

Cytoplasmic Dynein Plays a Role in Mammalian Mitotic Spindle Formation

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Abstract. The formation and functioning of a mitotic spindle depends not only on the assembly/disassembly of microtubules but also on the action of motor enzymes. Cytoplasmic dynein has been localized to spindles, but whether or how it functions in mitotic processes is not yet known. We have cloned and expressed DNA fragments that encode the putative ATP-hydrolytic sites of the cytoplasmic dynein heavy chain from HeLa cells and from *Dictyostelium*. Monospecific antibodies have been raised to the resulting polypeptides, and these inhibit dynein motor activity in vitro. Their injection into mitotic mammalian

cells blocks the formation of spindles in prophase or during recovery from nocodazole treatment at later stages of mitosis. Cells become arrested with unseparated centrosomes and form monopolar spindles. The injected antibodies have no detectable effect on chromosome attachment to a bipolar spindle or on motions during anaphase. These data suggest that cytoplasmic dynein plays a unique and important role in the initial events of bipolar spindle formation, while any later roles that it may play are redundant. Possible mechanisms of dynein's involvement in mitosis are discussed.

THE segregation of chromosomes during mitosis is accomplished by a series of temporally and spatially organized movements. In mammalian cells the most prominent of these events are the separation of the spindle poles in prophase, chromosome attachment to the spindle followed by congression to its equator during prometaphase, movement of chromosomes toward the poles in anaphase A, and spindle elongation in anaphase B. All these movements are to some extent microtubule dependent, and at least some of them appear to be mediated by microtubule-dependent mechanochemical enzymes. Some members of the kinesin-like protein superfamily of mechanoenzymes have been localized to the mitotic spindle and are thought to be involved with the motile events during spindle formation and function (reviewed in Sawin and Scholey, 1991; McIntosh and Pfarr, 1991).

Cytoplasmic dynein is a distinct motor enzyme, also capable of generating movement along microtubules (Paschal et al., 1987; Lye et al., 1987). It has been localized to membranous vesicles, including lysosomes in interphase cells (Lin and Collins, 1992) and vesicles in axonal processes (Hirokawa et al., 1990). These localizations, and some in vitro experiments (Schnapp and Reese, 1989; Schroer et al., 1989; Lacey and Haimo, 1992) suggest that cytoplasmic dynein

acts as a motor for vesicle transport. During the onset of mitosis, dynein distributes to the mitotic spindle and to the kinetochores of chromosomes (Steuer et al., 1990; Pfarr et al., 1990), suggesting that it may also play a role in mitosis. Certainly a number of indirect observations have implicated a minus end directed microtubule motor in several mitotic events (reviewed in McIntosh and Hering, 1991). While dynein's localization is consistent with many long-held ideas of how mitosis may "work," there is little direct evidence of dynein's contribution to mitotic events.

To investigate the functional roles of cytoplasmic dynein in the mitotic apparatus, we have generated two preparations of affinity-purified antibodies that inhibit dynein's action in vitro. In this paper we describe the microinjection of these antibodies into mammalian tissue culture cells and their effect on the formation and functioning of the mitotic spindle.

Materials and Methods

DNA Cloning Techniques and Antigen Expression

Unless otherwise indicated, the molecular methods were performed as described in Sambrook et al., (1989). Sequencing was performed using the dideoxy chain termination method and Sequenase 2.0 (U.S. Biochem. Corp., Cleveland, OH). The 2085-bp fragment of *Dictyostelium* cytoplasmic dynein heavy chain (CDHC)¹ was obtained by a BglII digestion of a λ -gt11 clone (Koonce et al., 1992).

1. *Abbreviations used in this paper:* CDHC, cytoplasmic dynein heavy chain; DHC, dynein heavy chain.

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For Northern blotting, 1 μ g of poly (A)⁺ RNA from HeLa cells (see below) was separated on a 0.8% formaldehyde-denaturing agarose gel, transferred to nitrocellulose, then hybridized with labeled DNA. Hybridization for Northern and Southern blotting was performed using the ECL kit (Amersham Corp., Arlington Heights, IL) as suggested by the manufacturer, except that ³²P-labeled probe was used instead of the peroxidase-DNA conjugate.

Preparation of cDNA and the PCR

A 438-bp clone (Hp22) of human CDHC, was generated by reverse transcription of HeLa mRNA, followed by PCR (Compton, 1990). Two degenerate primers were used: (a) TATGGATCC(T/C)TNACNGA(T/C)(A/C)-GNTG(T/C)TA, which encodes the LTDRCY sequence common to sea urchin flagellar and *Dictyostelium* cytoplasmic DHCs and contains an engineered BamHI restriction site at its 5' end; and (b) CACGAATCCC-NGC(A/G)TANCCNGG(A/G)TTCA, which encodes the MNPYAG sequence and contains an EcoRI site at its 5' end. Restriction enzyme recognition sites were included to facilitate subsequent identification and subcloning of PCR DNA products.

Poly(A)⁺ mRNA was purified from HeLa cells using a Fast Track mRNA purification kit (Invitrogen, San Diego, CA). 1 μ g of mRNA was reverse transcribed with AMV reverse transcriptase (Promega, Madison, WI) in a Taq polymerase buffer, supplemented with 2 mM MgCl₂, 0.5 mM of each of four deoxynucleotides, 0.5 U of RNasin (Promega) and with a sequence-specific degenerate oligonucleotide primer (primer 2). After the completion of the reverse transcription, AMV polymerase was inactivated by heating to 95°C for 5 min, then the second sequence-specific oligonucleotide primer (primer 1) and Taq polymerase (Promega) were added directly to the reaction mixture. Five cycles of amplification were performed with an annealing temperature of 37°C, followed by 30 cycles with annealing at 48°C. The entire reaction product was electrophoresed on an agarose gel, and a band of ~440-bp was excised, glass purified, and cloned into the pCR1000 vector (Invitrogen) according to the manufacturer's instructions. Resulting clones were analyzed for presence of BamHI and EcoRI restriction sites, and only those containing both of these sites were selected for further analysis.

Production of Antibodies and Immunoblotting

For antibody preparation, *Dictyostelium* and human DNA sequences were subcloned into the pET5c expression vector, and protein was expressed in the lysogenic *Escherichia coli* host BL23(DE3) (Studier et al., 1990).

Inclusion bodies containing the expressed protein were isolated as described by Lin and Cheng (1991) and fractionated by gel electrophoresis (Laemmli, 1970). After electrophoresis, proteins were transferred from the polyacrylamide gel to nitrocellulose. Bands of expressed proteins were excised, air dried, dissolved in a minimum volume of DMSO, then mixed with an equal volume of Freund's adjuvant, and used to immunize rabbits.

