Antibodies to the Kinesin Motor Domain and CENP-E Inhibit Microtubule Depolymerization-dependent Motion of Chromosomes In Vitro

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Abstract. Chromosomes can move with the ends of depolymerizing microtubules (MTs) in vitro, even in the absence of nucleotide triphosphates (Coue, M., V. A. Lombillo, and J. R. McIntosh. 1991. *J. Cell Biol.* 112:1165–1175.) Here, we describe an immunological investigation of the proteins important for this form of motility. Affinity-purified polyclonal antibodies to kinesin exert a severe inhibitory effect on depolymerization-dependent chromosome motion. These antibodies predominantly recognize a polypeptide of M_r ~250 kD on immunoblots of CHO chromosomes and stain kinetochores as well as some vesicles that are in the chromosome preparation. Antibodies to CENP-E, a kinetochore-associated kinesin-like protein, also recognize a 250-kD electrophoretic component, but they stain only the kinetochroe region of isolated chromosomes. Polyclonal antibodies that recognize specific domains of the CENP-E polypeptide affect MT disassembly-dependent chromosome motion in different ways; antibodies to the head or tail portions slow motility threefold, while those raised against the neck region stop motion completely. Analogous antibodies that block conventional, ATP-dependent motility of cytoplasmic dynein (Vaisberg, G., M. P. Koonce, and J. R. McIntosh. 1993. *J. Cell Biol.* 123:849–858) have no effect on disassembly-dependent chromosome motion, even though they bind to kinetochores. These observations suggest that CENP-E helps couple chromosomes to depolymerizing MTs. A similar coupling activity may allow spindle MTs to remain kinetochore-bound while their lengths change during both prometaphase and anaphase A.

THE idea that tubulin dynamics might generate forces sufficient to move objects attached to cytoplasmic microtubules (MTs)[1] has been considered for many years. The "dynamic equilibrium" model for mitotic chromosome movement constitutes one of the most important expressions of this idea (for review see Inoue, 1981). Early evidence for the concept came from experiments which suggested that the state of polymerization of MTs could affect chromosome movements within cells. For example, the direction of chromosome movement reversed during anaphase when tubulin assembly was promoted after chromatid separation (Bajer et al., 1982; Sheldon and Wadsworth, 1992). Experiments in vitro have demonstrated that growing MTs can do work by deforming membranes when grown within liposomes (Miyamoto and Hotani, 1989) and by elongating membranous networks in *Xenopus* extracts (Waterman-Storer, personal communcation). Shortening MTs are also able to provide sufficient force to move chromosomes in vitro, as was initially suggested by the work of Koshland et al. (1988). Real time observations have shown that depolymerizing MTs can exert forces greater than 1 pN on chromosomes bound at or near their ends, even when the nucleotide triphosphate concentrations are too low to support the activity of MT-dependent motor enzymes (Coue et al., 1991). Analogous depolymerization-dependent phenomena have also been seen in sea urchin ooplasm with MTs growing from axonemes added to this system (Glicksman and Salmon, 1993). It is therefore well established that MT dynamics can generate force.

An assay developed in our lab for studying depolymerization-dependent chromosome motion in vitro uses polymers of bovine brain tubulin initiated by the basal bodies within a *Tetrahymena* "pellicle," i.e., the cortex that remains after exhaustive detergent lysis of this ciliate (Lombillo et al., 1993). Pellicles will bind tightly to glass coverslips, providing not only an initiator of MTs with known structural polarity (Heidemann and McIntosh, 1981), but also an anchor to hold the minus ends of the MTs to a fixed position on the microscope stage during continuous perfusion in a

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1. *Abbreviations used in this paper*: AMP-PNP, 5' adenylyl-imidodiphosphate, a non-hydrolyzable analogue of ATP; KLP, kinesin-like protein; MT, microtubule; pAb, polyclonal antibody; MAP, microtubule-associated protein.

