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Kinesin-like molecules involved in spindle formation

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SUMMARY

To study the possible involvement of kinesin-like molecules in mitosis a polyclonal antibody against the head domain of *Drosophila* kinesin heavy chain (HD antibody) was microinjected into PtK_1 cells at the prophase-prometaphase transition. Progress of the cell through mitosis was recorded for subsequent detailed analysis. Cells injected with pre-immune IgG progressed through mitosis at rates similar to those for noninjected cells. After HD antibody injections, chromosomes failed to congress to an equatorial plane and cells failed to form a bipolar spindle. Rather, the spindle poles came together, resulting in a monopolar-like configuration with chromosomes arranged about the poles in a rosette. Sometimes the monopolar array moved to the margin of the cell in a way similar to anaphase B movement in normal cells. Antibody-injected cells progressed into the next cell cycle as evidenced by chromosome decondensation and nuclear envelope reformation. Anti-tubulin immunofluorescence confirmed the presence of a radial monopolar array of microtubules in injected cells. HD antibody stained in a punctate pattern in interphase and the spindle region in mitotic PtK_1 cells. The antibody also reacted with spindle fibers of isolated mitotic CHO spindles and with kinetochores of isolated CHO chromosomes. Immunoblotting indicated that the major component recognized by the antibody is the 120 kDa kinesin heavy chain. At higher protein loads the antibody recognized also a 34 kDa polypeptide in PtK_1 cell extracts, a 135 kDa polypeptide in a preparation of CHO spindles and a 300 kDa polypeptide in a preparation of CHO mitotic chromosomes. We conclude that a kinesin-like molecule is important for the formation and/or maintenance of the structure of mitotic spindle.

Key words: kinesin, microtubule motor, mitosis, spindle, microinjection

INTRODUCTION

The mitotic spindle is a complex structure that is necessary for equal distribution of genetic material between daughter cells. The main constituents of the mitotic spindle are microtubules, two spindle poles or asters and chromosomes with kinetochores. The assembly and function of the mitotic spindle can be described as a highly ordered sequence of movements of the spindle poles relative to each other and of chromosomes relative to the spindle poles. After nuclear envelope breakdown (NEB), chromosomes attach to astral microtubules and move to the nearest aster, then display 'congression' movement towards the spindle equator as well as oscillating towards and away from the poles and, finally, in anaphase, sister chromatids separate and move to the spindle poles. Asters, which sometimes locate close to each other, move apart to form the mitotic spindle after NEB and, at the onset of anaphase, spindle poles continue their separation (see McIntosh and Koonce, 1989; Mitchison, 1989; McIntosh and Hering, 1991; Rieder, 1991; Sawin and Scholey, 1991, for recent reviews).

Though the molecular mechanisms of movement of the spindle poles and chromosomes are not yet understood, there is now little doubt that microtubule-based translocator proteins must be involved in mitosis. Known microtubule motors include cytoplasmic dynein, which supports movement along microtubules towards their minus ends (Paschal and Valle, 1987) or towards the poles in terms of the spindle structure, and kinesin and the members of kinesin family of proteins, most of which support transport along microtubules in the opposite direction (reviewed by Endow, 1991). Kinesin itself has been shown to not be involved in mitosis at least in *Drosophila* cells (Saxton et al., 1991; Wright et al., 1993), but multiple kinesin-like proteins that share substantial sequence similarity with kinesin in its motor domain were demonstrated to be important for different mitotic events. A number of genes, encoding kinesin-like proteins involved in mitosis have been identified in lower eukaryotes. The most common effect of mutations in these genes was reported to be the failure of formation of a bipolar mitotic spindle. Mutation in the *bimC* gene, which encodes a kinesin-like protein, prevents the separation of the spindle pole bodies in *Aspergillus* (Enos and Morris, 1990). Similar effects have been described for

mutation in the *cut7* gene of *Schizosaccharomyces pombe* (Hagan and Yanagida, 1990), and double mutation in *cin8/kip1* genes of *Saccharomyces cerevisiae* (Hoyt et al., 1992; Roof et al., 1992; Saunders and Hoyt, 1992).

Recently, kinesin-like proteins, CENP-E (Yen et al., 1992) and MKLP-1 (Nislow et al., 1992), have been localized to mitotic spindles of mammalian cells as well and their significance for mitosis was suggested by antibody-blocking experiments (Yen et al., 1991; Nislow et al., 1990). However, the effect of injections of antibodies to CENP-E and MKLP-1 was different from the effect of genetic knock out of kinesin-like genes in yeast and *Aspergillus*; a bipolar spindle formed in injected mammalian cells but the cells were blocked in metaphase. These observations raise the possibility that multiple kinesin-like proteins serve different function in mitosis or that the functions of kinesin-like motor molecules in mitosis in the cells of lower eukaryotes and in mammalian cells are different.

To study further the role of kinesin-like translocators in mitosis in mammalian cells we used antibody blocking experiments. The HD antibody used in the present work was raised against the motor domain of *Drosophila* kinesin heavy chain. As the members of the kinesin family show a high degree of similarity in amino acid sequences of their motor domains (see Endow, 1991; Goldstein, 1991; for recent reviews) it seemed reasonable to use HD antibody for the study of involvement of kinesin-like proteins in mitosis. The results of microinjection experiments showed that, like the cells of lower eukaryotes, kinesin-like proteins are involved in mitotic spindle formation in mammalian cells.

MATERIALS AND METHODS

Tissue culture

PtK_1 and CHO cells were grown in HAM's F-10 medium supplemented with 10% fetal bovine serum (Hy Clone Laboratories, Logan, UT) and antibiotics. Cells were cultured on glass coverslips mounted with silicon vacuum grease (Dow Corning Corp., Midland, MI) in 35 mm tissue culture dishes. If the injected cells were used for immunostaining we used coverslips with photoetched locator grids (Belco, Vineland, NJ).

Microinjections

HD antibody was prepared and affinity purified as described previously (Rodionov et al., 1991). Preimmune IgG was precipitated from the serum by 50% saturation with ammonium sulfate and purified on a DEAE-cellulose column (Whatman DE-52, Whatman Instruments, Maidstone, UK). Both HD antibody and preimmune IgG were concentrated up to 20 mg/ml and stored at −70°C. Just prior to use a sample of the antibody was thawed and spun at 20,000 ***g*** for 30 minutes to remove particulates.