For affinity column preparation, inclusion bodies were washed with water and solubilized in 100 mM Na-borate buffer, pH 9.0, containing 0.5% SDS. Solubilized protein was coupled to BrCN-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) at room temperature according to the manufacturer's protocol, except that 0.5% SDS was present in the coupling buffer and during the subsequent washes. Affinity chromatography was performed as described by Harlow and Lane, (1988). Fractions containing antibodies were immediately neutralized with 1 M tris/Cl, pH 8.0, and diluted with 5 vols of 0.1 M tris/Cl, pH 8.0. The IgG fractions of affinity purified antibodies and of the preimmune antisera were purified on a column of protein A-Sepharose (Pharmacia Fine Chemicals). Purified immunoglobulins were concentrated and transferred into 50 mM potassium glutamate, 0.5 mM glutamic acid, 0.5 mM MgCl₂, pH 6.5, using a Centricon 30 device (Amicon Corp., Danvers, MA).

For Fab₁ preparation, IgGs from Ddl serum were purified on a column of protein A-Sepharose CL-4b (Pharmacia Fine Chemicals) and digested with papain (1:100 wt/wt) in 100 mM tris/HCl, pH 8.0, 1 mM DTT at 37°C until no intact IgG heavy chain was visible on an overloaded Coomassie blue-stained SDS-polyacrylamide gel. The reaction was stopped by addition of iodoacetamide (Sigma Chemical Co., St. Louis, MO) to 20 mM, followed by incubation for 30 min at room temperature. Fab fragments were further purified by affinity chromatography and concentrated as described above.

Preparation of Dynein from HeLa Cells and In Vitro Motility Assay

HeLa cell extract, microtubule pellet and ATP extract from the microtubule

pellet were prepared as described by Hollenbeck (1989) for chicken brain, except that 20 μ M taxol was present during ATP extraction, and microtubules were removed from the extract by centrifugation at 150,000 g for 45 min. For dynein preparation, an ATP extract was prepared as described above, except that AMPNP was omitted. Dynein was further purified by 5–20% sucrose gradient centrifugation, and motility assays were performed as described (Lye et al., 1987). To assay antibody inhibition, coverslips with preadsorbed dynein were incubated with the antibody for 15 min at room temperature in a humid atmosphere and rinsed with buffer before addition of taxol-stabilized microtubules and ATP. Microtubule gliding rates were measured using the Metamorph image processing system (Universal Imaging Corporation, West Chester, PA). Displacements during four 5 s intervals were averaged to obtain a velocity for each microtubule.

Cell Culture and Microinjection

HeLa cells were grown in spinner culture, using MEM (Sigma Chemical Co.) supplemented with 7% horse serum (GIBCO BRL, Gaithersburg, MD), 100 U/ml of penicillin G and 100 μ g/ml of streptomycin sulfate.

PtK₁ cells were grown at 37°C in Ham's F-12 medium supplemented with 50 U/ml of penicillin G and 50 μ g/ml of streptomycin sulfate antibiotics. Cells were plated on etched-glass coverslips (Bellco Glass, Inc., Vineland, NJ) and allowed to grow for 24–36 h before microinjection. Microinjections were performed as described by Nislow et al. (1990). Antibodies were transferred into buffer containing 50 mM potassium glutamate, 0.5 mM glutamic acid and 0.5 mM MgCl₂, pH 6.5. For some experiments fluorescent dextran (FITC-dextran, 10 kD; Sigma Chemical Co.) was included in the injection buffer to a final concentration of 1 mg/ml to simplify identification of injected cells. Dextran fluorescence was abolished after fixation with methanol or permeabilization with saponin (see below), and thus did not interfere with subsequent staining of cells for immunofluorescent microscopy.

For direct observation of mitotic cells immediately after injection, coverslips with injected cells were placed on slides with parafilm spacers, sealed with wax, leaving gaps for medium changes, and kept on the microscope stage at 35 \pm 1°C. Cells were imaged with a Zeiss Photomicroscope II (Carl Zeiss, Inc., Thornwood, NY) equipped with phase-contrast optics and an MTI NC-67M video camera (DAGE-MTI, Inc.), connected to S-VHS recorder (HS-423UR, Mitsubishi). The medium in the chamber was replaced every 30 min.

For nocodazole disruption experiments, cells were incubated at 0°C for 45–55 min in medium containing 10 μ g/ml nocodazole, then in nocodazole for 40–45 min at room temperature (while injections were performed). Drug was removed by several washes with fresh medium, and cells were allowed to recover in fresh medium at 37°C.

Immunofluorescent Microscopy

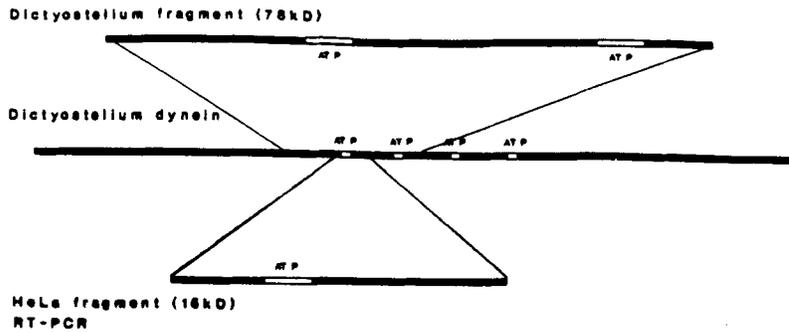
For immunofluorescence, cells were fixed with cold methanol (–20°C) for 6 min, or permeabilized with 0.05% saponin in 100 mM Pipes, 2 mM MgCl₂, 2 mM EGTA, 1 mM DTT, pH 6.9, for 5 min and fixed with 2% paraformaldehyde and 0.1% glutaraldehyde in the same buffer. Immunostaining was performed as described (Nislow et al., 1990). Centrosomes were visualized by staining with the Human CREST serum 5051 (Calarco-Gillam et al., 1983), kindly provided by Dr. Patricia D. Calarco (University of California, San Francisco, CA).

Results

Cloning of *Dictyostelium* and Human Dynein Heavy Chain Fragments

The predicted primary structure of CDHC from *Dictyostelium* (Koonce et al., 1992) contains regions with significant similarity to corresponding domains of sea urchin sperm flagellar β -dynein (Gibbons et al., 1991; Ogawa, 1991). The segment with the greatest similarity contains the putative site for ATP hydrolysis (Koonce et al., 1992). Such conservation suggests a functionally significant region of this protein, one that might elicit crossreactive and possibly inhibitory antibodies.