flow cell. Chromosomes and other particles can be introduced into the chamber and allowed to bind to the pellicle-initiated MTs, so their motile properties can be studied. The pellicle-initiated MTs will depolymerize upon either tubulin dilution or calcium addition, and the bound chromosomes move with the disassembling plus end, even in the absence of ATP. This motion of chromosomes in association with shortening MTs implies that kinetochore proteins can couple chromosomes to tubulin polymers in such a way that subunit depolymerization provides the energy necessary for movement against a load. The question arises, how do objects remain coupled to a disassembling polymer? Motor enzymes located on the chromosomes are plausible candidates for such coupling factors, but this activity of motors would be unusual, since it is independent of ATP. Motors that act as coupling factors may require, for example, a continuous state of weak binding to the shortening MT polymer. Precedent for such loose-binding states of motor enzymes and MTs is seen in the ability of both flagellar dynein (Vale et al., 1989) and some kinesin-like proteins (KLPs) (Stewart et al., 1993; Chandra et al., 1993) to bind MTs and display one-dimensional (1-D) diffusive movements without ATP hydrolysis.

Here, we investigate the identity of the chromosome-associated proteins that bind to MTs in our assay. There are several likely candidates for chromosome-MT coupling factors: cytoplasmic dynein, which is associated with the corona region of kinetochores (Pfarr et al., 1990; Steuer et al., 1990; Wordeman et al., 1991) and the members of the kinesin super family, which have been localized to both kinetochores and membrane-bound vesicles (Schroer et al., 1988; Yen et al., 1992; Wordeman and Mitchison, 1994). Function-blocking antibodies have recently become available as probes with which to study the roles of these proteins in a specific motility event. For example, polyclonal antibodies (pAbs) raised against a recombinant fragment encompassing the motor domain of conventional kinesin block motility of MTs in vitro and MT-dependent vesicle motion in vivo (Rodionov et al., 1991). In addition, pAbs to bacterially expressed polypeptides that contain dynein's putative active site can block ATP-dependent gliding of MTs over glass and early mitotic centrosome separation in PtK1 cells (Vaisberg et al., 1993). To probe the role of dynein and kinesin in the chromosome motility we observe in vitro, we have treated chromosomes with these antibodies and others raised against kinetochore KLPs, including CENP-E. Our results strongly suggest that a KLP is involved in disassembly-dependent chromosome motility in vitro.

Materials and Methods

In Vitro Assay for MT Depolymerization-driven Chromosome Motion

The procedures for chromosome isolation, for pellicle preparation, and for initiating disassembly-dependent motion are described briefly in Coue et al. (1991), and more completely in Lombillo et al. (1993).

Chemical Reagents

Pipes used in our buffers was purchased from Boehringer Mannheim Corp. (Indianapolis, IN) and unless otherwise specified all other reagents were purchased from Sigma Chem. Co. (St. Louis, MO).

Antibodies

DD1. Function-blocking antibodies to dynein were isolated from the blood of rabbits immunized with a bacterially expressed polypeptide of 70 kD that included the putative ATP hydrolytic domain of cytoplasmic dynein from *Dictyostelium discoideum*. Dynein-specific antibodies were collected by affinity chromatography, using antigen immobilized on cyanogen bromide–activated–Sepharose (Vaisberg et al, 1993) and used at a final concentration of 1.25 mg ml^{-1}. This concentration blocks ATP-dependent MT motility in vitro but does not affect the binding of dynein to MTs (Vaisberg et al., 1993).

Kin2/HD. Function-blocking antibodies to kinesin were raised in rabbits against a bacterially expressed fragment of the *Drosophila* kinesin motor domain (Rodionov et al., 1991). They were affinity purified on a column containing immobilized antigen, concentrated on a protein-A column, and eluted by reducing the buffer pH to ~2.0, and then neutralizing it to a pH 7.0 with phosphate buffer.

