Gaglio et al., 1995). Tubulin was detected using the mAb DM1α (Blose et al., 1984). Eg5 was detected using an affinity purified rabbit polyclonal antibody (Sawin et al., 1992a). Dynactin was detected using either an mAb directed against the Arp1 subunit (mAb45A; Schafer et al., 1994) or an mAb specific for the p150B subunit (mAb150B; Gill, S.R., and T.A. Schroer, unpublished results). Cytoplasmic dynein was detected using an mAb specific for IC74 intermediate chain (mAb70.1; Steuer et al., 1991). Finally, pericentrin was detected using a rabbit polyclonal antibody generated against the purified recombinant pericentrin protein (Doxsey et al., 1994).

Immunological Techniques

Indirect immunofluorescence microscopy was performed on cultured cells by immersion in microtubule stabilization buffer (MTSB; 4 M glycerol, 100 mM Pipes, pH 6.9, 1 mM EGTA, and 5 mM $MgCl_2$) for 1 min at room temperature, extraction in MTSB + 0.5% Triton X-100 for 2 min, followed by MTSB for 2 min. Cells were then fixed in −20°C methanol for 10 min. Indirect immunofluorescence microscopy on mitotic asters assembled in the cell free mitotic extract was performed by dilution of 5 μl of the extract into 25 μl of KHM buffer (78 mM KCl, 50 mM Hepes, pH 7.0, 4 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT; Burke and Gerace, 1986). The diluted sample was then spotted onto a poly-L-lysine–coated glass coverslip and fixed by immersion in −20°C methanol. Both the fixed cells and mitotic asters were rehydrated in TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 1% albumin and all antibody incubations and washes were performed in TBS + 1% albumin. Each primary antibody was incubated on the coverslip for 30 min followed by 5-min washes in TBS + 1% albumin, and the bound antibodies were detected using either fluorescein- or Texas red–conjugated species-specific secondary antibodies at dilutions of 1:500 (Vector Labs, Inc., Burlingame, CA). The DNA was detected using 4',6-diamidino-2-phenylindole (DAPI) at 0.4 μg/ml (Sigma Chemical Co., St. Louis, MO.). After a final wash, the coverslips were mounted in FITC-guard mounting medium (Testog, Inc., Chicago, IL) and observed on a microscope equipped for epifluorescence (model Optiphot; Nikon Inc., Melville, NY).

Proteins from the mitotic extracts were solubilized directly with SDS-PAGE sample buffer. The proteins were then separated by size using SDS-PAGE (Laemmli, 1970) and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore Corp., Bedford, MA). The membranes were blocked in TBS containing 5% nonfat milk for 30 min at room temperature, and the primary antibody incubated for 6 h at room temperature in TBS containing 1% nonfat milk. Nonbound primary antibody was removed by washing five times for 3 min each in TBS, and the bound antibody was detected using either horseradish peroxidase–conjugated protein A or horseradish peroxidase–conjugated goat anti–mouse (Bio-Rad Labs., Hercules, CA). The nonbound secondary reagent was removed by washing five times for 3 min each in TBS and the signal detected using enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

Microinjection

CV-1 cells growing on photo-etched alpha-numeric glass cover slips (Bellco Glass Co., Vineland, NJ) were microinjected following the procedures of Compton and Cleveland (1993) and Capecchi (1980). For the antibody microinjection experiments, interphase cells were microinjected in the cytoplasm with either a preimmune IgG or the immune IgG and monitored by phase contrast microscopy as they progressed into mitosis. Cells were followed for up to 4 h after injection and then processed for immunofluorescence microscopy.

For expression of the chicken 50-kD subunit of dynactin, the full-length cDNA contained on a single 1.5-kb EcoRI fragment was inserted into the unique EcoRI site of the cytomegalovirus (CMV) expression vector (Compton and Cleveland, 1993). This plasmid was purified by CsCl centrifugation and diluted to 100 μg/ml in 10 mM Tris-HCl, pH 7.4, 1 mM

EDTA. The injected cells were then followed by phase contrast microscopy as they progressed into mitosis and were then processed for immunofluorescence microscopy.

Immunodepletion of Mitotic Extracts

Mitotic extracts from HeLa cells were prepared according to Gaglio et al. (1995). HeLa cells were synchronized in the cell cycle by double block with 2 mM thymidine. After release from thymidine block, the cells were allowed to grow for 6 h and then nocodazole was added to a final concentration of 40 ng/ml. The mitotic cells that accumulated over the next 4 h were collected by mitotic shake off and incubated for 30 min at 37°C with 20 μg/ml cytochalasin B. The cells were then collected by centrifugation at 1,500 rpm and washed twice with cold PBS containing 20 μg/ml cytochalasin B. Cells were washed one last time in cold KHM buffer containing 20 μg/ml cytochalasin B and finally Dounce homogenized (tight pestle) at a concentration of ∼3 × 10^{7} cells/ml in KHM buffer containing 20 μg/ml cytochalasin B, 20 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin. The crude cell extract was then subjected to sedimentation at 100,000 g for 15 min at 4°C. The supernatant was recovered and supplemented with 2.5 mM ATP (prepared as Mg^{2+} salts in KHM buffer) and 10 μM taxol, and the mitotic asters were stimulated to assemble by incubation at 30°C for 30 min. After incubation, samples were processed for indirect immunofluorescence microscopy as described above, and the remainder of the extract containing the assembled mitotic asters was subjected to sedimentation at 10,000 g for 15 min at 4°C. The supernatant and pellet fractions were both recovered and solubilized in SDS-PAGE sample buffer for immunoblot analysis.

Immunodepletions from the extract before aster assembly were carried out using 100 μg of either a preimmune rabbit polyclonal IgG, anti-NuMA rabbit polyclonal IgG, anti-Eg5 affinity purified rabbit polyclonal IgG, mAb 45A, which is specific for the Arp1 subunit of dynactin, or mAb 70.1, which is specific for the IC74 intermediate chain of cytoplasmic dynein. Each antibody was adsorbed onto ∼25 μl of either protein A- or protein G-conjugated agarose (Boehringer Mannheim, Indianapolis, IN). The 70.1 mAb against cytoplasmic dynein intermediate chain was coupled to protein A-conjugated agarose using goat antimurine IgM-specific antibody (Vector Labs, Burlingame, CA). The antibody-coupled agarose was washed in KHM buffer and then packed by centrifugation to remove the excess fluid. Efficient depletion of the target protein was routinely achieved by sequential depletion reactions in which the total quantity of packed agarose did not exceed 40 μl per 100 μl of extract. First, half of the antibody-coupled agarose was resuspended with the mitotic extract and incubated with agitation for 1 h at 4°C. After this incubation, the agarose was removed from the extract by sedimentation at 15,000 g for 10 s and saved. Next, the extract was recovered and used to resuspend the other half of the antibody coupled agarose and another incubation performed with agitation for 1 h at 4°C. After this incubation, the agarose was removed by sedimentation at 15,000 g for 10 s and pooled with the agarose pellet from the initial depletion reaction. In all cases, immunoblot analysis indicates that this depletion protocol results in nearly 100% efficient depletion of the target protein in experiments where only one protein was depleted, and >98% depletion of the target proteins when more than one protein was depleted (see results). The depleted extract was recovered and microtubule polymerization induced by the addition of taxol and ATP and incubation at 30°C for 30 min. During all depletion experiments, the extract remained fully mitotic as judged by histone H1 kinase assay as previously determined (Gaglio et al., 1995). Each depletion experiment was performed at least three times, and in all cases the data shown are representative of the microtubule structures we observed.
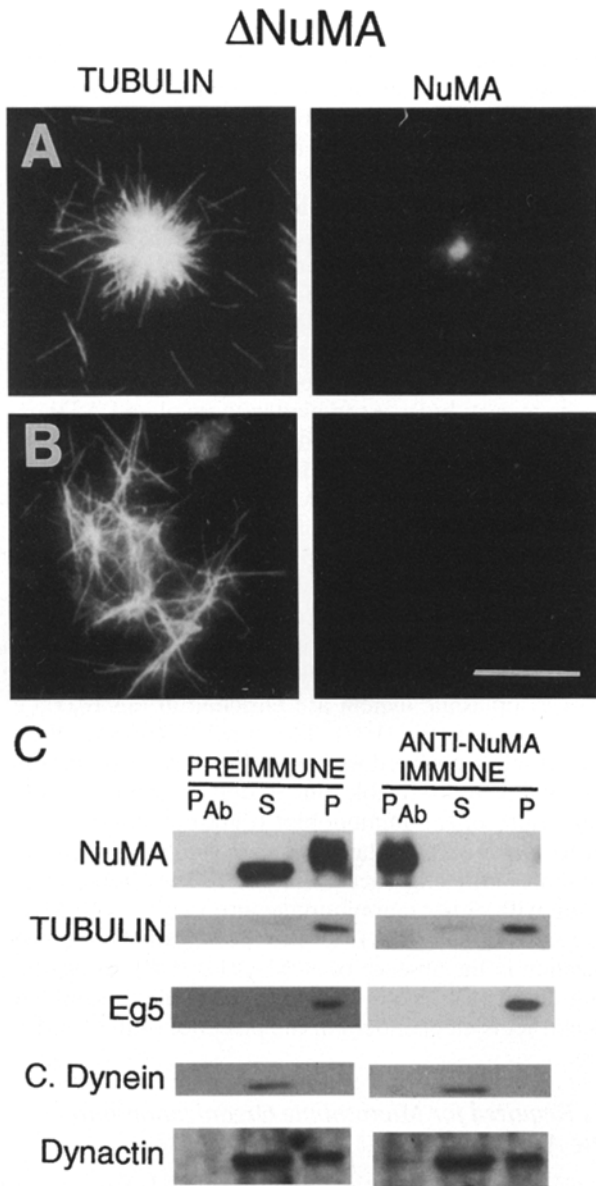
Cytoplasmic dynein and dynactin were purified from bovine brain. Briefly, both proteins in a high speed supernatant were concentrated and enriched by two successive rounds of ion exchange chromatography on fast-flow S-Sepharose (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) and velocity sedimentation into a 5–20% sucrose gradient. Dynein and dynactin copurified through these steps of the purification. The final sucrose gradient fractions enriched for dynein and dynactin were then separated on a MonoQ ion exchange column (Pharmacia LKB Biotechnology, Inc.). Both proteins had the expected polypeptide compositions and were >98% pure as judged by Coomassie blue staining of overloaded gels. Protein straight off the MonoQ column was cryoprotected by addition of 1.25 M sucrose and then flash frozen in 5-μl aliquots in liquid nitrogen and stored at −70°C. The final concentrations of dynein and dynactin after this procedure were 0.8 and 1.4 mg/ml, respectively.