A 2.1-kb fragment of *Dictyostelium* CDHC cDNA that encodes the first two putative phosphate-binding consensus se-



A

	1					50
Human	LTDRCYLMT	QALEARIGGS	PFPGAGTGKT	ESVKALGHQL	GRFVLVFNCD	
Dictyostelium	-----L-	---S-M--N	-P-----	-T-----S-	-----C--	
Sea Urchin	-----I-L-	-S-HLVMS-A	-A-----	-TT-D--RA-	-IM-Y----S	
	51					100
Human	ETPDFQAMGR	IFVGLCQVGA	WGCFDEFNRL	EERMLSAVSQ	QVQCIQEALR	
Dictyostelium	-G--L---S-	-----C--	-----I-	---I-----	-I-T--V--K	
Sea Urchin	-QM-YKSC-N	-YK--S-T-	-----I	SVEV--V-AV	--K-V-D-I-	
	101					142
Human	EHSNPNYDKT	SAPITCELL	NKQVKVSPDM	AIFITMNPGY	AG	
Dictyostelium	-N-K.....EV---G	G-NISLHQ--	G--V-----	--	
Sea Urchin	DKKERFN...FM	GEEISLI-SV	G-----	--	

B

Figure 1. Cloning summary of the *Dictyostelium* and human (Hp22) cytoplasmic dynein heavy chain fragments (A) and alignment of predicted amino acid sequence of the human CDHC fragment and corresponding fragments of *Dictyostelium* cytoplasmic (Koonce et al., 1992) and sea urchin flagellar DHC (Gibbons et al., 1991; Ogawa, 1991) (B); identical amino acids relative to the HeLa sequence are represented as dashes. The consensus sequence for the P-loop of the ATP-binding site is underlined. The nucleotide sequence of the human cDNA clone Hp22 is available from GenBank under accession number L23958.

quences of the CDHC (Fig. 1) was subcloned into a protein expression vector and used to generate antigen for immunizations (Fig. 1).

The high degree of sequence conservation in this region of the protein also allowed us to design oligonucleotide primers with which to amplify and clone a corresponding fragment of a human DHC (Fig. 1). Two degenerate primers were synthesized—one encoding the LTDRCY sequence located 17 amino acids upstream from the first phosphate-binding consensus sequence (GPAGTGKT) of the *Dictyostelium* enzyme (Koonce et al., 1992). The second primer encoded MNPGYAG, a sequence located 95 amino acids downstream from the GPAGTGKT site. Reverse transcription was performed on poly(A)⁺ mRNA isolated from HeLa cells using the downstream oligonucleotide as a

primer; PCR was used to amplify the resulting cDNA. Products of the PCR reaction were cloned into the pCR1000 vector and sequenced (see Materials and Methods for details).

We examined 62 independent clones derived from four independent PCR reactions (made with 2 HeLa mRNA preparations). 31 clones contained an identical 438-bp sequence (Hp22) which encodes the amino acid sequence shown in Fig. 1 B. The encoded polypeptide is 70% identical and 84% similar to the corresponding region of the *Dictyostelium* CDHC (Fig. 1 B) and 100% identical to the corresponding region of CDHC from rat brain (Mikami et al., 1993). Four additional clones differed in only one nucleotide, and one differed in two nucleotides, presumably as a result of polymerase mistakes during PCR. The remaining clones showed no similarity to a CDHC sequence.

Northern blotting analysis of HeLa mRNA revealed a single band of ~15-Kb (Fig. 2). Southern blot analysis of HeLa genomic DNA probed with Hp22 DNA at either high or low stringency (Fig. 2 and not shown) was consistent with presence of a single hybridizing component in the human genome, if at least two introns are present in the genomic fragment corresponding to Hp22 cDNA clone.

Characterization of the Antibodies

Like the 2.1-kb *Dictyostelium* CDHC fragment, the 438-bp human DNA fragment was expressed in bacteria. Both resulting polypeptides were purified and used as immunogens to elicit antibodies in rabbits. The antibodies to the human peptide (Dh1) and to the *Dictyostelium* peptide (Dd1) were affinity purified on columns of their respective fusion proteins.

The specificity of Dd1 and Dh1 antibodies was assessed by immunoblotting against extracts of HeLa and PtK₁ cells. Both antibodies react with a single polypeptide that comigrates with sea urchin flagellar DHC (Fig. 3). This antigen

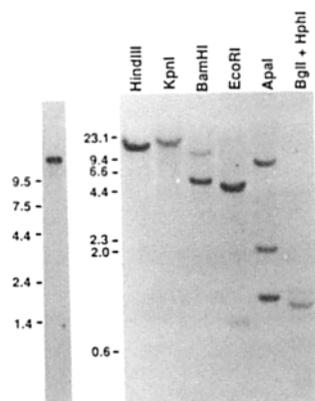


Figure 2. Northern (left panel) blot analysis of HeLa mRNA probed with Hp22 DNA and Southern (right panel) blot analysis of human genomic DNA probed with Hp22 DNA at high stringency. Similar results were obtained at low stringency (not shown). Positions of molecular weight markers (kb) are indicated on the left on each figure. Restriction enzymes used for the digestion of genomic DNA are listed at the top of each lane on the right panel. Hp22

cDNA clone does not contain sites for HindIII, KpnI, BamHI, EcoRI, ApaI, and BglI enzymes. Digestion with HphI should result in the production of three fragments of 389, 33, and 6 bp.

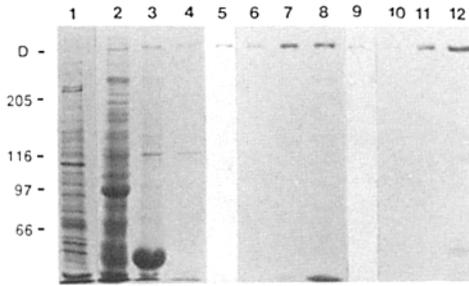


Figure 3. Immunoblot analysis with the Ddl and Dh1 antibodies. (Lanes 1–4) Coomassie blue-stained SDS-polyacrylamide gel; (lanes 5–8) immunoblotting with Ddl antibody and 9–12-Dhl antibody. (Lanes 1, 5, and 9) PtK₁ cell extract; (lanes 2, 6, and 10) HeLa cell extract; (lanes 3, 7 and 11) HeLa microtubule pellet; (lanes 4, 8, and 12) ATP-extract of HeLa microtubule pellet. Positions of molecular weight markers and sea urchin flagellar dynein (D) are indicated on the left. Kinesin heavy chain is represented as a major band of ~120 kD on lanes 3 and 4.

binds to microtubules in the absence of ATP and is released by Mg-ATP, properties characteristic of dynein (Paschal et al., 1987; Lye et al., 1987). Minor bands of lower molecular weight were seen with both antibodies in ATP-extracted material (Fig. 3, lanes 8 and 12). These bands were not observed in whole cell extracts or microtubule pellets and were more evident after longer extraction of the microtubule pellets with ATP-containing buffer (not shown), suggesting that they resulted from partial hydrolysis of CDHC during extraction of microtubule pellets at 37°C. Interestingly, the pattern of these minor bands differed for the two antibodies, implying that they react with different epitopes on the human cytoplasmic dynein heavy chain molecule.