anti-CENP-E. A mouse monoclonal antibody (mAb-177) that recognized CENP-E was raised using salt washed chromosomal scaffolds as antigen (Yen et al., 1991). Rabbit pAbs were generated against three non-overlapping subdomains of CENP-E. Antibodies Not and 1.6 were raised against the motor (amino acids 1–256) and the neck (amino acids 256–817) regions, respectively. The appropriate restriction fragments that encode these two regions of CENP-E were cloned and expressed in the pMAL expression vector (New England Biolabs, Beverly, MA). HX antibody is directed against the COOH-terminal portion of the central rod domain (amino acids 1601–1889), which was expressed from a pATH vector as a trpE fusion protein. A schematic illustrating the subdomains recognized by these CENP-E antibodies is shown in Fig. 7. Expression of these fusion proteins in bacteria was performed as described in Rattner et al. (1993). All CENP-E polyclonal IgGs were purified by binding to protein A, eluted as described above for kin2/HD, and further concentrated by ion exchange chromatography.

anti-MCAK. Linda Wordeman (University of Washington) kindly supplied a mAb (used at 12 mg ml^{-1}) and pAbs (used at 4.7 mg ml^{-1}) that were raised and affinity purified against a recombinant portion of MCAK, an inner kinetochore 90 kD KLP (Wordeman and Mitchison, 1994).

Immediately before use, a sample of concentrated chromosomes was thawed, mixed with antibodies (at a ratio of 1:10, vol:vol, chromosomes:antibody) and incubated on ice for 1–5 h. Antibodies that inhibited chromosome motion (kin2/HD and 1.6) did so after as little as one hour of incubation; longer incubations of up to 6 h with each antibody yielded results similar to those obtained after only one hour.

UV-VO_4 Cleavage of Kinetochore Dynein

25-μl samples of chromosomes (in 10 mM Pipes, pH 7.2, 1 mM EDTA, 2 mM $MgCl_2$, 0.25 mM spermidine, 0.1 mM spermine, 0.1% β-mercaptoethanol, 50% sucrose + 5 μg/ml α-macroglobulin and a cocktail of other protease inhibitors listed in Lombillo et al., 1993) were made 100 μM with sodium orthovanadate and 1 mM with MgATP, and then placed on parafilm on ice. The chromosomes were exposed to light from a long wave (365 nm) UV lamp (model EN-280L, Spectroline Corp., Westbury, NY) at a distance of 1 cm for up to 1.5 h. This exposure is sufficient to cleave flagellar dynein (Gibbons and Gibbons, 1987). The treated chromosomes were used immediately for motility in the MT-depolymerization assay or were boiled in SDS-PAGE sample buffer for subsequent analysis by electrophoresis.

Immunofluorescence

Chromosomes were allowed to attach to coverslips for 25 min in a humid chamber on ice. These coverslips were then rinsed by gentle immersion in PME buffer (80 mM Pipes, pH 6.9, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT) and fixed in methanol at −20°C for 10 min, followed by acetone (also at −20°C) for another 10 min. After three washes in PBS, for 5 min each, the coverslips were incubated with primary antibody in a humid chamber for 1 h at 37°C. The coverslips were washed three times in PBS, and then overlaid with secondary antibody (goat-anti-rabbit) conjugated to Texas red (Jackson Labs, West Grove, PA). Chromosomes were stained with 10 μg ml^{-1} DAPI for 1 min. Finally, samples were rinsed in PBS and mounted on slides for observation on a Zeiss Universal microscope equipped with epi-illumination and Texas red or DAPI filters made by Chroma Technologies (Brattleboro, VT).

SDS-PAGE and Immunoblots

ATP-extracted microtubule-associated proteins (MAPs) were prepared from HeLa cells exactly as described in Vaisberg et al. (1993). MAPs and isolated chromosomes were separated by SDS-PAGE using 7% polyacrylamide and electroblotted to Immobilon-P membranes (Millipore, Bedford, MA). Chromosomal proteins were probed with primary antibodies used at roughly 1 $\mu g/ml^{-1}$. Secondary antibodies were diluted at 1:10,000 and visualized with the enhanced chemiluminescence kit from Amersham Corp. (Arlington Heights, IL).