Results

The plus end–directed kinesin-related protein Eg5, the minus end–directed motor protein cytoplasmic dynein, dynactin, and NuMA have each been shown to be involved in the organization of spindles using different experimental systems (Verde et al., 1991; Compton and Cleveland, 1994; Echeverri et al., 1996; Walczak and Mitchison, 1996). In this article, we have used specific antibodies to examine the role of each of these proteins or protein complexes in the formation of mitotic asters formed in a cell free extract prepared from synchronized HeLa cells. Under our standard assay conditions (Gaglio et al., 1995), in which the extract is untreated or mock depleted using a nonimmune antibody, microtubules assembled with taxol and ATP organize into aster-like arrays with NuMA concentrated at the central core (Fig. 1 A). To determine whether Eg5, cytoplasmic dynein, or dynactin are enriched on the mitotic asters that form in this extract, we collected the asters by sedimentation at 10,000 g for 15 min and analyzed the soluble and insoluble fractions by immunoblot (Fig. 1 C). Typically, both NuMA and Eg5 associate with the mitotic asters and are enriched in the insoluble pellet (60–95%; Fig. 1 C). In contrast, only 25–40% of dynactin and ∼5% of cytoplasmic dynein are enriched in the 10,000 g insoluble fraction (Fig. 1 C). The small fraction of cytoplasmic dynein associated with the mitotic asters in this extract is highly reproducible but is only detectable upon over exposure of the immunoblot (data not shown). The extent to which each of these proteins associates with the microtubules in this extract is not detectably altered after depletion with either a nonimmune antibody or a NuMA-specific antibody (Fig. 1 C), despite the lack of microtubule organization in the absence of NuMA (Fig. 1 B). Thus, the association of these proteins with microtubules assembled in this extract is independent of the presence of the NuMA protein.
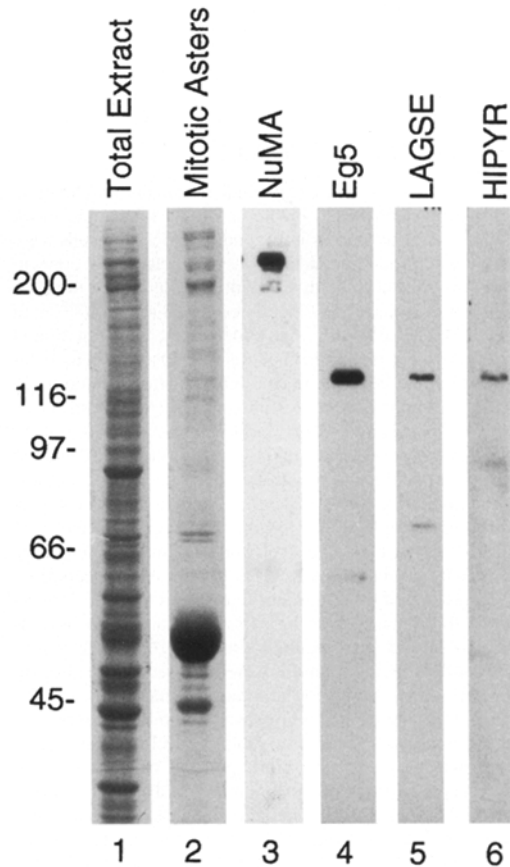
Eg5 Is Required for Microtubule Organization into Mitotic Arrays

To determine whether other kinesin-related proteins in addition to Eg5 might be associated with the mitotic asters, we examined the asters for proteins recognized by two broadly reactive antisera against kinesin-related proteins (Fig. 2; Sawin et al., 1992b). For this experiment, mitotic asters were purified by sedimentation at 10,000 g through a 25% sucrose cushion. This procedure enriches for the asters and minimizes contamination of the aster-associated proteins with soluble components from the extract (such as the abundant proteins of 30 and 85 kD; see Fig. 2, lane 1). As expected, immunoblot analysis of this enriched fraction demonstrates that both NuMA and Eg5 are components of the mitotic asters. Immunoblot analysis with antibodies directed against the highly conserved peptides LAGSE and HIPYR in the kinesin motor domain reveals only one major protein that is reactive with both antibodies. This band most likely corresponds to Eg5, as the human Eg5 protein sequence contains both the LAGSE and HIPYR peptides (Blangy et al., 1995) and the immunoreactive species has the same apparent molecular weight as Eg5. There were also two additional minor proteins reactive with the peptide-specific antisera. One of these mi-

Figure 1. Association of Eg5, cytoplasmic dynein, and dynactin with mitotic asters. (*A* and *B*) Immunofluorescence microscopy for both tubulin and NuMA after treatment of the cell free mitotic extract with protein A–conjugated agarose containing either IgG from a preimmune rabbit (*A*) or IgG from a NuMA-specific rabbit serum (*B*). (*C*) The protein A–agarose from each sample was recovered (P_{Ab}) and the extract was separated into 10,000 g soluble (*S*) and insoluble (*P*) fractions after the induction of microtubule polymerization. These fractions were subjected to immunoblot analysis using antibodies specific for NuMA, tubulin, Eg5, the intermediate chain of cytoplasmic dynein, and the p150^{Glued} subunit of dynactin as indicated. Bar: (*A* and *B*) 10 μm.

Figure 2. The kinesin-related protein Eg5 is an integral component of mitotic asters formed in a cell free mitotic extract. Coomassie blue (lanes *1* and *2*) and immunoblot analyses (lanes *3–6*) of the total mitotic extract (lane *1*) or the mitotic asters purified by sedimentation through 25% sucrose (lanes *2–6*). The mitotic aster proteins were subjected to immunoblot analysis using antibodies specific for NuMA (lane *3*), Eg5 (lane *4*), the LAGSE peptide (lane *5*), or the HIPYR peptide (lane *6*). The migration position of myosin (*200*), β-galactosidase (*116*), phosphorylase B (*97*), bovine serum albumin (*66*), and ovalbumin (*45*) are indicated in kD.

nor proteins, which migrates at ∼68 kD, is positive with only the LAGSE-specific antibody, while the other, migrating at 95 kD, is positive with only the HIPYR-specific antibody. These results suggest that, although minor amounts of other members of the kinesin family may be present within these mitotic asters, Eg5 is the most abundant kinesin-related protein associated with the mitotic asters assembled in this mitotic extract.

To test if Eg5 is required for the organization of the mitotic asters assembled in this cell free system, we used the Eg5-specific antibody to deplete the Eg5 protein from the extract before the assembly of mitotic asters (Fig. 3). In the absence of Eg5, the microtubules are organized in astral arrays, but these astral arrays are larger than asters assembled under control conditions; they have an expanded central core (i.e., the microtubule density in the central region appears reduced), and the NuMA protein is organized in a ring-like structure that has a diameter of ∼5 μm (Fig. 3 *A*). Immunoblot analysis of the immunoprecipitate and the soluble and insoluble fractions obtained after aster assembly in the absence of Eg5 demonstrates that neither NuMA, cytoplasmic dynein, nor dynactin coimmunoprecipitated with Eg5, and that each of these proteins associates with microtubules to the same extent as in control extracts, suggesting that these interactions are independent of Eg5 (Fig. 3 *B*). Analysis of the immunoprecipitate after the depletion of Eg5 by SDS-PAGE and silver staining failed to reveal any detectable coprecipitating proteins, in-

ΔEg5

TUBULIN   NuMA

B
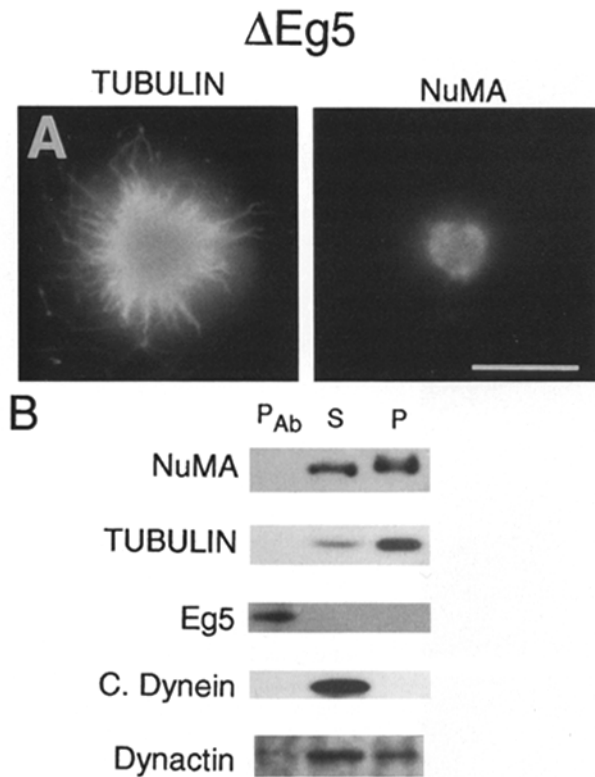
Figure 3. Eg5 is required for the organization of mitotic asters in the cell free mitotic extract. The cell free mitotic extract was treated with protein A–conjugated agarose containing IgG specific for the Eg5 protein. Microtubule polymerization was then stimulated by the addition of taxol and ATP and incubation at 33°C, and the resulting structures were analyzed by immunofluorescence microscopy using antibodies specific for tubulin and NuMA as indicated (*A*). The protein A–agarose was recovered (P_{Ab}) and the extract was separated into 10,000 g soluble (*S*) and insoluble (*P*) fractions after the induction of microtubule polymerization. These fractions were subjected to immunoblot analysis using antibodies specific for NuMA, tubulin, Eg5, the intermediate chain of cytoplasmic dynein, and the p150^{Glued} subunit of dynactin as indicated (*B*). Bar, 10 μm.

dicating that the Eg5 protein is not present in a complex before microtubule polymerization, although we can not rule out the possibility of putative Eg5-associated proteins with a molecular mass of 50–60 kD because of the presence of the antibody heavy chains (data not shown). Thus, these data suggest that Eg5 is necessary for the development of a focused central core in the mitotic asters consistent with previous observations in extracts prepared from *Xenopus* eggs (Sawin et al., 1992a), although it does not appear to be required for the convergence of the microtubule minus ends into asters.