PtK_1 cells in late prophase with fully condensed chromosomes were selected for microinjection. The nuclear envelope often broke down during the manipulations or immediately after injection. Thus, all injections if not otherwise indicated were made during the prophase/prometaphase transition not earlier than 1 minute before and not later than 1 minute after NEB. Microinjections were performed by means of a Leitz micromanipulator (E. Leitz, Inc., Rockleigh, NJ) and an inverted microscope (Nikon Inc., Garden City, NY). During micromanipulation and subsequent observation, the temperature was kept at 37(±1)°C. Immediately after injection, oil (Wesson) was layered over the tissue culture medium to prevent evaporation. Phase-contrast images (×100 objective NA 1.3) of all injected cells, except a few cells that were definitely injured, were collected through a VENUS videocamera and recorded with an optical memory disk recorder (OMDR) (Panasonic, Sucaucus, NJ) as 8-frame averages in the time-lapse mode (1 frame/4 s) for the subsequent determination of the timing of individual stages of mitosis.

Preparation of CHO mitotic spindles and CHO mitotic chromosomes

CHO mitotic spindles were isolated by the method of Kuriyama et al. (1984). Mitotic chromosomes were obtained from vinblastine-blocked CHO cells using the polyamine method as described by Mitchison and Kirschner (1985), with minor modifications. Briefly, cells were blocked for 12 hours with 10 μM vinblastine, shaken off the substratum, and collected by centrifugation at 500 ***g*** for 10 minutes. Cells were then resuspended in 100 ml of a buffer containing 5 mM PIPES, pH 7.2 (adjusted with KOH), 5 mM NaCl, 5 mM $MgCl_2$, and 0.5 mM EDTA at 4°C. All subsequent steps were performed at 0 to 4°C. After 10 minutes of swelling cells were pelleted again at 500 ***g*** for 2 minutes and resuspended in a lysis buffer containing 10 mM PIPES, pH 7.2 (adjusted with KOH), 2 mM EDTA, 0.1% 2-mercaptoethanol, 1 mM spermidine HCl and 0.5 mM spermine HCl. The lysis buffer was saturated with digitonin prior to use by addition of digitonin up to 0.1%, stirring for 30 minutes at 4°C and pelleting the excess of unsolubilized detergent by centrifugation. A macroglobulin was added to the lysis buffer to a final concentration of 2 μg/ml just prior to use. Cells were then disrupted in a glass-glass Dounce homogenizer and cell debris was pelleted at 250 ***g*** for 1 minute. Chromosomes were then purified in a 20% to 60% sucrose gradient by centrifugation at 2,500 ***g*** for 15 minutes at 4°C. Sucrose solutions were prepared on 10 mM PIPES, pH 7.2 (adjusted with KOH), 1 mM EDTA, 0.1% 2-mercaptoethanol, 0.5 mM spermidine and 0.25 mM spermine, supplemented with 1 μM α-macroglobulin. Chromosomes formed a white flocculent mass at about 50% sucrose. Chromosomes were collected and either used immediately or stored in liquid nitrogen.

Mitotic spindles and chromosomes were pelleted at 20,000 ***g*** for 30 minutes and dissolved in SDS sample buffer or adsorbed on polylysine-coated coverslips by centrifugation at 500 ***g*** for 10 minutes and used for immunostaining.

Immunofluorescence microscopy

For immunostaining of cultured cells, we used the protocol of Steuer et al. (1990) with minor modifications. Briefly, cells were washed 3 times with PBS, 3 times with PEEM (0.1 M PIPES, pH 6.9, 1 mM $MgSO_4$, 0.1 mM EDTA, and 2 mM EGTA) and once with PEEM containing 1 mM dithiothreitol, and 10 μg/ml of each of the protease inhibitors (leupeptin, pepstatin, aprotinin, Na-*p*-tosyl-L-arginine methyl ester (TAME), and soybean trypsin inhibitor were added). Cells were either fixed immediately with methanol containing 1.0% formaldehyde at −70°C for 30 minutes or first permeabilized with PEEM supplemented with 1 mM DTT, the mixture of protease inhibitors, 10 μM taxol (a gift from Dr N. R. Lomax, Drug Synthesis and Chemistry Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD) and one of the following detergents: 0.5% Brij-58, 0.02% digitonin or 0.01% saponin. Coverslips were then rehydrated in TTBS (20 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 0.01% sodium azide and 0.05% Tween-20) and incubated overnight in TTBS with 10% normal goat serum at 4°C.

CHO chromosomes were fixed with 1% formaldehyde for 30 minutes, adsorbed on polylysine-coated coverslips by centrifugation at 500 ***g*** for 10 minutes and postfixed with methanol at

−70°C. Mitotic spindles were spun onto polylysine-coated coverslips and fixed with either 1% glutaraldehyde on PBS for 20 minutes or methanol at −70°C for 6 minutes. If glutaraldehyde was used as a fixative, coverslips were washed with PBS and incubated in 2 changes of 1 mg/ml sodium borohydride solution. Coverslips with adsorbed spindles and chromosomes were then blocked with 1% solution of bovine serum albumin on TTBS, and incubated with primary and secondary antibodies. Antibody solutions were prepared on TTBS with 2 mg/ml bovine serum albumin. Coverslips were incubated with each of the antibody solutions at 37°C for 30 minutes and washed for 15 minutes with 3 changes of TTBS between incubations. In some experiments Hoechst 33258 at a concentration of 0.1 μg/ml was included in one of the final TTBS washes to stain chromosomes. For double immunostaining, coverslips were sequentially incubated with each of the primary and with each of the secondary antibodies.

Primary antibodies used were HD antibody at a concentration of 0.02-0.2 mg/ml, anti-β-tubulin (Amersham) diluted 1:250, and CREST serum (a gift from Dr W. Earnshaw, Johns Hopkins University School of Medicine, Baltimore, MD). Secondary antibodies were TRITC-anti-rabbit IgG, FITC-anti-mouse IgG and FITC-anti-human IgG.

A cooled charge coupled device (CCD) camera (model 200, Photometrics Ltd., Tucson, AZ) was used to collect fluorescence images. Digital image CCD files were stored on WORM drive optical disks (model 3363, IBM Corp.). Image processing was performed with the Image-1 system (Universal Imaging Corp., Westchester, PA). Negatives for photographs were obtained with a 4000 line film recorder.

Analytical methods

SDS-gel electrophoresis was performed according to the method of Laemmli (1970) in 4% to 12% gradient gels at a 37.5:1 (v/v) acrylamide/methylene-bisacrylamide ratio. For the preparation of PtK_1 and CHO extracts, cells were washed 3 times with PBS containing the mixture of protease inhibitors and dissolved immediately in SDS sample buffer. Partially purified HeLa kinesin (a gift from Dr G. Hering) was used as a marker for the mobility of kinesin heavy chain. Gels were stained with Coomassie Blue R-250. Immunoblotting was performed according to the method of Towbin et al. (1979). Protein concentration was determined by the method of Bradford (1976).