Results

An Antibody Raised against the Putative ATP Hydrolytic Domain of Cytoplasmic Dynein Does Not Affect Depolymerization-induced Motility of Chromosomes or Vesicles

We have examined the role of cytoplasmic dynein in depolymerization-dependent chromosome motions in vitro by using DD1, an affinity purified anti-dynein antibody known to block both ATP-dependent MT gliding in vitro and centrosome separation in PtK cells (Vaisberg et al., 1993). Purified DD1 antibody binds both native and chemically fixed dynein; it localizes exclusively to the kinetochores of isolated CHO chromosomes (Fig. 1). After incubation in a concentration of DD1 that disrupted dynein function both in vivo and in vitro (1.25 mg/ml^{-1}), chromosomes and vesicles were still able to bind pellicle-initiated MTs and move as the MTs depolymerized. Even 5 h of incubation with DD1 antibodies did not affect chromosome motility after tubulin dilution. Antibody binding to kinetochores of incubated chromosomes was confirmed by immunofluorescence (not shown). DD1-treated chromosomes moved all the way to the pellicle surface, and the mean distance of movements was within the range observed in control experiments (not shown). Mean rates of chromosome movement after incubation in DD1 were statistically indistinguishable from untreated controls (Fig. 2). In addition, the frequency with which bound objects moved was not altered by the DD1 antibody; as reported earlier (Coue et al., 1991), approximately half of the bound chromosomes moved during any given experiment.

We have previously shown that adding sodium orthovanadate to our assays, which is a potent inhibitor of dynein ATPase activity, does not alter depolymerization-dependent chromosome motility in vitro (Coue et al., 1991). To further test dynein's role in disassembly-dependent chromosome movement, we asked whether UV-vanadate induced cleavage (Gibbons and Gibbons, 1987) of kinetochore dynein in situ might affect motility in our assay. Most of the chromosomal dynein was cleaved, as determined by its faster mobility on SDS-PAGE after treatment (not shown). Chromosomes exposed to UV-ATP-vanadate induced cleavage were still able to bind to MTs and move in our assay (Fig. 2). Taken together, these results suggest that if dynein has a role in the depolymerization-dependent motility of chromosomes, its role does not depend on the conventional mechanochemical properties of this enzyme.

Antibodies Raised against Kinesin's Motor Domain Inhibit Depolymerization-dependent Chromosome Movement

We have also used antibodies to examine kinesin's involvement in coupling cellular objects to the ends of shortening MTs. KLPs have been localized to kinetochores (Yen et al., 1991; Rodionov et al., 1993; Wordeman and Mitchison, 1994), so it is plausible that a KLP could contribute to chromosome-MT binding. Although conventional kinesin is a plus-end directed MT motor (Vale et al., 1985), and the motions we have observed are minus-end directed, several KLPs such as NCD (Walker et al., 1988; McDonald et al., 1990) and KAR3 (Endow et al., 1994) are minus-end directed. A KLP is therefore a reasonable candidate for coupling MT disassembly to chromosome movement. As a first step to test this possibility, we used kin2/HD, an antibody that recognizes a broad range of kinesins and KLPs and effectively blocks kinesin-dependent motility both in vitro and in vivo (Rodionov et al., 1991; Gyoeva and Gelfand, 1991; Rodionov et al., 1993*a*,*b*).

Chromosomes were incubated with affinity-purified kin2/HD antibody at 1.4 mg ml^{-1}, a concentration that reduces MT binding and inhibits motility in vitro. Standard assays for depolymerization-dependent motility were then per-

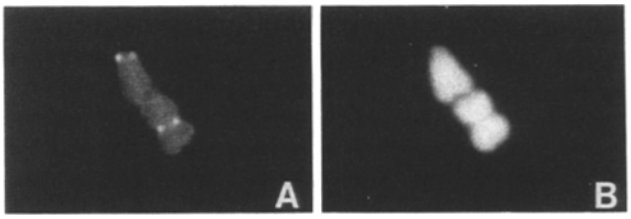

Figure 1. Dynein stains kinetochores of isolated CHO cells. *A* is an image of two chromosomes, one of which is telocentric, stained with the same affinity-purified antibody (DD1) used to test dynein's role in depolymerization-dependent motion. *B* is the image visualizing DNA with DAPI.