It was recently reported that microinjection of an Eg5-specific antibody into cultured cells prevented the completion of mitosis. Immunofluorescence analysis of injected cells demonstrated that the centrosomes had failed to separate, resulting in the formation of a monastral spindle (Blangy et al., 1995). To determine the fate of the NuMA protein under these conditions, we microinjected affinity purified Eg5-specific antibodies into CV-1 cells and followed the progression of each injected cell through mito-

sis. Most of the cells that entered mitosis after microinjection with the Eg5-specific antibody failed to complete mitosis for up to 2 h (92%; $n = 45$), compared with cells injected with a nonimmune antibody, which typically complete mitosis within an hour. Indirect immunofluorescence microscopy of cells microinjected with Eg5 antibody shows that the mitotic spindle is organized in a radial array rather than a bipolar array consistent with a failure in centrosome separation (Sawin et al., 1992a; Blangy et al., 1995; Fig. 4, *A* and *B*). Interestingly, the NuMA protein is associated with the microtubules and is concentrated at the center of these astral arrays, but it is organized in a ring-like structure with a diameter of approximately 5 μm instead of the typical crescent-shaped pattern observed in a control injected cell (compare Fig. 4, *A* and *B*, with *C*). These data, in combination with the results of the depletion of Eg5 from our cell free system, support the hypothesis that the functional activity of Eg5 is necessary for the organization of the microtubules at the mitotic spindle pole.

Cytoplasmic Dynein and Dynactin Are Required for the Organization of Microtubules into Mitotic Arrays

To determine if cytoplasmic dynein is required for mitotic aster formation in our cell free system derived from HeLa cells, and to determine the fate of the NuMA protein in the absence of cytoplasmic dynein, we used an mAb against the intermediate chain of cytoplasmic dynein (mAb 70.1) to deplete cytoplasmic dynein from the extract. In the absence of cytoplasmic dynein, microtubules fail to organize into mitotic asters and are randomly dispersed in a similar fashion to when NuMA is depleted from the extract (Fig. 5 *A*). Eg5, NuMA, and dynactin associate with microtubules in the absence of cytoplasmic dynein to approximately the same extent as in control extracts (Fig. 5 *C*); however, NuMA shows a punctate staining pattern along the length of many of the microtubules and does not become concentrated at a single point (Fig. 5 *A*). This result is similar to what we observed when we attempted to assemble mitotic asters in the absence of ATP or in the presence of vanadate or AMP-PNP (Gaglio et al., 1995).

To verify that the failure of the mAb 70.1–treated extract to form asters was due specifically to the removal of cytoplasmic dynein from the sample, we added various concentrations of cytoplasmic dynein purified from bovine brain back to the depleted extract. Addition of purified cytoplasmic dynein allowed microtubules to become organized into asters in which NuMA was concentrated at the central core (Fig. 5 *B*), although addition of approximately five times the endogenous level of cytoplasmic dynein was needed to reconstitute this process (Fig. 5 *C*). Thus, cytoplasmic dynein is not only required for the organization of microtubules into mitotic asters in this cell free system but is also required for the efficient concentration of the NuMA protein at the center of the asters, suggesting a dynein-dependent mechanism for movement of NuMA to microtubule minus ends.

The association of cytoplasmic dynein with its intracellular cargo is thought to be mediated by activating or accessory proteins such as dynactin (Schroer and Sheetz, 1991; Schroer, 1994a, b; Schroer et al., 1996; Vallee and
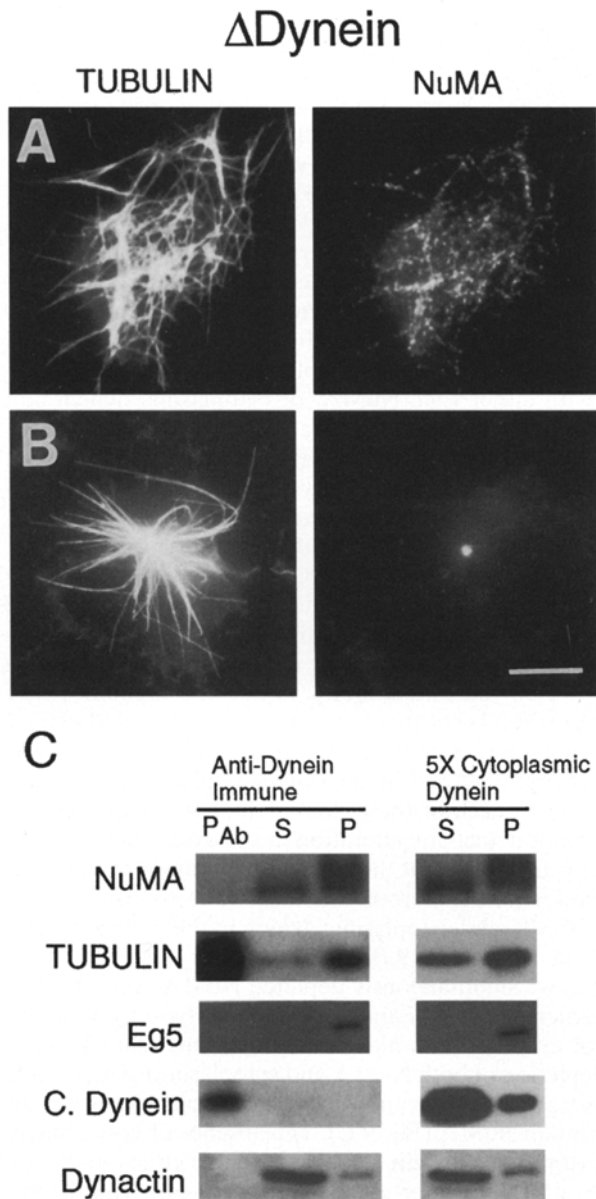
DAPI   TUBULIN   NuMA
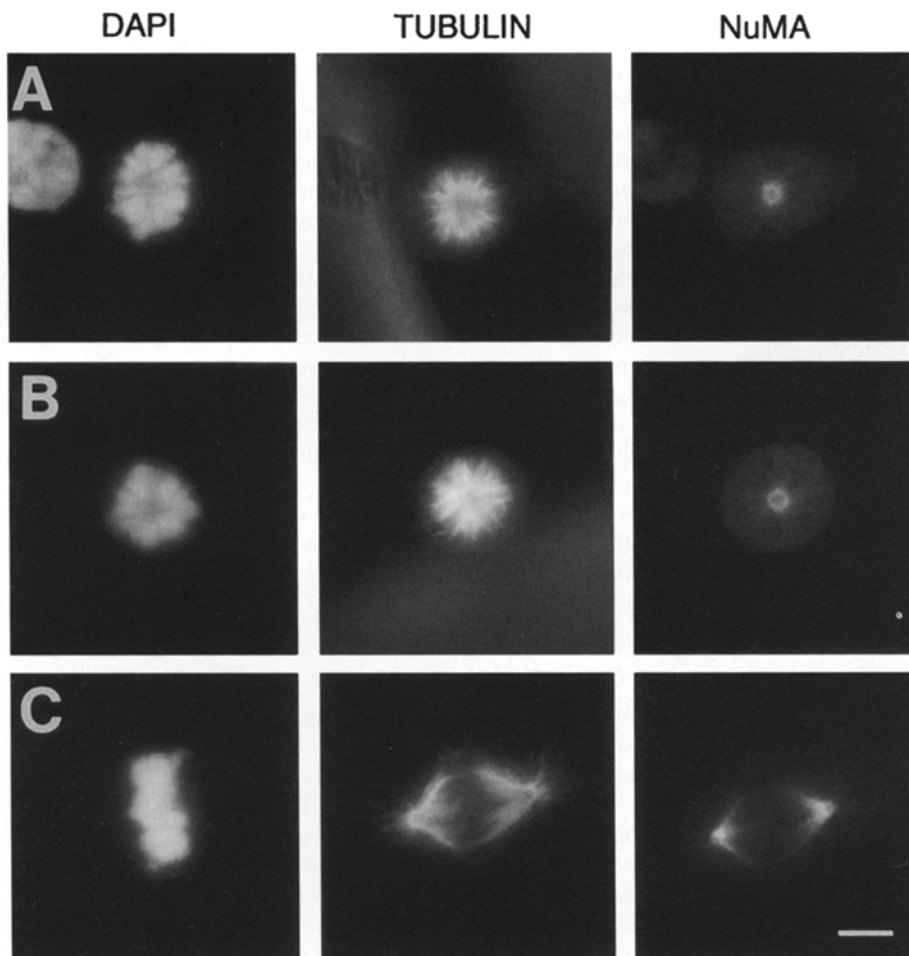
Figure 4. Organization of the mitotic spindle requires the Eg5 kinesin–related protein. Monkey CV-1 cells were monitored as they progressed through mitosis after microinjection of an Eg5-specific antibody (*A* and *B*) or a preimmune control antibody (*C*). The mitotic cells were fixed and processed for immunofluorescence microscopy using the DNA-specific dye DAPI, and antibodies specific for tubulin and NuMA as indicated. Bar, 10 μm.

Sheetz, 1996), and Echeverri et al. (1996) have recently shown that the mitotic spindle is poorly organized after the disruption of dynactin. To determine whether dynactin is necessary for the assembly of mitotic asters in our cell free system, we used an antibody directed against the Arp1 subunit of dynactin (mAb45A) to deplete dynactin from the extract. In the absence of dynactin, the microtubules fail to organize into aster-like arrays and are randomly dispersed similar to when the extract is depleted of either NuMA or cytoplasmic dynein (Fig. 6 *A*). Eg5, NuMA, and cytoplasmic dynein associate with microtubules in the absence of dynactin to approximately the same degree as in control extracts (Fig. 6 *C*), but the NuMA protein is distributed along the length of many of the microtubules in a punctate fashion similar to what is seen when cytoplasmic dynein is depleted from the extract (Fig. 5 *A*).