Neither Ddl nor Dh1 antibodies reacted with the kinesin present in the preparations (Fig. 3). Immunostaining of PtK₁ cells with Ddl and Dh1 antibodies showed localization patterns similar to those previously described for other anti-

dynein antibodies (Pfarr et al., 1990; Steuer et al., 1990). During interphase and early prophase, the antibodies revealed punctate staining all over the cytoplasm and bright staining of the centrosomal region. No association of dynein with microtubules was observed in interphase cells. During progression through prophase and prometaphase, increasing amounts of dynein were localized at the poles of the forming spindle and on the spindle microtubules. The kinetochores of condensed chromosomes were also clearly stained (not shown).

We characterized the ability of affinity purified Ddl and Dh1 antibodies to inhibit the motor activity of dynein purified from HeLa cells by assessing their effect on dynein-driven microtubule motility in vitro (Vale et al., 1985; Lye et al., 1987). At 1.2 mg/ml the Ddl antibody blocked such movements, while 0.6 mg/ml had little effect. Increasing concentrations of Dh1 antibody gradually decreased the rate of microtubule gliding, and almost complete inhibition was achieved at 8 mg/ml (Fig. 4). Thus both antibodies are capable of inhibiting dynein's motor activity, with Ddl antibody displaying inhibition at a concentration significantly lower than that required for the Dh1 antibody.

Neither antibody appeared to affect microtubule binding to dynein-coated glass, suggesting that they do not interfere with dynein's binding to microtubules (not shown). Neither set of preimmune antibodies had an effect on microtubule binding or motility, even when they were purified on protein A columns and concentrated in the same way and to the same extent as Ddl and Dh1 antibodies.

Injection of the Antibodies Blocks Bipolar Spindle Formation

PtK₁ cells at different stages of mitosis were injected with the IgG fraction of affinity purified anti-dynein antibodies and incubated at 37°C for different periods of time (usually 3 to 5 h) before fixation and immunostaining. Comparable cells were prepared as controls by injection with buffer, or with the IgG fraction of preimmune antisera purified and

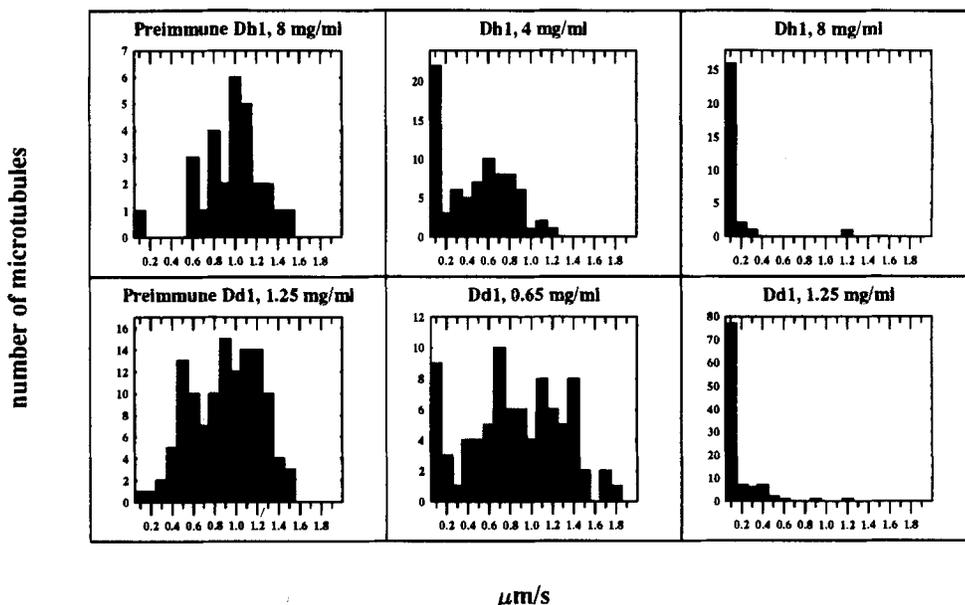


Figure 4. Effect of the antibodies on microtubule motility in vitro. The graphs represent the rate distribution of microtubule movement along dynein-coated coverslips pretreated with Ddl, Dh1, or preimmune antibodies. Antibody concentration is indicated on each graph. Motility assays with cytoplasmic dynein purified from HeLa cells were performed as described in Materials and Methods. Antibodies were applied to dynein-coated coverslips for 15 min before application of microtubules and ATP, and the rate of an individual microtubule's gliding was measured.