RESULTS

Microinjections of PtK_1 cells with HD antibody

The goal of the present work was to determine a possible role of kinesin-like translocator proteins in mitosis. We injected PtK_1 cells at the prophase/prometaphase transition (defined as immediately before or after NEB) with affinity-purified antibodies to *Drosophila* kinesin motor domain (HD antibody) and followed their progression through mitosis. Preimmune IgG was used for control injections. All injected cells were recorded for subsequent analysis of their mitotic behavior and determination of the duration of individual stages of mitosis. A few cells that were definitely injured were discounted from further analysis immediately after injection. To obtain reference information about the normal timing of mitosis in PtK_1 cells under our experimental conditions, normal non-injected cells were also recorded.

The results of injections of HD antibody and of preimmune IgG at a needle concentration of 20 mg/ml are shown in Fig. 1. Almost all (21 of 25) of the preimmune IgG-

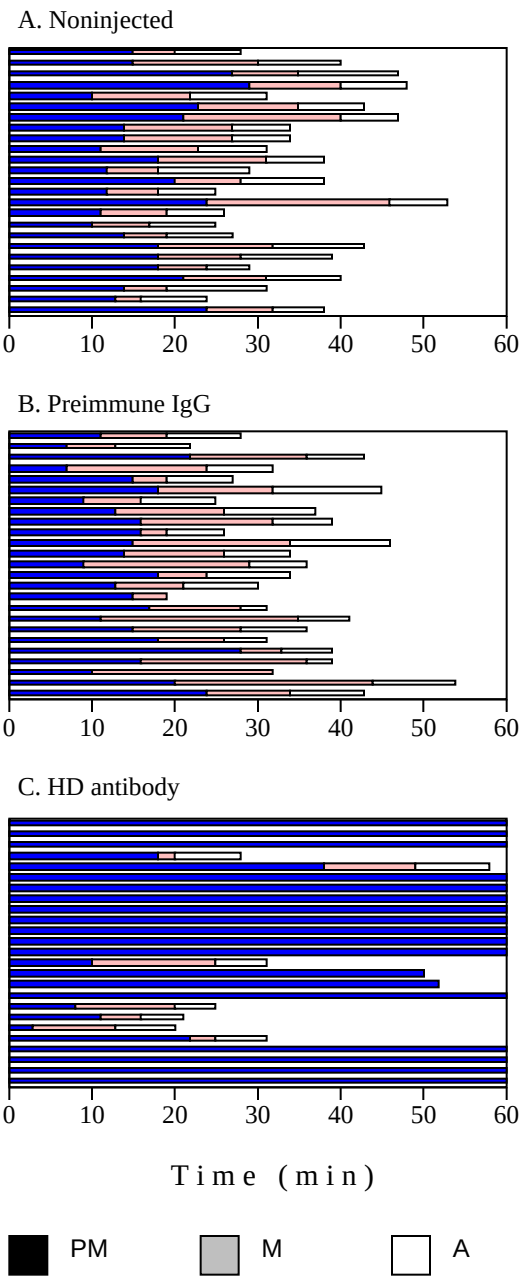

Fig. 1. Progression through mitosis of control noninjected PtK_1 cells, and PtK_1 cells injected with preimmune IgG or antibody HD. Preimmune IgG and antibody HD were injected at a needle concentration of 20 mg/ml right after NEB. Progression through mitosis of both injected and noninjected cells was recorded, the main timepoints of mitosis (the NEB, metaphase plate formation, the beginning of the anaphase chromosome separation, and the beginning of cleavage furrow formation) for each cell were determined from the playbacks and the duration of prometaphase, metaphase and anaphase were quantified. The bars in the panels correspond to individual cells. In Fig. 1B four cells (1, 2, 10, 18, counting from the bottom) showed abnormalities in anaphase chromosome segregation but divided with otherwise normal kinetics. (A) noninjected cells; (B) preimmune IgG-injected cells; (C) HD antibody-injected cells; PM, prometaphase; M, metaphase; A, anaphase.