To verify that dynactin is the only functional component depleted from the extract by mAb45A, we added various concentrations of dynactin purified from bovine brain to the dynactin-depleted extract. Addition of purified dynactin at levels equivalent to the concentration of endogenous dynactin restores the formation of mitotic asters that have NuMA concentrated at their central cores (Fig. 6 *B*). Interestingly, despite the fact that dynactin and cytoplasmic dynein do not appear to be associated with each other in the extract before microtubule assembly (as judged by the

fact that they are not coimmunoprecipitated), addition of excess exogenous dynactin to either the dynactin-depleted extract (Fig. 6 *D*) or the untreated extract (data not shown) leads to the increased association of cytoplasmic dynein with the mitotic asters. This effect was specific to cytoplasmic dynein because neither the morphology of the mitotic asters nor the extent to which Eg5 and NuMA associated with the microtubules were altered by adding excess dynactin in the extract (data not shown). Thus, these data demonstrate that dynactin is required for the assembly of mitotic asters in this cell free system and suggest that dynactin may facilitate and/or stabilize the association of cytoplasmic dynein with the asters consistent with the hypothesis that dynactin participates in the association of cytoplasmic dynein with its cargo.

Because both cytoplasmic dynein and dynactin (Waterman-Storer et al., 1995) possess microtubule-binding activity, it is possible that they may serve to cross-link microtubules, and the combination of minus end–directed motor activity and cross-linking activity has been postulated to drive the formation of spindle poles in meiotic systems (McKim and Hawley, 1995; Vernos and Karsenti, 1995). To determine whether dynactin might be contributing to aster formation by promoting microtubule cross-linking, we followed the course of mitotic aster formation in a dynactin-depleted extract that we reconstituted with purified dynactin after the initial stimulation of microtubule poly-

ΔDynein

TUBULIN   NuMA

A

B

C

	Anti-Dynein Immune			5X Cytoplasmic Dynein	
	P_{Ab}	S	P	S	P
NuMA					
TUBULIN					
Eg5					
C. Dynein					
Dynactin					

Figure 5. Cytoplasmic dynein is required for the organization of mitotic asters in the cell free mitotic extract. The cell free mitotic extract was treated with protein A–conjugated agarose containing IgM specific for the intermediate chain of cytoplasmic dynein (mAb70.1). Microtubule polymerization was then stimulated by the addition of taxol and ATP and incubation at 33°C either in the absence (A) or presence (B) of exogenously added purified bovine brain cytoplasmic dynein (5×), and the resulting structures were analyzed by immunofluorescence microscopy using antibodies specific for tubulin and NuMA as indicated (A and B). The protein A–agarose was recovered (P_{Ab}), and both the depleted extract and the extract supplemented with 5× the endogenous level of the purified bovine brain cytoplasmic dynein were separated into 10,000 g soluble (S) and insoluble (P) fractions after the induction of microtubule polymerization. These fractions were subjected to immunoblot analysis using antibodies specific for NuMA, tubulin, Eg5, the intermediate chain of cytoplasmic dynein, and the p150^{Glued} subunit of dynactin as indicated (C). The strong signal on the tubulin immunoblot in the lane labeled P_{Ab} is due to cross reactivity between murine reagents used for both immunodepletion and immunoblotting. Bar: (A and B) 10 μm.

merization (Fig. 7). In samples incubated for 15 min in the absence of dynactin, the microtubules were seen to be arranged in disorganized aggregates (Fig. 7 A). After addition of purified dynactin, the first observable effect was parallel bundling of the microtubules (Fig. 7 B, arrows). NuMA was distributed in a punctate fashion along many of these bundles. At later time points, the microtubule bundles became progressively more organized into aster-like structures, concomitant with the concentration of NuMA at sites of microtubule convergence (Fig. 7, C and D). Finally, the microtubules became organized into aster-like arrays with localized concentrations of NuMA, although the structures formed by the readdition of dynactin after microtubule polymerization tend to be highly interconnected networks that are less radially organized (Fig. 7 D) compared to the individual, isolated structures such as those seen in Fig. 6 B.

It was recently discovered that overexpression of the 50-kD (p50/dynamitin) subunit of dynactin perturbs the organization of the mitotic spindle by disrupting dynactin and dislocating cytoplasmic dynein from mitotic structures such as kinetochores (Echeverri et al., 1996). To determine if overexpression of p50/dynamitin alters the localization of the NuMA protein at spindle poles, we microinjected a plasmid driving high level expression of chicken p50/dynamitin into CV-1 cells and examined the morphology of their mitotic spindles. Overexpression of chicken p50/dynamitin causes disruption of dynactin structure as judged by the reduced sedimentation velocity of the p150^{Glued} subunit of dynactin (data not shown) similar to previous results (Echeverri et al., 1996). Cells overexpressing chicken p50/dynamitin fail to complete mitosis, and 96% ($n = 28$) of the mitotically arrested cells have aberrant mitotic spindle morphology as judged by indirect immunofluorescence microscopy (Fig. 8). The microtubules in these mitotic cells are highly dispersed and are not organized in a typical bipolar configuration compared to a control cell injected with the expression vector alone (compare Fig. 8, A and B, with C). The NuMA protein is associated with the microtubules in these p50/dynamitin overexpressing cells but does not become concentrated at any particular site and fails to attain the characteristic crescent-like distribution seen in control-injected cells. The centrosomes in these p50/dynamitin overexpressing cells (Fig. 8, A and D, arrows) appear to be both structurally intact, as judged by indirect immunofluorescence microscopy using an antibody against pericentrin, and functionally intact because they are actively nucleating microtubules. Thus, these data corroborate the findings of Echeverri and co-workers (1996) and suggest that, in vivo, cytoplasmic dynein and dynactin are necessary for both proper organization of microtubules at the mitotic spindle pole and for concentration of NuMA at the minus ends of spindle microtubules.

Antagonistic Activity of Motors During Mitotic Aster Assembly

We have thus far shown that four distinct proteins are required for the organization of mitotic asters in our cell free system: NuMA (Gaglio et al., 1995), Eg5, cytoplasmic dynein, and dynactin. Removal of each of these proteins
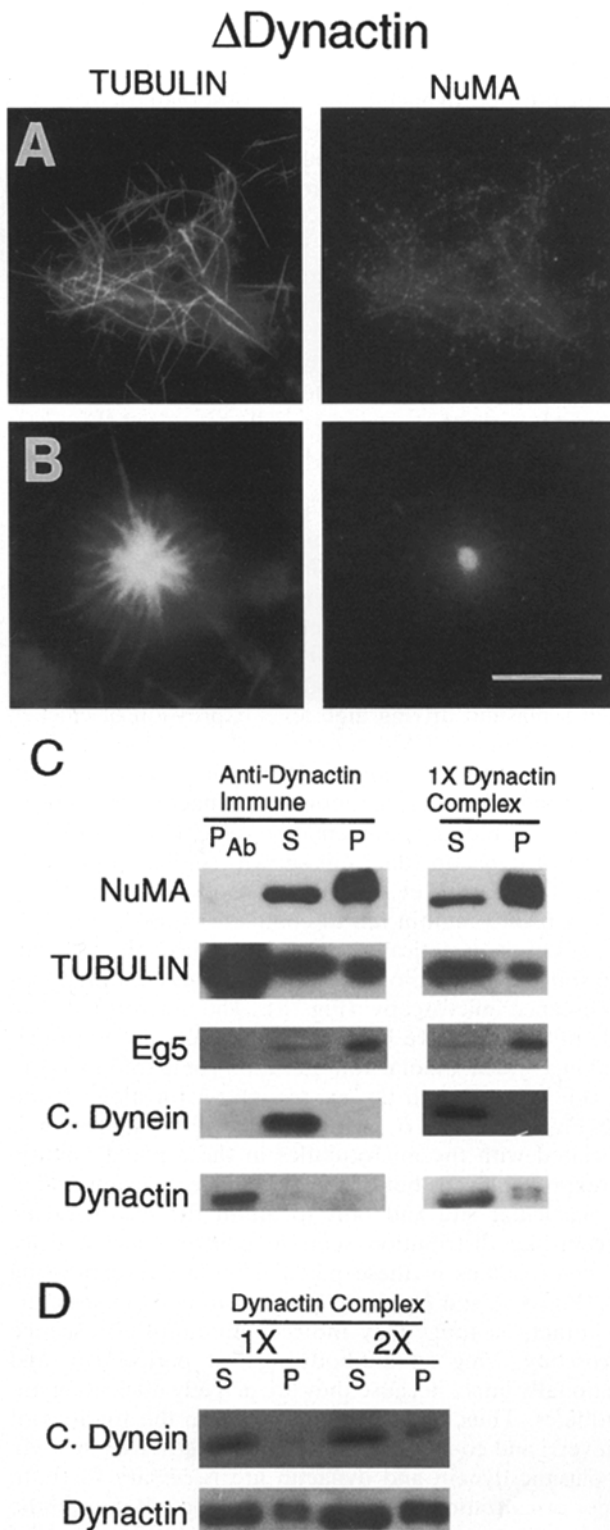
Figure 6. Dynactin is required for the organization of mitotic asters in the cell free mitotic extract. The cell free mitotic extract was treated with protein A–conjugated agarose containing mAb45A, an IgG specific for the Arp1 subunit of dynactin. Microtubule polymerization was then stimulated by the addition of taxol and ATP and incubation at 33°C either in the absence (A) or presence (B) of exogenously purified bovine brain dynactin complex, and the resulting structures were analyzed by immunofluorescence microscopy using antibodies specific for tubulin and NuMA as indicated (A and B). The protein A–agarose was re-

from the cell free system affects the organization of the microtubules into mitotic asters in a way that is strikingly similar to the way in which the perturbation of each protein in living cells affects the organization of the microtubules within the mitotic spindle. None of these proteins appear to be stably associated with one another in the mitotic extract before microtubule assembly because immunodepletion of any one does not detectably decrease the concentration of the others. Therefore, to explore possible functional relationships between these four proteins during aster formation, we performed a series of double immunodepletion experiments (Fig. 9).