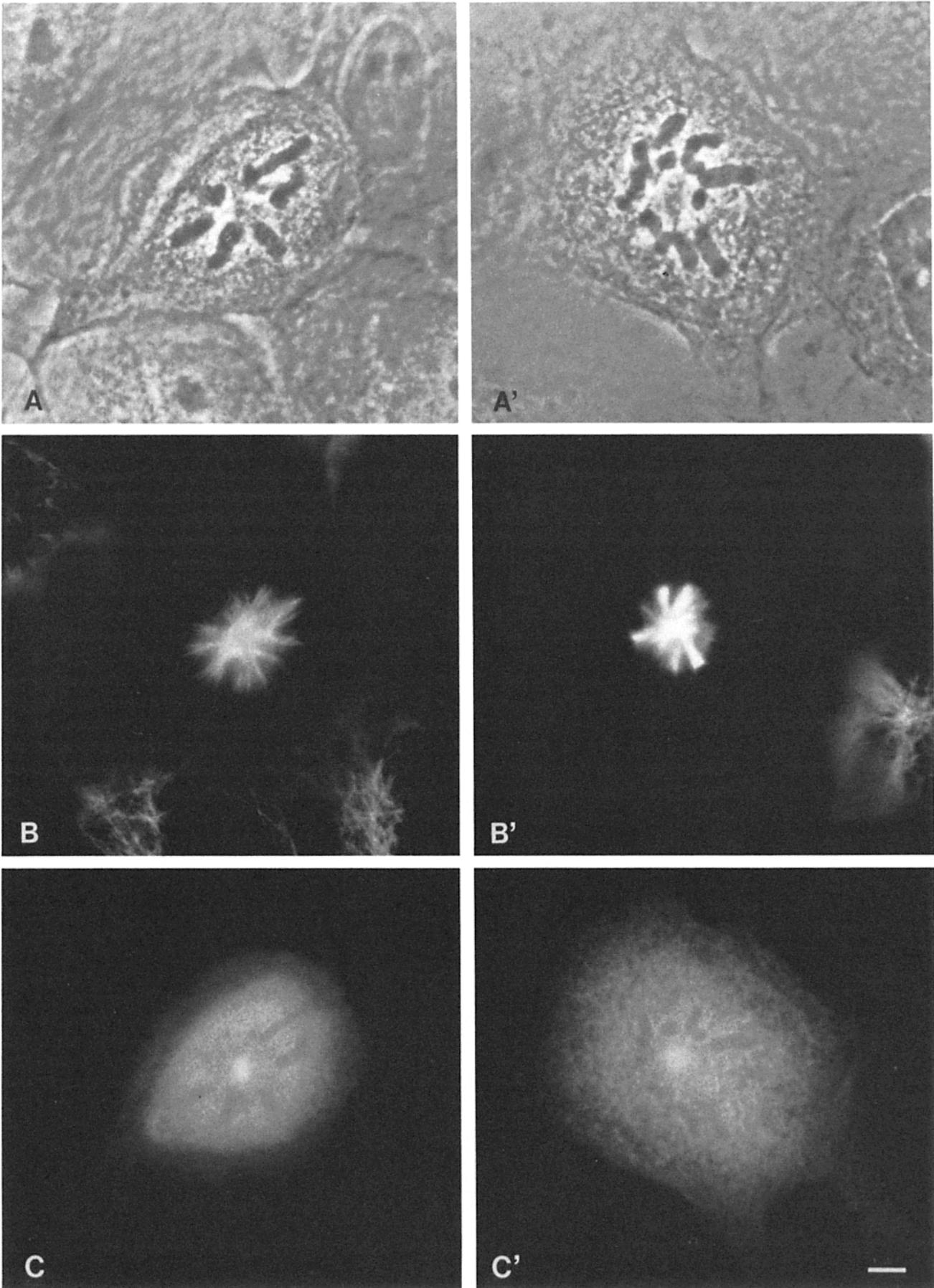


Figure 5. Effect of anti-dynein antibodies on mitotic progression in PtK1 cells. The two cells shown were injected during prophase with 12 mg/ml of Dd1 antibody, incubated at 37°C for 3 h after injection, then fixed and indirectly stained with a mouse anti-tubulin antibody and directly stained with a Texas red-labeled goat antibody against rabbit immunoglobulins (to reveal distribution of injected antibody); (*A* and *A'*) phase-contrast images; (*B* and *B'*) anti-tubulin staining; (*C* and *C'*) anti-rabbit IgGs staining. Bar, 2.5 μ m.

concentrated in the same way as the immune antibodies (see Materials and Methods). During that period all cells that were injected with buffer or preimmune antibody completed mitosis.

Mitotic progression was blocked in 73% of the prophase PtK₁ cells injected with Dd1 at 12 mg/ml ($n = 59$). Similarly 30% of the prophase cells injected with Dh1 at 20 mg/ml failed to continue mitosis ($n = 10$). Injection of preimmune antibodies at the same concentrations had no effect on spindle morphology or mitotic progression. Lower concentrations of antibodies (6 mg/ml of Dd1 or 10 mg/ml of Dh1) also had no obvious effect on mitotic progression (not shown).

All the arrested cells displayed a similar morphology: a radial array of microtubules emanated from a single focus, surrounded by a ring of condensed chromosomes (Fig. 5). In many but not all of the arrested cells, some of the microtubules formed bundles resembling kinetochore fibers (compare the two cells on Fig. 5). These bundles seemed to connect the unseparated centrosomes with the chromosomes, indicating that injected cells retained, at least to some extent, the ability to establish centrosome-chromosome connections. The arrested cells maintained condensed chromosomes for at least 16 h; not once did these cells recover and divide or reenter interphase. Injected antibody was evenly distributed throughout most of the cytoplasm but showed some concentration at the centrosomal region. There was no visible association of the injected antibody with the microtubules of monopolar spindle (Fig. 5, *c* and *c'*). The anticrosomal serum 5051 (Calarco-Gillam et al., 1983) stained one or two small spots at the center of the single aster (not shown). The diameter of the area stained was invariably $<2.5 \mu\text{m}$.

While the injected dynein antibodies appeared to block early events in spindle formation, they did not prevent a cell's entry into mitosis. When interphase cells were injected with 12 mg/ml Dd1 antibodies, 3 out of 10 cells entered mitosis during the subsequent 24 h, whereupon they arrested with the morphology characteristic of cells injected during prophase (not shown). During the same time 3 out of 10 cells injected with preimmune antibodies entered and successfully completed mitosis.

To address the possibility that the defects in spindle pole separation and spindle formation were a result of dynein's crosslinking or precipitation by bivalent antibodies, we prepared Fab₁ fragments of the Dd1 antibody. When these were injected into prophase PtK₁ cells at 8.1 mg/ml, they too caused the formation of monopolar spindles and mitotic arrest (6 out of 15 injected cells).

Injection of the Antibodies Does Not Affect Chromosome Motility during Prometaphase and Anaphase

Cells injected with either Dd1 or Dh1 antibody during prometaphase, metaphase, or anaphase completed mitosis with no detectable delay. To look for subtle effects of dynein antibody on chromosome motility, ten cells were injected with Dd1 antibody during prometaphase and imaged continuously until the completion of mitosis. Recorded video images were later analyzed for perturbation of the timing and rates of the various mitotic stages. We could detect no changes in chromosome motility in these cells, as compared

to noninjected cells or cells injected with buffer. Prometaphase was normal; while the timing of anaphase onset was variable, it was similar for injected and control cells; and anaphase A and B were normal in both their rates and extent (data not shown).

Antibodies Prevent Normal Spindle Formation during Recovery from Microtubule Depolymerization

The experiments described above suggest that dynein plays an essential role in producing a bipolar spindle. To investigate whether dynein might also contribute to spindle reformation during later mitotic stages, we treated cells with nocodazole (10 $\mu\text{g/ml}$) and cold (0°C) for 1 h to disassemble the cell's microtubules, then we microinjected affinity purified Dd1 antibody (12 mg/ml) into cells whose chromosome arrangement identified them as prophase, prometaphase, and metaphase. After injection, cells were placed in fresh medium lacking the drug and returned to 37°C for 3–5 h. During that time all noted mitotic cells that were not injected or were injected with preimmune antibodies ($n = 73$) restored their spindle and completed mitosis.