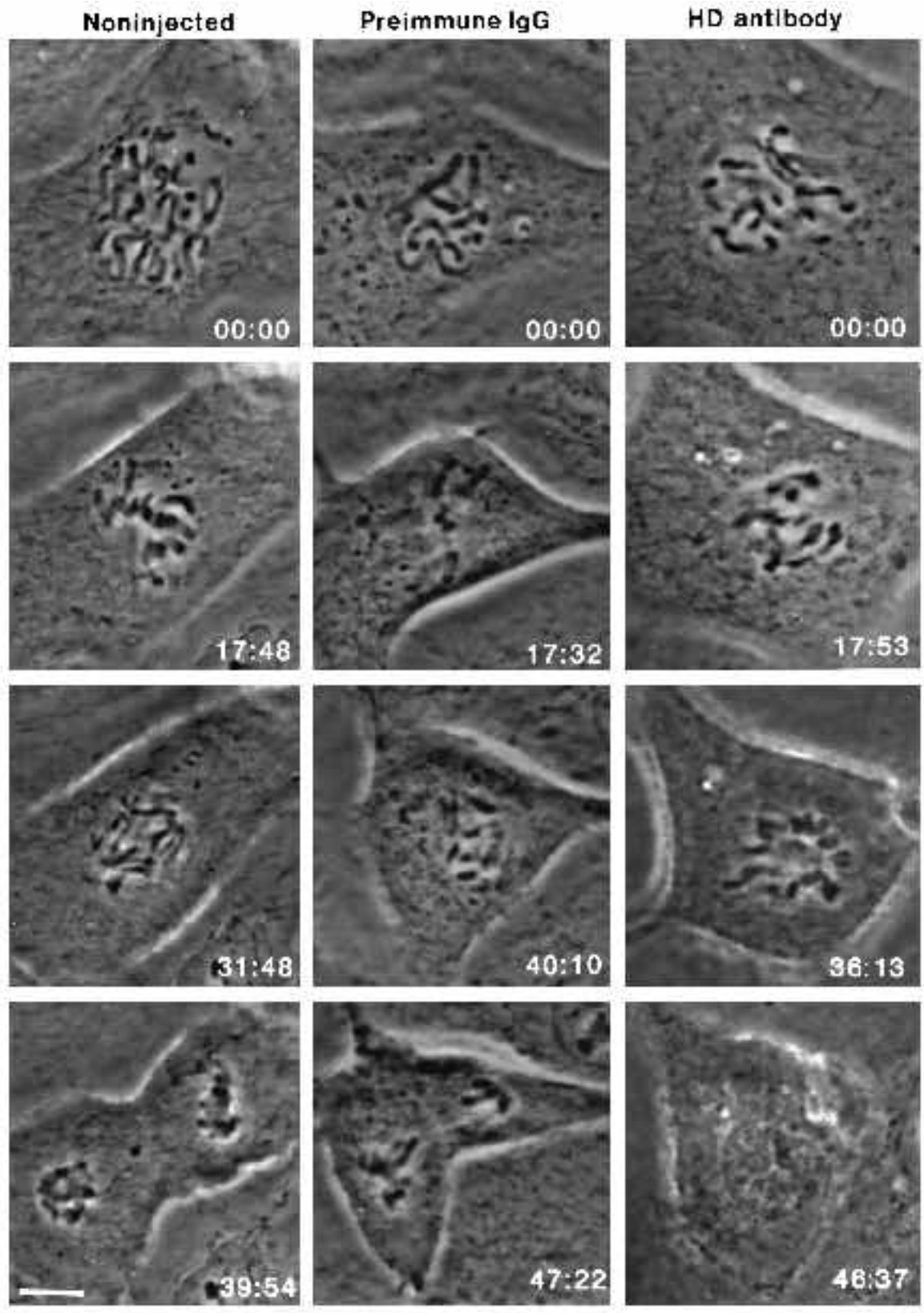

Fig. 2. The progression through mitosis of a noninjected cell, preimmune IgG-injected cell, and HD antibody-injected cell. Preimmune IgG and HD antibody were injected at 20 mg/ml right after NEB. Phase-contrast images of each cell were obtained immediately after injection, at early metaphase, at the beginning of anaphase, and at the start of cytokinesis. Numbers indicate time in min:s after injection. Bar, 10 μm.

injected cells progressed through mitosis and divided normally (Fig. 1B). Moreover, injections of the preimmune IgG did not induce any detectable delays in the progression of the cells through the individual stages of mitosis: the values for the duration of prometaphase, metaphase and anaphase in noninjected and in the preimmune IgG-injected cells were not significantly different: 17.0±5.4 and 15.0±5.1, 10.0±4.6 and 12.3±6.5, and 8.4±1.9 and 8.0±2.5 minutes (mean ± s.d.), respectively. The remaining preimmune IgG cells (4 of 25) also divided but showed some minor abnormalities in chromosome segregation; that is, a few of the chromosomes appeared sticky and failed to separate completely. In contrast, most (18 of 25) cells injected with HD antibody gave a distinctive phenotype and were blocked in prometaphase (Fig. 1C). Some HD-injected cells (7 of 25) were not blocked and these cells showed behavior not significantly different from non-injected ones. The reason for this failure of blocking is not clear but may be due to the variation in the amount of antibody injected.

Fig. 2 (right column) illustrates the phenotype displayed by the majority of the HD antibody-injected cells. The progression through mitosis of a noninjected (left column) and of a preimmune IgG-injected (middle column) cell are shown for comparison. The chromosomes of cells injected with HD antibody failed to congress to the metaphase plate, but rather scattered throughout the cytoplasm (right column, 17 minutes time point). Later on, when noninjected and preimmune IgG-injected cells entered anaphase, chromosomes in the HD antibody-injected cells became arranged in a rosette (Fig. 2, right column, 36 minutes time point). Some of the HD-injected cells demonstrated even more unusual behavior (Fig. 3). In these cells, chromosomes clearly formed a monopolar array, which moved at a velocity of 1.8-2.0 μm/min towards the margin of the cell. Such chromosome behavior resembled their usual movement in anaphase B except that all chromosomes moved to the same margin of the cell. Although antibody-injected cells did not divide, their chromosomes ultimately decondensed, the nuclear envelope re-formed, cells respread and entered the next cell cycle (Fig. 2, right column, and Fig. 3).

To characterize further the effect of HD antibody we followed chromosome and spindle pole distribution in injected cells more closely. Fig. 4 shows an example of chromosome distribution in an HD-injected cell. A short period of time after NEB chromosomes appeared to be mono-oriented with their kinetochores facing the poles and their arms radiating away from the poles (Fig. 4, 4 minute time point) as in non-injected cells. Moreover, at this time one could sometimes observe a sudden fast movement of a distantly located chromosome towards the nearest pole (not shown). Such fast prometaphase chromosome movements were shown to take place after attachment of astral microtubules to distantly located chromosomes (Hayden at al., 1990). This observation suggests that in injected cells, like in intact ones, astral microtubules appear to contact kinetochores of chromosomes. Unlike non-injected cells, in HD antibody-injected cells it became impossible to locate two separate spindle poles later on (Fig. 4, 12 and 16 minute time points), and by 30 minutes after NEB, most of the cells showed a rosette distribution of chromosomes (Fig. 4, 22 minute time point). Anti-tubulin staining of 15 of the HD-injected cells fixed at this time showed that 11 of them had a monoaster of microtubules rather than a bipolar mitotic spindle (Fig. 4, bottom right). The remaining four cells had a bipolar spindle that appeared to be structurally normal. The proportion of monopolar and bipolar cells as assayed by anti-tubulin fluorescence was approximately the same as the proportion of blocked or dividing cells as assayed by