First, we simultaneously depleted dynactin in combination with either Eg5, NuMA, or cytoplasmic dynein and induced the assembly of mitotic asters as before. In the absence of both dynactin and Eg5 (Fig. 9 A), dynactin and NuMA (data not shown), or dynactin and cytoplasmic dynein (data not shown), the microtubules fail to become organized into astral arrays and are randomly dispersed, with NuMA (when applicable) distributed along the length of the microtubules, much like what is seen when just dynactin is depleted from the extract (Fig. 6 A). Addition of purified dynactin to the dynactin-Eg5–depleted extract caused the microtubules to become organized into structures similar to those seen in an Eg5-depleted extract (data not shown), indicating that the mitotic extract was not otherwise altered by the double depletion of Eg5 and dynactin. In each of these double depletion experiments, we did not detect any alteration in the extent to which the proteins that were not the targets of immunodepletion associated with the microtubules relative to control extracts (e.g., NuMA and cytoplasmic dynein in the dynactin-Eg5–depleted extract; Fig. 9 B).

Next, we simultaneously depleted NuMA and cytoplasmic dynein (Fig. 9, C and D). Consistent with the depletion of either NuMA alone or cytoplasmic dynein alone, the depletion of both NuMA and cytoplasmic dynein leads to disorganized microtubule arrays that, as expected, do not contain NuMA (Fig. 9 C). The absence of both NuMA and cytoplasmic dynein had no detectable effect on the extent to which both Eg5 and dynactin associate with the microtubules in this extract (Fig. 9 D).

Next, we simultaneously depleted NuMA and Eg5 (Fig. 9, E and F). We expected to see randomly dispersed microtubules because the previous single and double depletions of NuMA, cytoplasmic dynein, and dynactin suggest

covered (P_{Ab}) and both the depleted extract and the extract supplemented with 1× the endogenous level of the purified bovine brain dynactin complex were separated into 10,000 g soluble (S) and insoluble (P) fractions after the induction of microtubule polymerization. These fractions were subjected to immunoblot analysis (C) using antibodies specific for NuMA, tubulin, Eg5, the intermediate chain of cytoplasmic dynein, and the p150^{Glued} subunit of dynactin as indicated. (D) Immunoblot analysis of the 10,000 g soluble (S) and insoluble (P) fractions obtained after the induction of microtubule polymerization for dynactin and cytoplasmic dynein in dynactin-depleted mitotic extract supplemented with either 1 or 2× quantities of purified dynactin. The strong signal on the tubulin immunoblot in the lane labeled P_{Ab} is due to cross reactivity between murine reagents used for both immunodepletion and immunoblotting. Bar: (A and B) 10 μm.
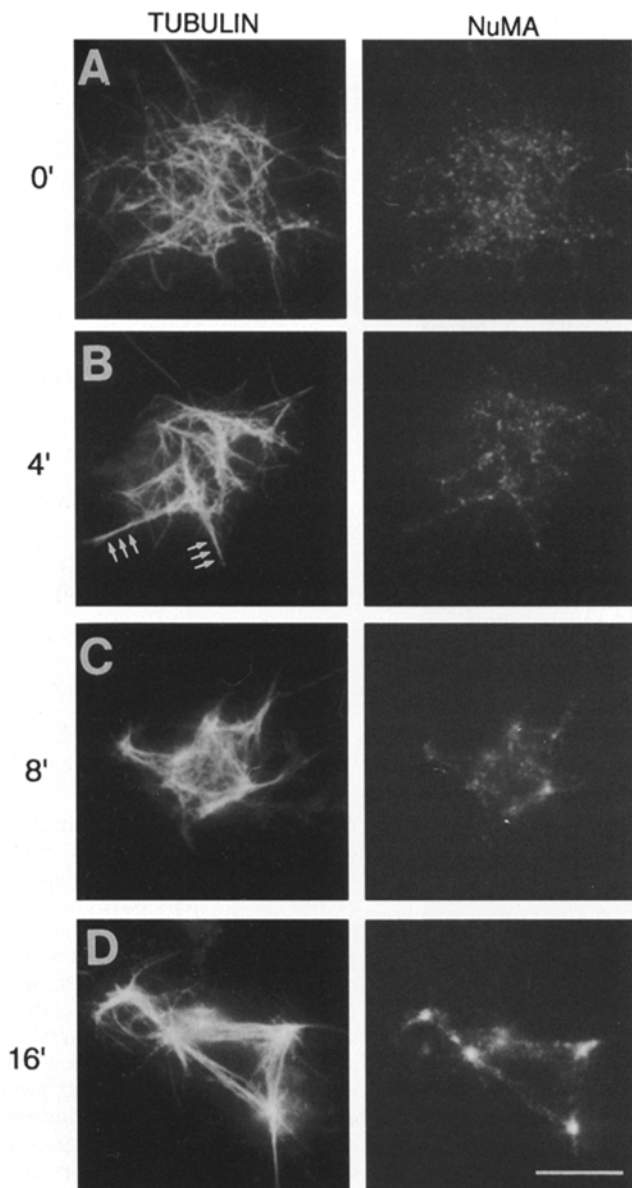
Figure 7. Aster formation in dynactin-depleted mitotic extracts reconstituted with purified dynactin. Microtubule polymerization was induced in dynactin-depleted mitotic extract for 15 min (A). The purified dynactin was added (1× concentration) at this time, and the progression of microtubule organization was monitored at the indicated times by immunofluorescence microscopy using antibodies specific for tubulin and NuMA as indicated (B–D). Arrows denote regions of microtubule bundling. Bar, 10 μm.

that each of these three proteins are required components of a single minus end–organizing activity. To our surprise, we found that in the absence of both NuMA and Eg5, the microtubules were still organized into astral arrays emanating from an expanded central core (Fig. 9 E). These astral arrays resemble those seen after depletion of Eg5 alone (Fig. 3), but they do not contain detectable quantities of NuMA. The absence of both NuMA and Eg5 had no detectable effect on the extent to which cytoplasmic dynein and dynactin associated with the microtubules (Fig. 9 F).

Finally, we depleted both cytoplasmic dynein and Eg5

expecting to see randomly dispersed microtubule arrays for similar reasons outlined in the preceding paragraph (Fig. 9, G and H). Again, we were surprised to find that in the absence of cytoplasmic dynein and Eg5, the microtubules had become organized into astral arrays with an expanded central core (Fig. 9 G) much like those formed in extracts depleted of just Eg5 or of both NuMA and Eg5. NuMA and dynactin associate with the microtubules of these astral arrays to a similar degree as in control extracts (Fig. 9 H), but NuMA does not appear to be efficiently concentrated at their centers and does not form a ring-like structure like those observed after depletion of Eg5 alone (compare Figs. 3 A and 9 G). Extreme overexposure of the immunoblot for cytoplasmic dynein in this double depletion experiment failed to reveal any detectable quantities of cytoplasmic dynein verifying that cytoplasmic dynein has been depleted from the extract to nearly 100% in this double depletion (data not shown).

Taken together, the results of these double depletion experiments lead to three conclusions. First, the observation that mitotic asters form when both cytoplasmic dynein and Eg5 are depleted from the extract suggests that there must be a second, undefined, minus end–directed motor that is sufficient to drive mitotic aster formation in this system. Second, because relatively normal mitotic asters form in the absence of both cytoplasmic dynein and Eg5, but not in the absence of cytoplasmic dynein alone, these data suggest that the plus end–directed motor activity of Eg5 must be precisely balanced by minus end–directed motor activities during mitotic aster formation. Otherwise, disorganized microtubule arrays will result such as those observed in extracts depleted of cytoplasmic dynein alone. Finally, these data suggest that NuMA is associated with a minus end–directed motor activity that is required for mitotic aster formation in this system, since the depletion of NuMA, either alone or in combination with Eg5, results in similar patterns of microtubule organization as those observed when the minus end–directed motor cytoplasmic dynein is depleted either alone or in combination with Eg5.

However, because removal of NuMA, cytoplasmic dynein, or dynactin, either alone or in combination, yields the same type of disorganized microtubule arrays, these data do not distinguish between two possibilities. The first possibility is that NuMA, cytoplasmic dynein, and dynactin act together to provide a single minus end–directed motor activity that is independent of the second, undefined minus end–directed motor activity. Second, it is possible that NuMA is associated with the second minus end–directed motor activity, and this NuMA–motor complex acts independently, and in parallel, to the minus end–directed activity of cytoplasmic dynein and dynactin. To discriminate between these two possibilities, we simultaneously depleted Eg5, NuMA, and cytoplasmic dynein expecting, if the first possibility holds true, to observe asters similar to those seen after the double depletion of either NuMA and Eg5 or cytoplasmic dynein and Eg5. Fig. 10 A shows that in the absence of Eg5, NuMA, and cytoplasmic dynein, the microtubules fail to organize into astral arrays and are randomly dispersed. Addition of purified cytoplasmic dynein (using similar conditions to the reconstitution of the dynein-depleted extract; Fig. 5) is sufficient to restore the organization of the microtubules into astral ar-
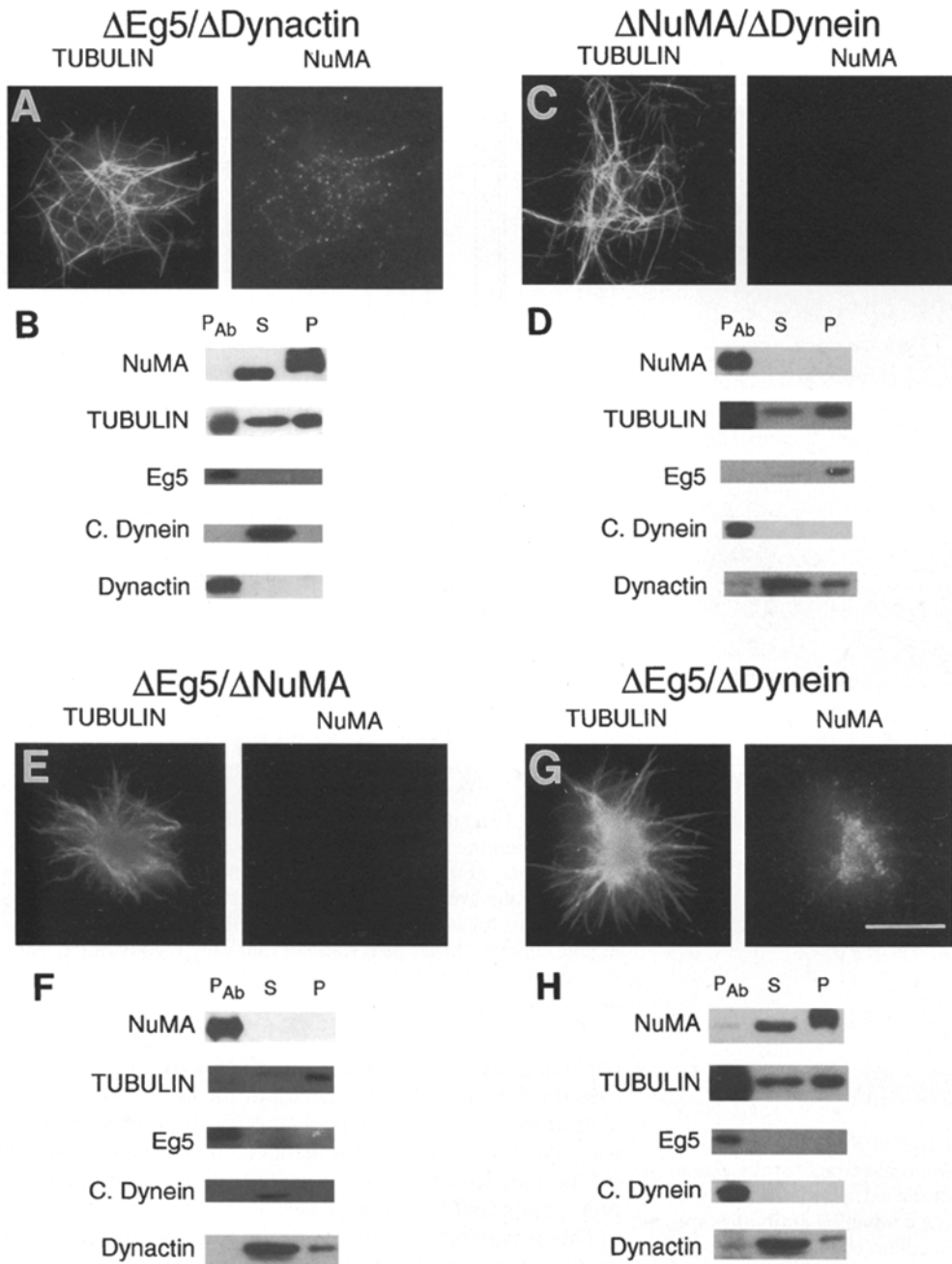
DAPI   TUBULIN   NuMA