59% of the drug and cold-treated cells injected during prophase ($n = 44$) became arrested with a morphology almost indistinguishable from untreated cells injected with Dd1 antibody during prophase. Surprisingly, however, 57% of the drug and cold treated cells injected during prometaphase and metaphase ($n = 103$) arrested with the morphology seen after prophase injection (Fig. 6). The only detectable difference from cells injected during prophase was a slightly more rounded cell shape. As with cells arrested after prophase injection, staining with anticrosomal serum revealed a spot (or sometimes two closely spaced spots) at the center of the microtubule array (Fig. 6 *c*). This result shows that dynein plays an important role in the initial stages of spindle morphogenesis, whether it occurs during a normal prophase or during recovery from microtubule disruption.

Injection of Anti-Dynein Antibodies Early in Spindle Formation Leads to a Collapse of the Interpolar Distance

There are two major pathways that might plausibly lead to the formation of a monopolar spindle after anti-dynein's injection. One possibility is that dynein is involved in the earliest stages of spindle pole separation and is not necessary after this separation has been accomplished. Another is that dynein activity is necessary not only at the onset of pole separation, but at later stages as well, probably until the central spindle forms during prometaphase. The first scenario predicts that bipolar spindle formation will be prevented only if antibody injection occurs before centrosome separation. In the second case, the antibody should cause a collapse of the distance between previously separated centrosomes. To distinguish between these possibilities we have measured the intercentrosomal distance in uninjected PtK₁ cells during prophase or in prometaphase and metaphase cells treated with 10 $\mu\text{g/ml}$ nocodazole at 0°C for 1 h, as well as in uninjected cells during recovery from nocodazole treatment (Fig. 7).

In 82% of the untreated and uninjected prophase PtK₁ cells, centrosomes were separated by a distance $>2.5 \mu\text{m}$ ($n = 73$; Fig. 7 *A*). In prophase cells arrested by dynein anti-

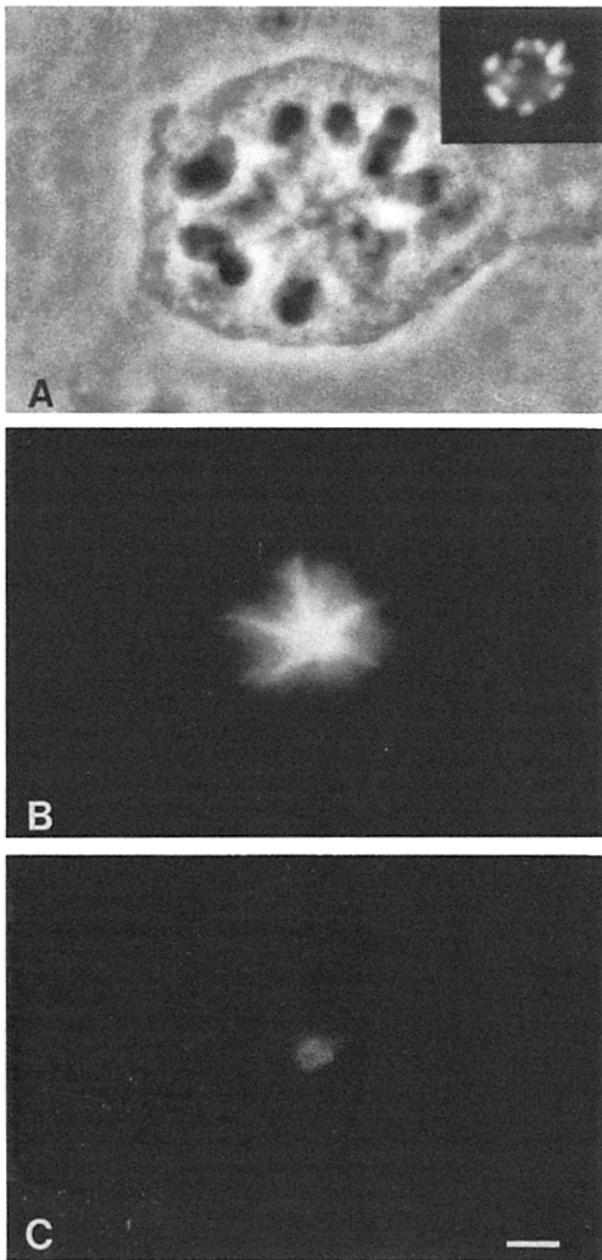


Figure 6. Effect of antibody injection on spindle formation in cells pretreated with a microtubule depolymerizing regimen. The cell was incubated for 60 min in 10 $\mu\text{g/ml}$ nocodazole at 0°C, injected during middle-late prometaphase with 12 mg/ml of Ddl antibody in the presence of nocodazole, and incubated at 37°C in nocodazole-free medium for 3 h before fixation. (A) phase-contrast image; (B) antitubulin staining; (C) staining with anticentrosomal serum (5051). (A, inset) DNA, revealed by DAPI staining. Bar, 2.5 μm .

bodies, on the other hand, centrosomal staining was confined to a region with a diameter $<2.5 \mu\text{m}$ ($n = 20$). If the anti-dynein antibody was able only to prevent centrosome separation but not to reverse it, the maximal number of cells with a monopolar spindle should have been $<18\%$, while the observed number of cells arrested after injection of Ddl antibody during prophase was $>70\%$ (see above). These results

imply that the antibody injection causes a collapse of the intercentrosomal distance.

This interpretation is corroborated by results obtained from cells treated with nocodazole before anti-dynein injection. In a majority of these uninjected prometaphase and metaphase cells, the distance between centrosomes immediately after incubation with the drug (Fig. 7 B) and during the recovery from drug treatment (Fig. 7 C) is significantly greater than the maximal diameter of the region stained with anticentrosomal antibody in cells arrested upon anti-dynein injection. Thus, injection of anti-dynein prior to recovery from microtubule depolymerization leads to a decrease of the distance between centrosomes, just like that seen for cells injected during prophase.

Discussion

We have used DNA sequences that encode portions of the heavy chains from two cytoplasmic dyneins to express polypeptides likely to be part of this enzyme's hydrolytic site. Antibodies to these protein fragments block dynein's motility in vitro, and thus may serve as experimental tools with which to probe aspects of dynein's function in cells. Injection of these antibodies into cultured PtK₁ cells causes the two centrosomes of a forming spindle to collapse toward one another. These results are surprising because the maintenance of centrosome separation might have been thought to depend on a plus end-directed, microtubule cross-linking motor enzyme, not a minus end directed motor like dynein. Furthermore, the injections failed to perturb chromosome attachment to the spindle, prometaphase congression to the spindle equator, and the motions of anaphase A and B. Since dynein has been localized to kinetochores as well as to the centrosomes, our results pose as many questions as they answer. In the discussion that follows we address several points relevant to the data.