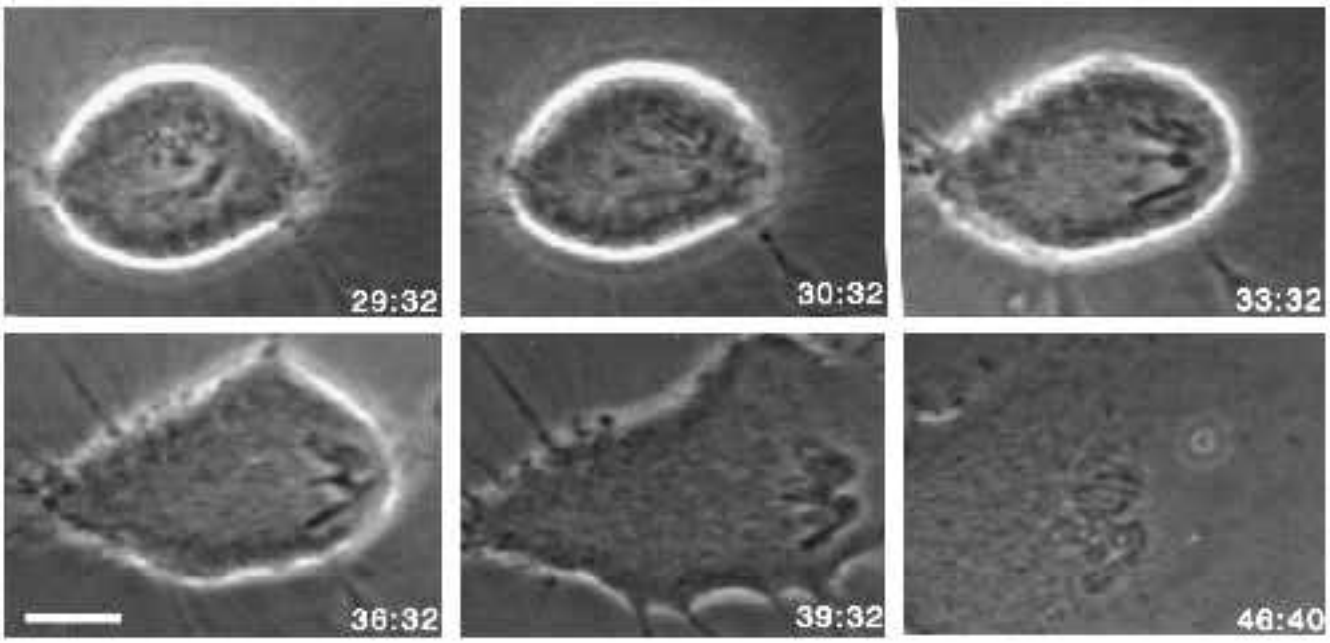

Fig. 3. The movement of chromosomes to a cell margin in a HD-injected cell. The cell was injected just before the NEB with HD antibody at 20 mg/ml; 30 minutes later, when noninjected cells usually entered anaphase, chromosomes started to move to a cell margin. Numbers on the micrographs show time in min:s after injection. Bar, 10 μm.

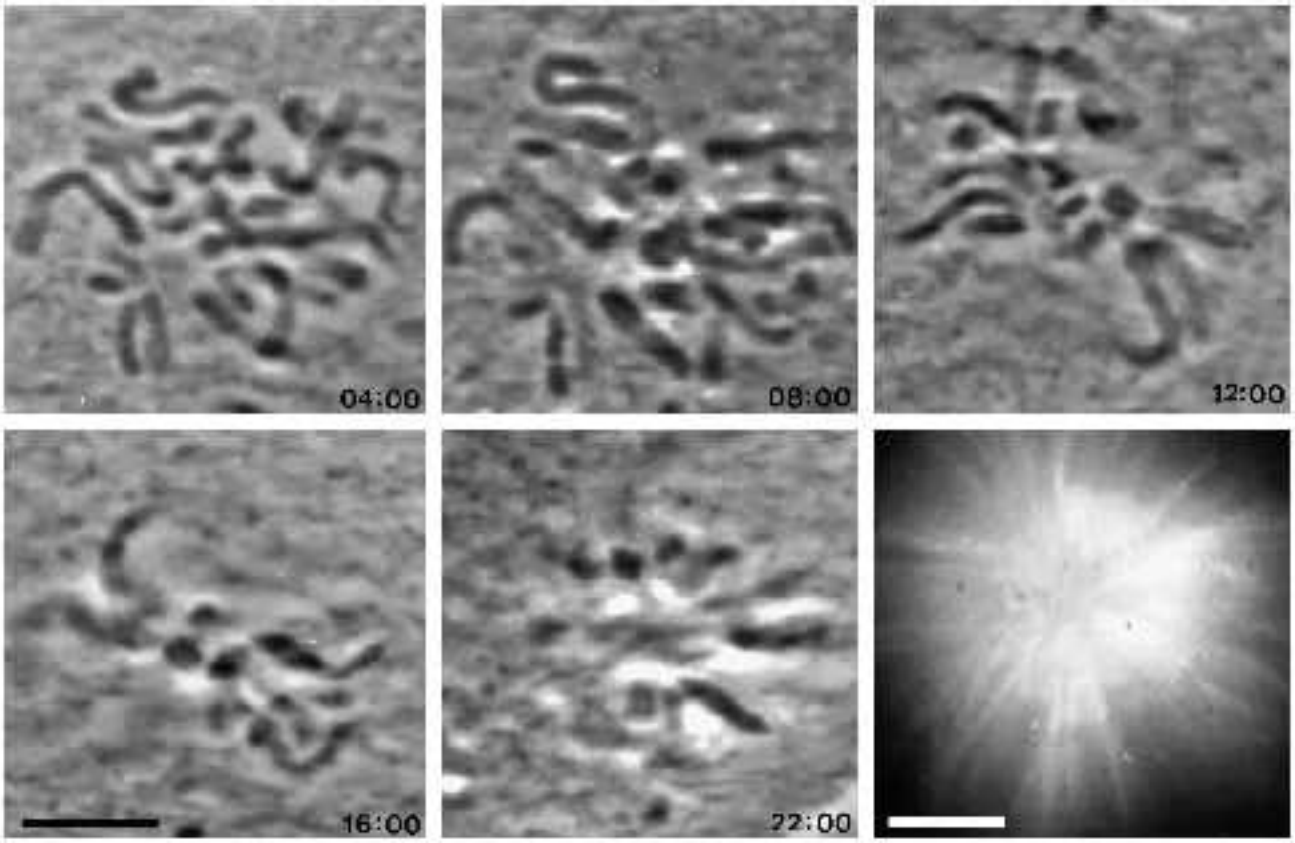

Fig. 4. The spindle poles and chromosomes in a HD-injected cell. HD antibody at 20 mg/ml was injected just before the NEB. Numbers on the micrographs indicate time in min:s after injection. Spindle poles could be seen shortly after injection (4 and 8 minutes), but their position became unclear later; 22 minutes after injection chromosomes arranged in a rosette; the cell was fixed 35 minutes after injection and stained with anti-tubulin antibody, which revealed a monoaster of microtubules (lower right). Bar, 5 μm.

time-lapse phase-contrast observation. We conclude that in the HD antibody-blocked cells, the spindle poles come together to form a monopolar microtubule aster.

The blocking effect of the HD antibody did not depend on whether a cell was injected just before or after NEB. However, antibody injections were without effect if made after metaphase plate formation. At lower concentrations (≤10 mg/ml) HD antibody had no discernable effect on mitosis and all injected cells divided (7 injections). Thus, our results showed that HD antibody induces a disorder of mitosis, acting in prometaphase, and its effect is dose dependent.