DAPI   TUBULIN   PERICENTRIN
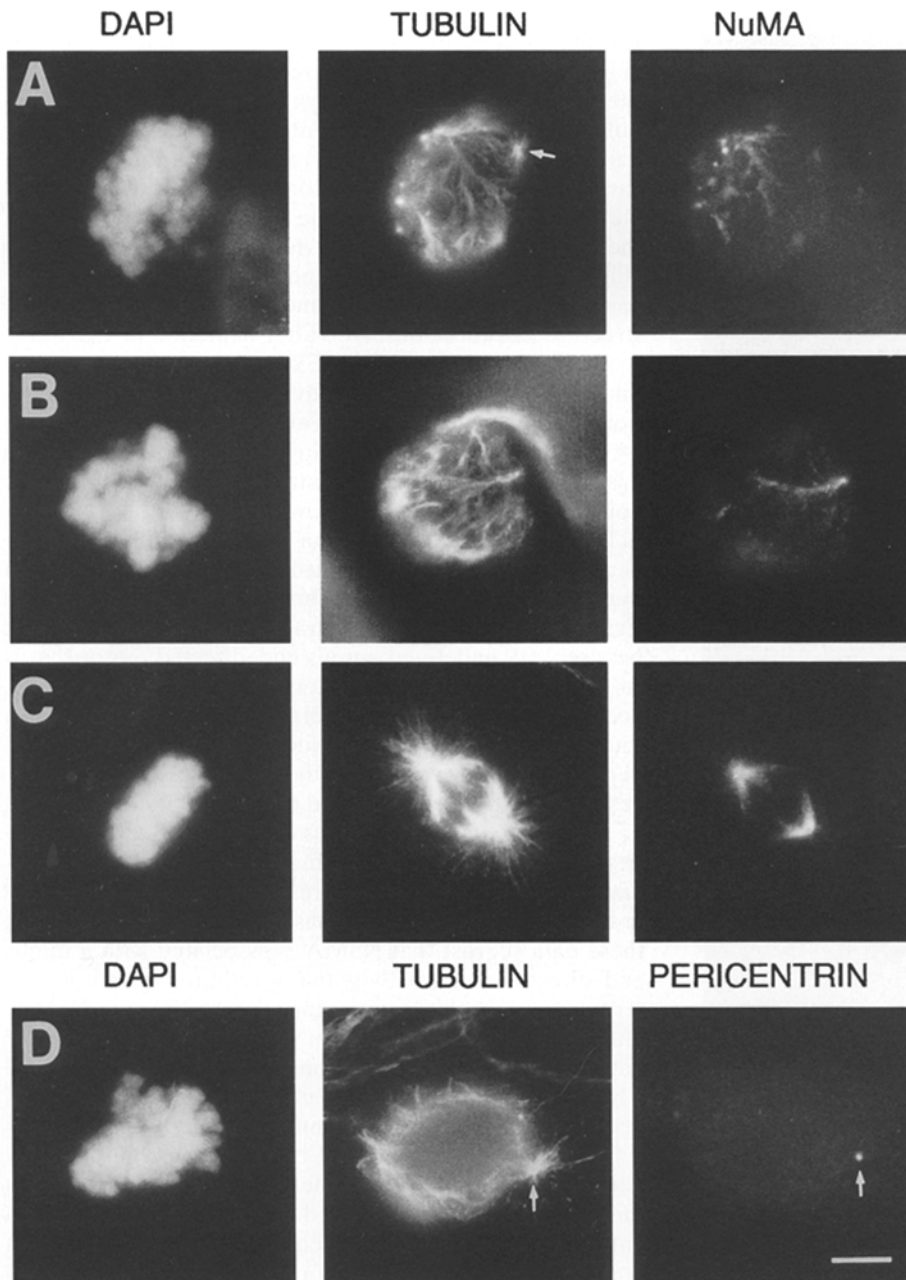
Figure 8. Cytoplasmic dynein and dynactin are required for the organization of the mitotic spindle. Monkey CV-1 cells were microinjected with a plasmid driving the expression of the 50-kD subunit of dynactin (p50/dynamitin) (*A, B,* and *D*) or a plasmid lacking any cDNA insert (*C*) and monitored as they progressed through mitosis. The mitotic cells were fixed and processed for immunofluorescence microscopy using the DNA-specific dye DAPI, and antibodies specific for tubulin, NuMA, or pericentrin as indicated. The centrosomes in some cells are indicated by arrows. Bar, 10 μm.

rays that are similar to those seen in the absence of Eg5 and NuMA, indicating that the mitotic extract has not been adversely affected by the simultaneous triple depletion of Eg5, NuMA, and cytoplasmic dynein (Fig. 10 *B*). Thus, despite the fact that NuMA requires cytoplasmic dynein activity to efficiently concentrate at the minus ends of the microtubules, the result of this triple depletion supports the hypothesis that NuMA is associated with a second minus end–directed motor activity distinct from cytoplasmic dynein and suggests that both cytoplasmic dynein and this NuMA-associated minus end–directed activity act independently to balance the plus end–directed activity of Eg5 during mitotic aster formation.

Finally, to estimate the "aster forming" capacity of the mitotic extracts during these complex depletion experiments, we have counted the total number of mitotic asters

in 20 randomly selected microscope fields. Fig. 11 summarizes the results of these analyses and demonstrates that the aster forming capacity of extracts depleted of Eg5, Eg5 and NuMA, or Eg5 and cytoplasmic dynein is comparable to that of a control extract. On the other hand, if NuMA, cytoplasmic dynein, dynactin, NuMA, and cytoplasmic dynein, Eg5 and dynactin, or Eg5, NuMA, and cytoplasmic dynein are depleted from the extract, the capacity of the extract to form mitotic asters is severely inhibited. The reduction of the mitotic aster–forming capacity of these depleted extracts is restored to values ranging from 51.6% (cytoplasmic dynein reconstitution of the Eg5-NuMA-cytoplasmic dynein depletion) to 89.3% (dynactin reconstitution of the dynactin depletion) relative to the control extract by addition of purified proteins. Thus, the individual microtubule arrays depicted in Figs. 1, 3, 5, 6, 9, and 10

ΔEg5/ΔDynactin
TUBULIN | NuMA

A

B | P_{Ab} S P
NuMA
TUBULIN
Eg5
C. Dynein
Dynactin

ΔNuMA/ΔDynein
TUBULIN | NuMA

C

D | P_{Ab} S P
NuMA
TUBULIN
Eg5
C. Dynein
Dynactin

ΔEg5/ΔNuMA
TUBULIN | NuMA

E

F | P_{Ab} S P
NuMA
TUBULIN
Eg5
C. Dynein
Dynactin

ΔEg5/ΔDynein
TUBULIN | NuMA

G

H | P_{Ab} S P
NuMA
TUBULIN
Eg5
C. Dynein
Dynactin

Figure 9. Simultaneous double depletion of Eg5 and dynactin (*A* and *B*), NuMA and cytoplasmic dynein (*C* and *D*), Eg5 and NuMA (*E* and *F*), or Eg5 and cytoplasmic dynein (*G* and *H*) from the cell free mitotic extract for the assembly of mitotic asters. The cell free mitotic extract was treated with protein A– (or G–) conjugated agarose containing antibodies specific for the indicated combinations of proteins or protein complexes and analyzed by immunofluorescence microscopy after the induction of microtubule polymerization using antibodies specific for tubulin and NuMA as indicated (*A, C, E,* and *G*). In each case, the protein A (or G–) agarose was recovered (P_{Ab}) and the extract was separated into 10,000 *g* soluble (*S*) and insoluble (*P*) fractions after the induction of microtubule polymerization. These fractions were subjected to immunoblot analysis using antibodies specific for NuMA, tubulin, Eg5, the intermediate chain of cytoplasmic dynein, and the 150-kD subunit of dynactin as indicated (*B, D, F,* and *H*). The strong signal on the tubulin immunoblot in the lane labeled P_{Ab} is due to cross reactivity between murine reagents used for both immunodepletion and immunoblotting. Bar: (*A, C, E,* and *G*), 10 μm.

are representative of the total aster-forming capacity of each extract.