Cloning of a Human Cytoplasmic Dynein Heavy Chain Fragment

Comparisons between the primary structure of flagellar dynein and cytoplasmic isotypes of the enzyme revealed regions of substantial similarity over the carboxy-terminal two thirds of the coding sequence, suggesting that this region comprises the mechanochemical domains of the protein. We have used such sequence information to identify a region in the *Dictyostelium* CDHC with a particularly high degree of identity to a flagellar DHC. It surrounds the first P-loop in the cytoplasmic sequence, which is likely to represent an important functional domain for ATP hydrolysis in this protein (Gibbons et al., 1991; Ogawa, 1991).

Degenerate PCR, using highly conserved primer sites in this region, has led to the identification of a fragment of a DHC expressed in HeLa cells. HeLa cells lack axonemes, save those that might be found in primary cilia, which lack dynein arms (reviewed in Sorokin, 1982). As expected, the HeLa sequence is more similar to CDHCs than to DHCs from flagellar.

Degenerate PCR with conserved primers has revealed an increasingly large family of kinesin-like proteins in a number of diverse organisms (for example see Endow and Titus, 1992). One might imagine that this approach would identify different isotypes of cytoplasmic dynein if they were ex-

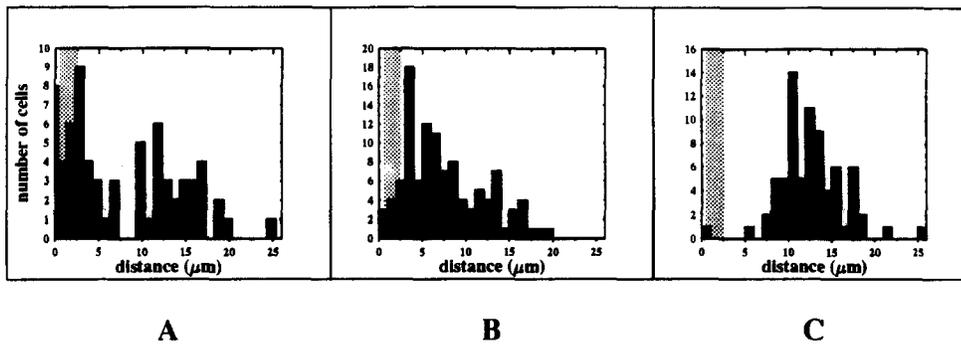


Figure 7. Distance between centrosomes in uninjected PtK₁ cells. Cells were fixed with cold methanol and stained with 5051 antiserum (Calarco-Gillam et al., 1983). Cells at different stages of mitosis were identified using phase-contrast microscopy, and the distance between centrosomes in these cells was measured. (A) Untreated prophase cells; (B) prometaphase and metaphase cells after

15 min recovery from nocodazole treatment (when the distribution of microtubules in both mitotic and interphase cells is mostly restored). Shaded areas represent the maximum diameter of the anticentrosome-reactive area in cells arrested upon antibody injection.

pressed in HeLa cells. However, sequence analysis of multiple independent clones derived from several PCR reactions revealed only one dynein heavy chain isotype in HeLa cells. The most likely interpretation of this result is that only one CDHC is expressed in HeLa cells. There are, however, alternative explanations for our results. It is possible, for example, that the sequence conservation in the region used for primer design is not preserved among different dynein isoforms. Alternatively, the level of isotype expression may differ significantly, and the less abundant isoforms may have escaped screening. Further study using different molecular biological, genetical and/or biochemical approaches may clarify the question of whether there are different isoforms of cytoplasmic dynein in a single cell type.

Effect of Dynein Inhibition on Centrosome Separation and Spindle Formation

The ability of two independently raised, affinity-purified antibodies to cause a seemingly identical defect in spindle formation strongly suggests that their effect is due to their interaction with dynein. The effect of injection was stronger with Dd1 antibody at 12 mg/ml than with Dh1 at 20 mg/ml, suggesting that Dd1 is more active in preventing spindle formation. This correlates with its greater potency in inhibiting dynein-driven motility *in vitro* (Fig. 4). Moreover, if we assume that the volume of solution injected was 10% of the cell volume (Zavortnik et al., 1983), the Dd1 antibody inhibited bipolar spindle formation at concentrations similar to those which inhibited dynein-driven microtubule motility *in vitro*. Furthermore, the same result was obtained with Fab fragments, suggesting that the effect is due to a specific inhibition, rather than to a precipitation or crosslinking of dynein.

The spindle morphology in cells affected by anti-dynein antibody suggests that the antibody interferes with the processes that cause centrosome separation at initial stages of spindle formation. However, our analysis of intercentrosomal distances, which shows that anti-dynein injections cause a decrease in intercentrosomal distances, suggests that dynein is also involved in the maintenance of centrosome separation at these early stages.

When injected into prometaphase or metaphase cells, antibodies had no obvious effect on spindle structure or chromosome behavior. Probably, the forming spindle, which by this time consists of bundles of kinetochore microtubules

and overlapping nonkinetochore microtubules originating from opposite poles, stabilizes the spacing between centrosomes. Thereafter, dynein activity may no longer be required to maintain the separation of the spindle poles. Experiments in which anti-dynein antibody was injected into cells with disassembled microtubules demonstrate, however, that the formation of a monopolar spindle can occur at prometaphase and metaphase as well as at prophase if experimental conditions require the spindle to form at these stages. Thus, dynein might contribute to the early stages of spindle formation whenever they occur.

Role of Dynein in Chromosome Motility

Antibody injection during prometaphase, metaphase or anaphase did not seem to affect chromosome motility. Neither immunofluorescent analysis, nor observation of live cells revealed any detectable abnormality. The lack of an effect of injected antibodies on prometaphase or anaphase chromosome movements can be interpreted to suggest that dynein plays no role in these mitotic events. However, the localization of dynein at the kinetochores of mammalian cells (Pfarr et al., 1990; Steuer et al., 1990) and the minus end-directed motility of chromosomes on microtubules, both *in vivo* (Rieder and Alexander, 1990) and *in vitro* (Hyman and Mitchison, 1991) has nurtured the idea that dynein is a motor for chromosome-pole movements. An alternative interpretation of our results is that cytoplasmic dynein is not the only motor involved in this kind of movement. Minus end-directed kinesin-like proteins have been found (Walker et al., 1990; McDonald et al., 1990), and Hyman et al. (1992) have failed to identify either dynein or kinesin-like proteins in preparations possessing minus end directed microtubule-translocator activity in association with centromeric DNA of *S. cerevisiae*. Thus, proteins other than dynein may contribute to minus end-directed chromosome motility during prophase and anaphase. Even if dynein normally plays a role in chromosome movement, other enzymes may complement its functions. An alternative possibility is that while dynein is essential for chromosome movements, the accessibility of dynein epitopes to antibodies is impeded in an assembled spindle. At present, we can not state that dynein does not play a role in chromosome movement, and we are currently working on other approaches to study possible roles of cytoplasmic dynein in chromosome motility.