Immunostaining of tissue culture cells, mitotic spindles and mitotic chromosomes with the HD antibody

To explain the effect of HD antibody injection it was important to localize the target protein(s) in the mitotic spindle. Because the half-spindles interact with each other through the interdigitating polar microtubules as well as through the kinetochore microtubules, and the HD antibody induces spindle collapse, we attempted to find out whether the corresponding antigen was located on the polar microtubules, on kinetochores, or on both. Mitotic and interphase PtK_1 cells were immunostained with HD antibody that had been demonstrated to be effective in microinjection experiments. Because previous studies showed anti-kinesin immunostaining to be sensitive to Triton extraction (Hollenbeck, 1989; Pfister et al., 1989), we used non-extracted cells, or cells extracted before fixation with mild detergents (0.5% Brij 58, 0.01% digitonin or 0.02% saponin), for the HD antibody staining.

Fig. 5A,B shows the double immunostaining of an interphase PtK_1 cell with the HD antibody and with the anti-tubulin antibody. As can be seen in Fig. 5A, HD antibody showed punctate staining, which corresponded presumably to cytoplasmic membrane vesicles. The immunoreactive particles were more abundant in central regions of the cytoplasm where microtubules were more numerous (Fig. 5B). This staining pattern was in a good agreement with the previously reported results of immunostaining of interphase mammalian tissue culture cells (Pfister et al., 1989) and sea urchin caelomocytes (Wright et al., 1991) with monoclonal antibodies to kinesin heavy and light chains. In mitotic PtK_1 cells some staining of the region of the spindle was often observed, but high background precluded detailed analysis of the spindle staining (Fig. 5C,E). The background could originate from the staining of membrane vesicles accumulated at the spindle region. The staining pattern was similar for prometaphase (not shown), metaphase (Fig. 5C,D) and anaphase (Fig. 5E,F) cells. The CREST staining for kinetochore localization in Fig. 5E identifies clearly that this cell was in late anaphase.

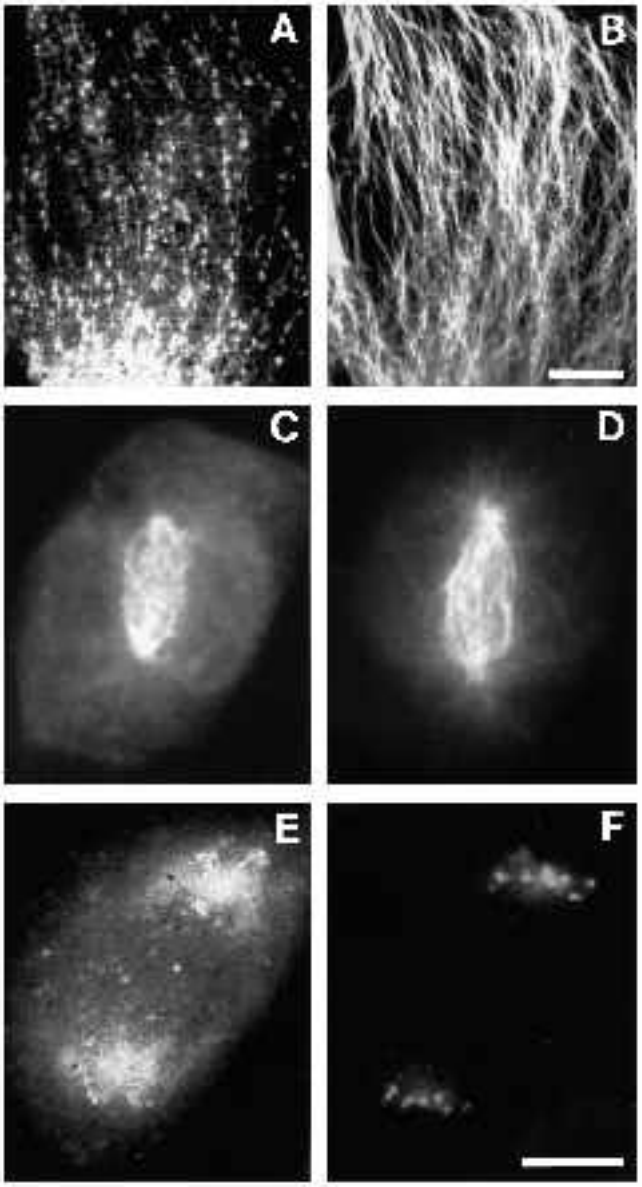

Fig. 5. Immunostaining of interphase and mitotic PtK_1 cells with HD antibody. An interphase PtK_1 cell double stained with HD antibody (A) and with anti-tubulin antibody (B). Mitotic PtK_1 cells in metaphase (C,D) and anaphase (E,F) of mitosis double stained with HD antibody (C,E) and either anti-tubulin antibody (D) or CREST serum (F), specific for kinetochores of chromosomes. For the immunostaining, cells were either briefly permeabilized with Brij 58 (A,B), or fixed with methanol without detergent permeabilization (C,D and E,F). Bars, 10 μm.

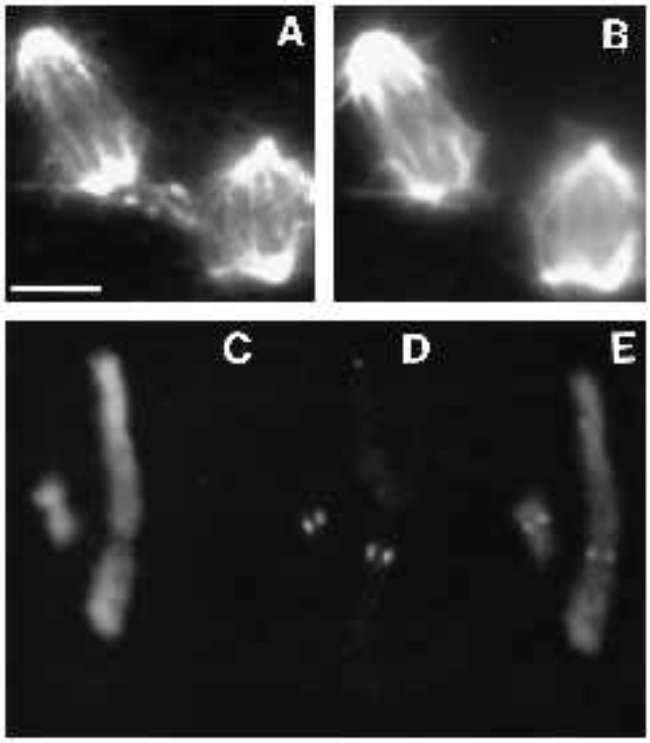

Fig. 6. Immunostaining of isolated mitotic CHO spindles and chromosomes with HD antibody. CHO spindles and chromosomes were isolated as described in Materials and Methods and adsorbed on coverslips. Spindles were double stained with HD antibody (a) and with anti-tubulin antibody (b). Chromosomes were triple stained with Hoechst 33258 (c) to reveal chromosome arms, with autoimmune CREST serum specific to kinetochores (d) and with HD antibody (e); note the staining of spindle fibers (a) and kinetochore staining (e) with HD antibody. Bars, 5 μm.