Discussion

In this article, we examine the role of both plus end– and minus end–directed microtubule motors in the organization of the minus ends of the microtubules into a mitotic spindle pole and the accumulation of the NuMA protein at the polar ends of the mitotic spindle. Consistent with recent observations of meiotic spindle assembly in *Drosophila* oocytes (Matthies et al., 1996), our results support a model for the organization of microtubules into mitotic asters in which the convergence of the microtubule minus ends is driven by two distinct minus end–directed motor

activities: cytoplasmic dynein and a second, previously undescribed minus end–directed motor associated with NuMA. Both of these minus end–directed motors are partially balanced during mitotic aster formation by the opposing plus end–directed activity of Eg5 (Fig. 12, *arrows*). In accordance with this model, in each depletion experiment where the net sum of forces in the extract is predominantly minus end–directed (i.e., untreated extract, Eg5-depleted extract, Eg5-NuMA–depleted extract, or Eg5-dynein–depleted extract), the microtubules are organized into astral arrays, although in all cases where Eg5 has been depleted, the unopposed minus end–directed activities appear to induce large asters with expanded central cores. If, on the other hand, the balance of forces is shifted so that the minus end–directed activities are not predominant (i.e., NuMA-

ΔEg5/ΔNuMA/ΔDynein
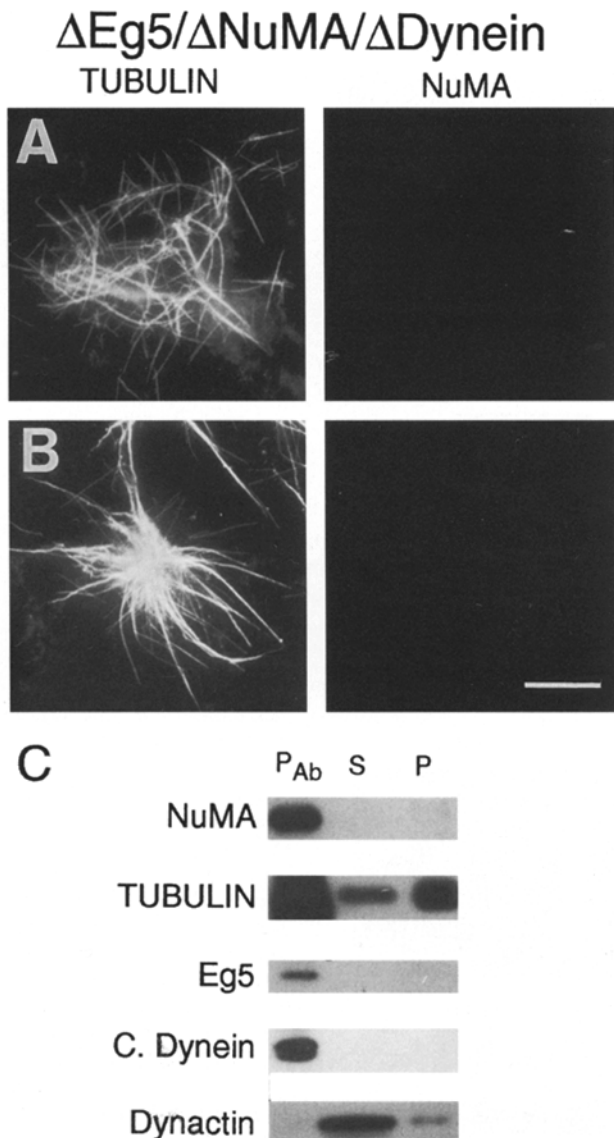
Figure 10. Simultaneous triple depletion of Eg5, NuMA, and cytoplasmic dynein from the cell free mitotic extract for the assembly of mitotic asters. The cell free mitotic extract was treated with protein A– and G–conjugated agarose containing antibodies specific for Eg5, NuMA, and the intermediate chain of cytoplasmic dynein. Microtubule polymerization was then stimulated by the addition of taxol and ATP and incubation at 33°C either in the absence (*A*) or presence (*B*) of exogenously added purified bovine brain cytoplasmic dynein, and the resulting structures were analyzed by immunofluorescence microscopy using antibodies specific for tubulin and NuMA as indicated (*A* and *B*). The protein A– and G–agarose was recovered (P_{Ab}) and the extract was separated into 10,000 g soluble (*S*) and insoluble (*P*) fractions after the induction of microtubule polymerization. These fractions were subjected to immunoblot analysis using antibodies specific for NuMA, tubulin, Eg5, the intermediate chain of cytoplasmic dynein, and p150^{Glued} subunit of dynactin as indicated (*C*). The strong signal on the tubulin immunoblot in the lane labeled P_{Ab} is due to cross reactivity between murine reagents used for both immunodepletion and immunoblotting. Bar: (*A* and *B*) 10 μm.
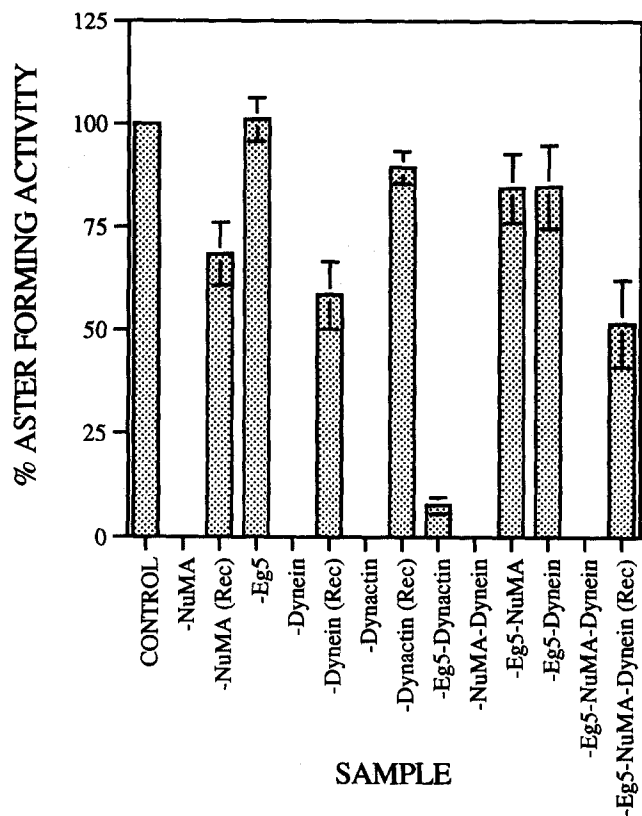
Figure 11. Total mitotic aster forming activity in mitotic extracts. The total number of mitotic asters in 20 randomly selected microscope fields were counted (magnification 400×). The values shown are the average of the counts derived from three separate experiments for each treatment and are normalized to 100% using the control extract. *Rec*, samples that were reconstituted using the purified protein that was depleted in each sample.

depleted, dynein-depleted, or NuMA-dynein–depleted), then the formation of organized mitotic asters is disrupted. Thus, the convergence of microtubule minus ends into mitotic asters in this system is achieved in a dynamic fashion by the balance of forces generated by opposing microtubule-dependent motor activities.

The antagonistic nature of the motor proteins involved in the formation of mitotic asters in this mitotic extract is analogous to the antagonistic activity reported for similar motor proteins in the organization of the mitotic spindle in *S. cerevisiae* (Saunders and Hoyt, 1992; Saunders et al., 1995). *CIN8* and *KIP1*, like Eg5, are members of the bimC family of kinesin-related motor proteins that provide plus end–directed motor activity that opposes the minus end–directed activity of both cytoplasmic dynein (Saunders et al., 1995) and *KAR3* (Saunders and Hoyt, 1992). Thus, the organization of microtubules into mitotic asters in this cell free system uses some of the same opposing motor activities that are required for the assembly of the mitotic spindle in *S. cerevisiae*. The striking difference between these two experimental systems, however, is that the motor activities act through microtubule structures in a geometrically different way. For example, in *S. cerevisiae*, cytoplasmic dynein is thought to interact with the astral microtubules at the cell cortex, in opposition to *CIN8* acting
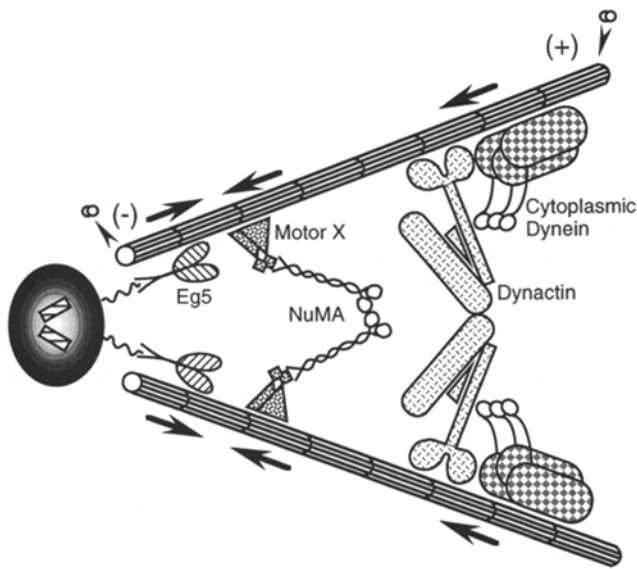
Figure 12. Hypothetical depiction of how Eg5, cytoplasmic dynein, dynactin, NuMA, and an unidentified motor (*Motor X*) might work together to organize the microtubules at the polar ends of the mammalian mitotic spindle. Formation of mitotic asters in our cell free system is independent of centrosomes and chromosomes and requires both microtubule motor and cross-linking activities. (We show a centrosome in this diagram to provide the reader with orientation.) Two minus end–directed motors, cytoplasmic dynein, and motor X (which is associated with and may require NuMA for function in this system) provide the predominant minus end–directed activity required for aster formation. These minus end–directed activities are partially balanced by the plus end–directed motor activity of Eg5. The direction that each of these motors is predicted to move along the microtubules is illustrated by arrows along the length of the microtubules. Upon depletion of either minus end–directed activity, the lack of predominant minus end–directed activity prevents aster formation. Two microtubule cross-linking activities are depicted; one involves NuMA and the other dynactin. In this model, the cross-linking activity associated with NuMA is not sufficient for aster formation, because no mitotic asters are observed after the double depletion of dynactin and Eg5. The tethering of Eg5 to the centrosome via an unidentified structural protein is based on the model proposed by Sawin and co-workers (1992*a*), although in our system this tethering event may not strictly require centrosomes. The plus (+) and minus (−) ends of the microtubules are indicated and tubulin subunit exchange as predicted by poleward microtubule flux is depicted (*arrowheads*).

within the body of the mitotic spindle inside the yeast cell nucleus. In the cell free system for the assembly of mitotic asters, the forces generated by cytoplasmic dynein and Eg5 act in an opposing manner on the same microtubules. In fact, the antagonistic nature of these motor activities is observed in the mitotic asters despite the absence of both chromosomes and conventional centrosomes. This implies that the mitotic asters assembled in vitro contain not only the motor proteins themselves, but also the structures that are necessary to counter balance each motor molecule during force production against the microtubule.