Possible Roles of Cytoplasmic Dynein in Spindle Formation

The results from the microinjection experiments suggest that cytoplasmic dynein does play an essential role in the early stages of mammalian spindle formation. Presumably, inactivation of dynein by antibody leads to the collapse of separated spindle poles and therefore to a failure in the formation of a normal bipolar spindle. The collapse of centrosomes caused by dynein inhibition supports the idea that proper spindle pole separation is a result of a balanced action of inward and outward directed forces, perhaps generated by microtubular motors with opposite polarity (Saunders and Hoyt, 1992; Fuller and Wilson, 1992).

There are several ways in which a minus end-directed, microtubule-dependent motor enzyme might contribute to the process of spindle pole separation. One, which probably has the strongest support from the cytological data, involves a pulling on the poles by minus end-directed motors via astral microtubules (for example see Waters, 1993) (Fig. 8 A). Though initially proposed as a mechanism for pole separation during anaphase B, it is equally plausible as a mechanism for earlier mitotic events. The astral forces model is strongly supported by the observations that asters can separate when there is no array of overlapping microtubules between them and that asters can move independently of each other (Aist et al., 1991; Bajer, 1982; Hamaguchi and Hiramoto, 1986; Hiramoto and Nakano, 1988; Waters et al., 1993). The movement of microtubule asters could be achieved if a minus end-directed motor, such as dynein, was anchored either to the cell cortex or to some distributed cytoplasmic structure that could provide the mechanical support to allow a pulling on microtubules due to dynein's motor activity (Fig. 8 A). Direct cytological evidence for this association is yet to be obtained. However, it is known that dynein is distributed throughout the cytoplasm in interphase cells, and that it localizes to spindle microtubules during very early prophase (Pfarr et al., 1990; Steurer et al., 1990). It has also been shown that dynein is capable of generating sliding movements of free microtubules over each other in extracts of *Xenopus laevis* eggs (Verde et al., 1991). This sliding might occur due to dynamic association of dynein molecules with microtubules (Fig. 8 B). Such activity of dynein occurs in mitotic but not in interphase extracts, suggesting that it is probably needed for building and/or supporting some mitosis-related structure. The mechanism discussed below presents an additional way in which the properties of dynein can contribute to the formation of a central spindle and to the separation of spindle poles.

Our model posits that a microtubule sliding similar to one outlined on Fig. 8 B occurs between microtubules that are already attached to adjacent centrosomes. Such sliding would lead to a gathering of these microtubules into the space between the centrosomes, forming a central spindle, as shown in Fig. 8 C. If we make an additional assumption that each dynein molecule is (temporarily) attached to a fixed point on one microtubule while it slides along a microtubule growing from the other pole, a net outward-directed force is applied to the centrosomes, pushing them apart (Fig. 8 D). It is important to notice, however, that this force can be generated only when the angle between intersecting microtubules ($\angle LKM$ on Fig. 8 D) is $<90^\circ$ (which is true for most microtubules at early stages of pole separa-

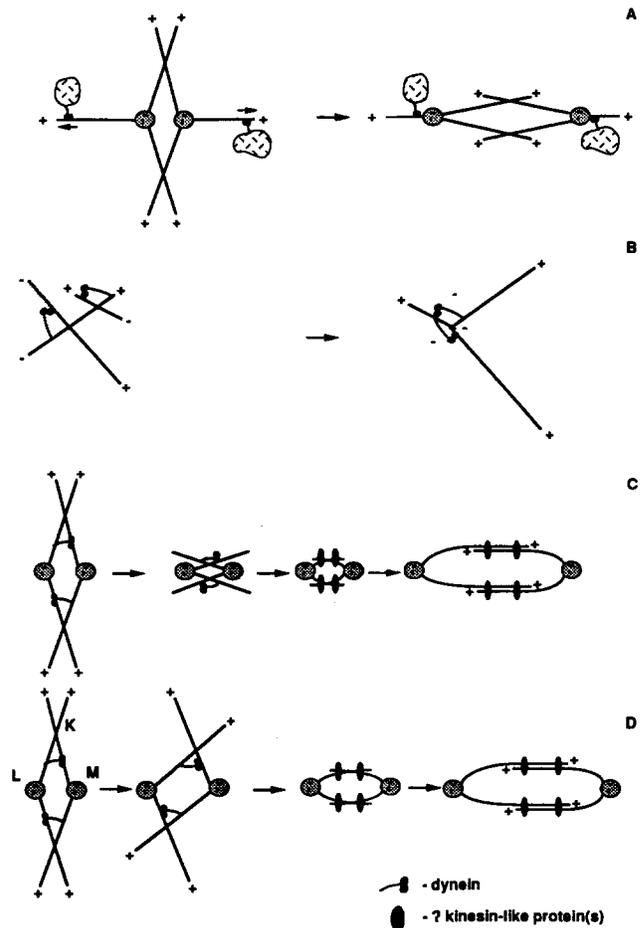


Figure 8. Models for spindle pole separation. (A) "Astral forces" model. Dynein anchored to cellular objects interacts with and attempts to move upon astral microtubules, generating a net outward force on the centrosome; (B) dynein-dependent aster formation in mitotic *Xenopus* extracts (adopted from Verde et al., 1991). Note, that for the sliding described here and below to occur, interaction between microtubules and dynein molecules should be dynamic, i.e., different populations of dynein molecules will be involved in sliding microtubules over each other at different times. For simplicity this feature is not reflected on the figure; (C) Sliding of microtubules originating from different spindle poles, analogous to that described in B, could lead to the formation of antiparallel microtubule arrays characteristic of central spindles; subsequent addition of plus end motors and changes of microtubule dynamics could contribute to spindle formation; (D) if a dynein molecule is temporarily attached to a fixed point on one microtubule (distance KL does not change for some time) while it slides along a microtubule from the other pole, the two closely spaced centrosomes will be forced apart (NB, diagram assumes that $\angle LKM$ is less than 90° , which is true for most microtubules at early stages of pole separation).

tion). This arrangement resembles to some extent the situation in axonemes, where flagellar dynein acts between parallel doublet microtubules.

Such a mechanism might act early in mitosis to help set up the interdigitating arrangement of microtubules. It also might work in conjunction with an "astral forces" mechanism. Other proteins, e.g., kinesin-like, plus end-directed motors, might then bind to the interdigitating microtubules, stabilizing the spindle and further separating the poles. We

are currently working on several approaches to confirm (or exclude) the involvement of sliding microtubules in the formation of the mitotic spindle.

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