To evaluate more clearly whether spindle fibers and/or kinetochores of chromosomes were stained with HD antibody we isolated mitotic spindles from CHO cells. We used CHO cells because PtK_1 cells do not lend themselves to synchronization and cannot be obtained in quantities sufficient to make spindle or chromosome preparations. Taxol-stabilized mitotic spindles were obtained after lysis of synchronized cells with NP-40 and sucrose gradient centrifugation (Kuriyama et al., 1984). Fig. 6A,B shows double immunostaining of isolated spindles with anti-tubulin and HD antibodies. HD antibody interacts with the spindle fibers recognized by anti-tubulin antibody.

CHO mitotic spindles purified by the method of Kuriyama et al. (1984) lack chromosomes, though they still contain kinetochore remnants that can be revealed by human autoimmune CREST serum staining. However, double immuno-staining of isolated spindles with CREST serum and HD antibody showed that HD antibody did not recognize kinetochores in our isolated spindles (not shown). One possible explanation is that the putative kinesin-like protein associated with kinetochores was removed during detergent extraction used for preparation of mitotic spindles. To check further whether the protein(s) recognized by HD antibody was bound to kinetochores, we isolated chromosomes by the polyamine method as modified by Mitchison and Kirschner (1985). This method did not require detergent extraction of mitotic cells, but rather cells were allowed to swell in a low ionic strength buffer and then were disrupted by homogenization.

Double immunostaining of mitotic chromosomes isolated by this method with HD antibody and CREST serum showed that HD antibody recognized kinetochores (Fig. 6E). Though kinetochore staining with HD antibody was weaker than with CREST serum (compare Fig. 6D and E)

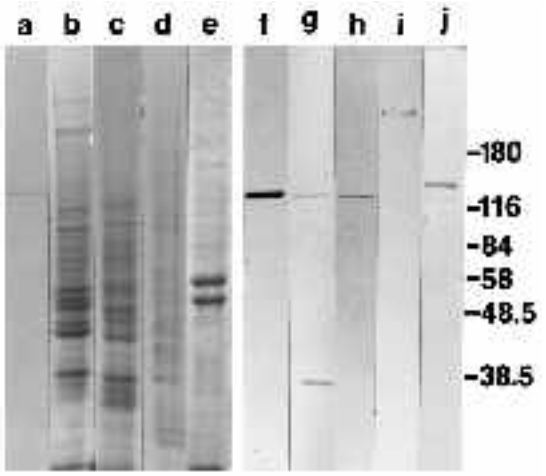

Fig. 7. Polypeptides, recognized by HD antibody in PtK_1 and CHO cells: lanes a-e, Coomassie-stained gels; f-j, corresponding blots; a,f, HeLa kinesin; b,g, PtK_1 extract; c,h, mitotic CHO extract; d,i, isolated CHO chromosomes; e,j, isolated CHO spindles.

and only 20-25% of all chromosomes were stained with HD antibody as compared to about 90% of chromosomes stained with CREST serum, this HD antibody staining was specific, as preimmune IgG taken at the same or higher concentration as HD antibody never stained kinetochores (not shown). The association of a putative kinesin-related protein recognized by HD antibody with kinetochores was not mediated by tubulin binding, because we used vinblastine for cell synchronization, and kinetochores of chromosomes in vinblastine-treated cells do not contain bound tubulin (Mitchison and Kirschner, 1985), as confirmed by lack of staining with anti-tubulin antibody (not shown).

Identification of polypeptides recognized by HD antibody in PtK_1 cells

To identify polypeptides recognized by HD antibody we used immunoblotting. Fig. 7 shows Coomassie-stained gels and corresponding blots with purified HeLa kinesin (a,f), PtK_1 cell extract (b,g), mitotic CHO cell extract (c,h), and preparations of chromosomes (d,i), and spindles (e,j). It is clear that the major polypeptide immunoreactive with HD antibody in PtK_1 and CHO cell extracts was the 120 kDa kinesin heavy chain, though a minor 35 kDa polypeptide was recognized by HD antibody in PtK_1 cell extracts as well. Kinesin was also contained in a preparation of mitotic spindles, though the predominant immunoreactive polypeptide in the mitotic spindles had a molecular mass of 135 kDa. In chromosome preparations, a 300 kDa polypeptide was the only species reactive with HD antibody.

DISCUSSION

In the present work we have shown that injections of the monospecific antibody to *Drosophila* kinesin motor domain (HD antibody) in PtK_1 cells inhibited mitosis. In the HD antibody-injected cells, the metaphase plate failed to form, spindle poles came together, and chromosomes became arranged in a rosette. Immunostaining of injected cells with anti-tubulin antibodies showed that a monoaster of microtubules rather than a bipolar spindle was present in most of the injected cells.

We believe that this effect of HD antibody injection was specific and could not be explained by cell damage caused by injection itself or by introduction of a large amount of immunoglobulins in the cytoplasm of dividing cells: (i) although injected cells failed to divide they remained fully viable - in all injected cells chromosomes ultimately decondensed, nuclear envelope re-formed, and cells entered a new interphase (see Figs 2, 3); (ii) the injection of preimmune IgG taken at the same concentration as HD antibody did not affect mitosis in the vast majority of cells; in those few cells that were affected, the inhibitory effect was clearly distinct from the effect of HD antibody; (iii) the effect of injection of HD antibody was also different from the effects on mitosis of antibody injections reported in a number of other studies, such as injections of antibodies against a microtubule-associated protein (Izant et al., 1983), 62 kDa phosphoprotein (Dinsmore and Sloboda, 1989), and KLPA (formerly CHO1 antigen) (Nislow et al., 1990), though CHO1 antibody was tried at a concentration as high as 50 mg/ml.