The results presented here also suggest that the NuMA protein interacts with two distinct minus end–directed microtubule motors on the mitotic spindle. This is consistent with the observation of the progressive accumulation of the NuMA protein at the polar ends of the mitotic spindle after the breakdown of the nuclear envelope at prometaphase (Compton et al., 1992). Based on the finding that NuMA fails to concentrate at microtubule minus ends after specific depletion of cytoplasmic dynein (Figs. 5 and 9 *G*), it is highly probable that one of the minus end–directed motor proteins that associates with NuMA is cytoplasmic dynein, although we do not currently have evidence for a direct physical interaction between these two proteins in our cell free system. This conclusion is consistent with immunofluorescence data showing that NuMA and cytoplasmic dynein have similar distributions at the polar ends of the mitotic spindle (Lyderson and Pettijohn, 1980; Gill et al., 1991; Kallajoki et al., 1991; Maekawa et al., 1991; Pfarr et al., 1991; Steuer et al., 1991; Tousson et al., 1991; Compton et al., 1992; Yang et al., 1992; Echeverri et al., 1996). This conclusion is further supported by the finding that the mitotic spindle is disrupted in indistinguishable ways if either NuMA (Yang and Snyder, 1992) or cytoplasmic dynein (Vaisberg et al., 1993) are perturbed by antibody microinjection or if either NuMA (Gaglio et al., 1995) or cytoplasmic dynein (Echeverri et al., 1996) are prevented from associating with the mitotic spindle. Indeed, microinjection of one NuMA-specific antibody was capable of inducing the collapse of a preassembled metaphase spindle, presumably, by imbalancing the forces exerted by microtubule-dependent motor proteins (Yang and Snyder, 1992). It is important to note, however, that under both in vitro and in vivo conditions, NuMA associates with the microtubules independently of cytoplasmic dynein (Figs. 5 and 8).

In addition to cytoplasmic dynein, the data presented here indicate that NuMA associates with another distinct minus end–directed motor activity. The identity of this putative minus end–directed motor is unknown at this time, although there are two obvious candidates. One is the human homologue of the CHO2 kinesin–related protein (Ando et al., 1994; Kuriyama et al., 1995), a member of the KAR3 family of kinesin-related proteins (Moore and Endow, 1996). Consistent with a role for CHO2, mitotic asters formed in our cell free system contain a ~68-kD protein that contains a LAGSE but not HIPYR peptide. Similar to the findings of Saunders and Hoyt (1992) in *S. cerevisiae*, the minus end–directed activity of the human homologue of the CHO2 would be predicted to antagonize the plus end–directed activity of the Eg5 protein. This prediction is further supported by the observation that *Drosophila* mutants in the ncd gene (another putative CHO2 homologue), in addition to meiotic defects, have disorganized mitotic spindles from which the centrosomes have detached (Endow, 1993; Endow et al., 1994) in a manner similar to that seen when either NuMA (refer to Fig. 9 in Gaglio et al., 1995) or cytoplasmic dynein (refer to Figs. 6 and 7 in Echeverri et al., 1996) are prevented from associating with spindle microtubules in mammalian cells.

Another candidate for a NuMA-associated minus end–directed motor activity is a novel isoform of cytoplasmic dynein that is not depleted from the extract by the 70.1 mAb. This possibility is supported by the recent identification of additional isoforms of the cytoplasmic dynein heavy chain that have different sedimentation velocities,

suggesting that they may have alternative subunit compositions relative to conventional 20S cytoplasmic dynein (Vaisberg et al., 1996). This possibility is particularly attractive in light of our results demonstrating that the depletion of dynactin appears to eliminate all minus end–directed, aster-forming activity (cytoplasmic dynein– and NuMA-associated) from our cell free system. Regardless of the identity of this additional minus end–directed motor activity, given that NuMA is capable of assembling into an extensive fibrous network (Saredi et al., 1996), it is possible that NuMA may be a structural component of the hypothetical mitotic spindle matrix that might serve to counterbalance the force exerted by this putative motor against the microtubules.

Finally, we show that dynactin is essential for the organization of the polar ends of the mitotic spindle. A large body of genetic and biochemical evidence suggests that an interaction between cytoplasmic dynein and dynactin is required for the proper function and/or organization of the mitotic spindle (Clark and Meyer, 1994; Muhua et al., 1994; Plamann et al., 1994; Echeverri et al., 1996; Tinsley et al., 1996; for reviews see Schroer, 1994b; Schroer et al., 1996). Consistent with genetic data showing that the phenotype of a strain carrying simultaneous disruptions of both the dynein heavy chain gene and the Arp1 gene or p150^{Glued} gene is similar to the phenotypes of strains carrying mutations in either the dynein heavy chain gene alone, the Arp1 gene alone, or the p150^{Glued} gene alone, we demonstrate that the depletion of either cytoplasmic dynein alone or dynactin alone is indistinguishable from the simultaneous depletion of both of these complexes. In addition, we show that the amount of cytoplasmic dynein that associates with the mitotic asters in our cell free system appears to be directly dependent on the quantity of dynactin present in the extract (Fig. 6). Thus, our data are consistent with the model that dynactin facilitates and/or stabilizes the interaction of cytoplasmic dynein with microtubules in the mitotic asters. Furthermore, given that each of these protein complexes is capable of interacting with microtubules and that cytoplasmic dynein and dynactin may physically interact with each other (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995), it is possible that a stable complex formed between dynactin and cytoplasmic dynein may cross-link microtubules within the mitotic spindle and promote the focusing of the minus ends of the microtubules as suggested by Vernos and Karsenti (1995).

Organization of the Mitotic Spindle Pole

Perhaps the most important concept emerging from this work is that all four of the proteins examined here (NuMA, Eg5, cytoplasmic dynein, and dynactin) are required for the organization of the microtubules at the polar ends of the mitotic spindle, yet none is an integral component of the centrosome. This fact emphasizes the idea that, although the centrosome serves an essential function in the nucleation and orientation of microtubules in the cell, the organization of microtubules into a mitotic spindle pole is a process that is superimposed onto the astral microtubule arrays in mitotic cells by a collection of noncentrosome accessory proteins. This concept is supported by a number of observations. First, meiotic spindles in many species have morphologically defined spindle poles but lack centrosomes (Rieder et al., 1993; McKim and Hawley, 1995; Vernos and Karsenti, 1995). Second, the minus ends of the microtubules converge into a morphologic pole within the mitotic spindle, but many of the minus ends of the microtubules terminate before reaching the centrosome and do not extend into the pericentriolar material (Rieder, 1981; Mastronarde et al., 1993). Third, the polar ends of the mitotic spindle remain organized, and in some instances fully functional, after the mechanical severing of the centrosome (Nicklas, 1989; Nicklas et al., 1989). Fourth, the minus ends of microtubules must be organized within the mitotic spindle pole, yet they must be able to detach from the centrosome so they can exchange tubulin subunits during the process of poleward microtubule flux (Mitchison, 1989a; Sawin and Mitchison, 1991). Finally, under experimental conditions where either NuMA or cytoplasmic dynein are prevented from associating with microtubules in the mitotic cell, the centrosomes continue to function normally by nucleating microtubules into astral arrays, but these centrosome-associated asters do not form morphologically identifiable spindle poles and remain detached from the bulk of the microtubules (Gaglio et al., 1995; Echeverri et al., 1996). Thus, the functional activities of proteins such as NuMA, Eg5, and cytoplasmic dynein are necessary to convert an astral array of microtubules nucleated from a centrosome into a functional mitotic spindle pole.

The idea that the minus ends of microtubules in the mitotic spindle are organized in a centrosome-independent manner by a collection of accessory proteins that includes significant minus end–directed motor activity raises the possibility that the mitotic spindle in vertebrate cells is organized, in part, in an "inside-out" fashion (i.e., from the chromosomes toward the centrosomes). This method of organizing the microtubules into a morphological pole is based on the mechanism of spindle assembly in meiotic cells that lack centrosomes (Theurkauf and Hawley, 1992; McKim and Hawley, 1995; Vernos and Karsenti, 1995; Matthies et al., 1996). During the assembly of meiotic spindles in some systems, the microtubules initially form loosely organized arrays around the chromatin. Then, presumably through the actions of minus end–directed microtubule motors (as well as chromatin-associated plus end–directed motors; Afshar et al., 1995; Vernos et al., 1995; Wang and Adler, 1995), the minus ends of the microtubules converge into a morphologically recognizable pole. This type of motor-driven convergence of microtubule minus ends also appears to operate in our cell free system for assembly of mitotic asters. Here, addition of purified dynactin to a dynactin-depleted mitotic extract after microtubule polymerization allows parallel bundles of microtubules to converge into mitotic asters (Fig. 7). While it is intuitively obvious why a meiotic cell lacking centrosomes would adopt this mechanism for the assembly of a bipolar spindle, the contribution of such a mechanism to the organization of a somatic mitotic spindle containing a centrosome is unclear. We can only point out that the detachment of the centrosomes from the body of the mitotic spindle that is induced by perturbations of either NuMA or cytoplasmic dynein (Fig. 9 in Gaglio et al., 1995; Figs. 6 and 7 in Echeverri et al., 1996) may be the manifestation

of the lack of this "inside-out" mechanism of mitotic spindle assembly in vertebrate cells (refer to Endow et al., 1994 for the description of a similar phenotype in nonvertebrate cells). Indeed, given that centrosome movements in vertebrate cells are intrinsic to each aster (Waters et al., 1993) and that mitotic centrosomes have been shown to "shed" microtubules (Belmont et al., 1990), it is possible that this minus end–directed activity is necessary to promote the assembly of a complex at the polar end of the mitotic spindle that sustains the association between the centrosome and the body of the mitotic spindle.

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