Recent genetic studies showed that kinesin itself is not involved in mitosis in *Drosophila* (Saxton et al., 1991), and the results of antibody microinjection experiments demonstrated that kinesin is not essential for spindle morphogenesis and mitosis in early sea urchin embryos (Wright et al., 1993). Our results also argue against kinesin participation in mitotic events. Indeed, the effect on mitosis was prominent at an HD antibody concentration of 20 mg/ml, while at 10 mg/ml the antibody had no effect. At the same time, inhibition of dispersion of melanosomes in melanophores (Rodionov et al., 1991) and collapse of vimentin intermediate filaments in fibroblasts (Gyoeva and Gelfand, 1991) were induced at a 2-3 times lower concentration of HD antibody. The most probable explanation of our results is that HD antibody, which blocks mitosis in PtK_1 cells, has a low affinity for a target kinesin-like protein or that the concentration of such a protein in the cytoplasm is very low. In PtK_1 cells extracts, the HD antibody recognized, besides the kinesin heavy chain, an additional polypeptide, which, however, had a molecular mass of only 35 kDa. This species may be the result of an unrelated cross-reaction as it appears to be too small to be a kinesin-like motor protein. The 135 kDa and 300 kDa polypeptides that were not detected by HD antibody in CHO extracts but were enriched in spindle and chromosome preparations are better candidates for an HD antibody target, though further work is needed for their identification as members of the kinesin family.

HD antibody had a prominent effect on mitosis if injected at the prophase/prometaphase transition but its injection in metaphase did not inhibit chromosome congression and anaphase B, though both processes have been considered to depend on plus-end-directed translocator(s) (reviewed by McIntosh and Pfarr, 1992). This finding may indicate that when HD antibody was injected in metaphase, the time for reaction with a target protein(s) responsible for congression and anaphase B pole separation was too short because the

duration of metaphase in PtK_1 cells was limited to 5-10 minutes. Alternatively, the target protein in metaphase may have been rendered inaccessible or unreactive to the antibody by a mitotic-stage-dependent change. Another possible explanation is that the HD antibody used in our experiments had limited cross-reactivity with the members of the kinesin family involved in mitotic events and that different kinesin-like molecules are involved in spindle formation and mitotic progression.

Several kinesin-like proteins involved in mitosis have been described recently. Among them is CENP-E, identified in mammalian cells (Yen et al., 1991, 1992), which is a 300 kDa protein with the N-terminal kinesin-like motor domain (Yen et al., 1992). This mitosis-specific translocator protein associates with kinetochores of chromosomes at late prometaphase/metaphase and redistributes to the midplate at or just after the onset of anaphase (Yen et al., 1991). HD antibody, used in our work, stained kinetochores and recognized a 300 kDa band in chromosome preparations. It thus seems likely that HD antibody cross-reacts with CENP-E. However, the effect on mitosis of anti-CENP-E antibody differed from the effect of HD antibody observed in our work: anti-CENP-E antibody blocked mitosis in metaphase rather than interfering with spindle formation. A possible explanation of this result is that HD antibody and anti-CENP-E antibody raised by Yen et al. (1991) recognized different epitopes on the CENP-E molecule.

Another kinesin-like translocator found in association with the mammalian mitotic spindle is MKLP1, a 110 kDa protein, localized to the spindle fibers early in mitosis and redistributed into the interzone in anaphase (Nislow et al., 1990, 1992). However, based on the phenotype induced by blocking antibodies, it seems possible that the responsible target of HD in our study is different from both CENP-E and MKLP1.

Most probably, HD antibody recognizes a translocator that belongs to another group of kinesin-like proteins. The members of this group are CUT7 from *Schizosaccharomyces pombe* (Hagan and Yanagida, 1990), *Saccharomyces cerevisiae* CIN8 and KIP1 (Hoyt et al., 1992; Roof et al., 1992; Saunders and Hoyt, 1992), BIMC from *Aspergillus* (Enos and Morris, 1990) and *Xenopus* Eg5 (Le Guellec et al., 1991). These proteins are more similar to each other in their N-terminal motor domain sequences than to other members of the kinesin superfamily and seem to perform similar functions in mitosis. In fact, mutation of *cut*7 (Hagan and Yanagida, 1990) and *bim*C (Enos and Morris, 1990) genes as well as double mutation of *kip*1 and *cin*8 (Hoyt et al., 1992; Roof et al., 1992; Saunders and Hoyt, 1992) prevented formation of bipolar mitotic spindle in yeast and *Aspergillus*. Anti-Eg5 antibody interfered with spindle formation in mitotic *Xenopus* egg extracts (Sawin et al., 1992). The effect of HD antibody injections appears to be very similar to these effects.

What is the mechanism of action of HD antibody? It is clear that the antibody injections influence the balance of forces established in the mitotic spindle. The inward force that tends to cause the spindle to collapse is possibly produced by dynein, localized recently to kinetochores of chromosomes (Pfarr et al., 1990; Stewer et al., 1990). Recent genetic experiments suggest the same function for certain members of the kinesin family of proteins: yeast KAR3 (Meluh and Rose, 1990; Saunders and Hoyt, 1992) and KLPA from *Aspergillus* (O'Connell et al., 1993; see Goldstein, 1993, for a discussion). The force produced by these translocators is possibly counteracted by the kinesin-like proteins of the CUT7/CIN8/KIP1/BIMc/Eg5 family. The simplest interpretation of our results is that the HD antibody reacts with and blocks the function of the mammalian analog of these proteins. Such a kinesin-like protein may be bound by its nonmotor tail domain to the antiparallel interdigitating astral microtubules (see Hogan and Cande, 1990, for a discussion) or to a hypothetical component of the spindle matrix (discussed by Mitchison, 1992). In any case, this translocator protein should play an important role in both the morphogenesis and the maintenance of integrity of mitotic spindle. Its inactivation would be predicted to prevent the formation or induce the collapse of the spindle.

Interestingly, Wang et al. (1983) described a mutant temperature-sensitive Syrian hamster cell line, ts-745. At nonpermissive temperature (39°C) mutant cells failed to form a normal metaphase spindle. Instead, the chromosomes were distributed in a spherical shell with microtubules radiating to the chromosomes from four closely associated centrioles near the center of the cell. Though it cannot be excluded that the mutation described by Wang et al. (1983) affects a nonmotor protein that is important for maintenance of the bipolar mitotic apparatus, such as NuMA protein (Yang and Snyder, 1992), it is also possible that the mutation described by Wang et al. (1983) affected a kinesin-like protein recognized by HD antibody.

Our data show that kinesin-like molecules are important for the formation of the mitotic spindle in mammalian cells and that the basic mechanisms of spindle morphogenesis in invertebrate and vertebrate cells are similar. The observation of Sawin et al. (1992), who found kinesin-like protein Eg5 to be important for spindle formation in *Xenopus* egg extracts, also confirms this conclusion. Further work should help to identify and characterize kinesin-like protein(s), possibly similar to Eg5, which is involved in mitosis in mammalian cells and whose functions were disrupted with HD antibody.

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