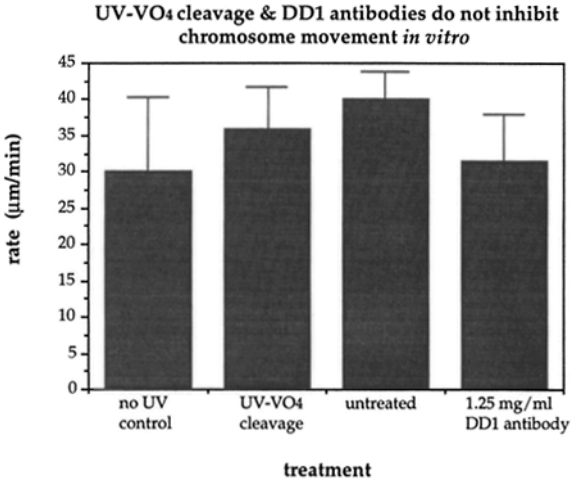

Figure 2. Histogram demonstrating that there is no significant effect on disassembly-dependent chromosome motility when kinetochore-bound dynein is either UV-VO_4 cleaved or bound by a known inhibitory dose of the function-blocking anti-dynein antibody, DD1 (Vaisberg et al., 1993). Control assays for the cleavage experiment were performed on chromosomes under identical conditions, but not exposed to UV. Controls experiments using anti-dynein were performed on untreated chromosomes in alternating assays.

formed with these chromosomes. Chromosome motility was dramatically reduced by the antibody treatment. Most chromosomes did not move at all after tubulin dilution and eventually washed off the pellicle-initiated MTs in the flow of buffer (e.g., Fig. 3). Only 3 chromosomes out of 16 moved and they traveled <1 μm before falling off the disassembling MT; the mean distance traveled by the chromosomes assayed was reduced from 7.1 μm (n = 5) in controls, to 0.25 μm (n = 16) by the kin2/HD antibody (Fig. 4). Although the normal efficiency of chromosome binding to pellicle-initiated MTs varies somewhat from assay to assay, there was no obvious effect of the antibodies on the frequency of chromosome binding.

We examined the inhibitory effect of the kin2/HD antibody further by performing identical experiments on chromosomes that had previously been incubated in: (*1*) the kin2/HD antibody affinity purified on a column with immobilized kinesin head domain. (*2*) The flow through from this affinity column, i.e., immune serum that was partially depleted of kin2/HD antibody. This flow-through fraction was then chromatographed on a column containing immobilized protein A. Preparation number 3 was the fraction that did not bind to the protein A column. Preparation number 4 comprised the immunoglobulins eluted from the protein A column, a fraction in which the kin2/HD IgGs that were present in the flow through were now concentrated. Chromosomes were treated with these four solutions without knowing their identity after a single blind experimental design, and then the treated chromosomes were tested for MT disassembly-dependent motility. Motility was unaffected by incubation in either solution 2 or 3, which contained little or no kin2/HD IgG. Chromosome motility was blocked by solutions 1 and 4, both of which contained concentrated kinesin antibodies (not shown). These experiments corroborate the data presented in Fig. 4.

To learn the subcellular location of the antigen affected by kin2/HD, we processed chromosomes treated with this antibody for immunofluorescence. Fig. 5 shows a panel of representative images of chromosomes stained with kin2/HD (Fig. 5 *A*) and DAPI (Fig. 5 *B*); both kinetochores and vesicles are stained by kin2/HD. The pattern of vesicle staining resembles that seen with the lipophilic dye $DiOC_5$ (Lombillo et al., 1993), so we infer that most of the vesicles that contaminate the chromosomes are associated with kinesin or a KLP. Given that dynein does not localize to this subset of vesicles (Fig. 1), it is likely that kinesin or KLPs are the principal MT-associated proteins responsible for the interactions of pellicle-initiated MTs with the arms of chromosomes, as described in a previous study (Coue et al., 1991). Since this antibody also binds to kinetochores, Kin2/HD may be disrupting chromosome motility by interfering with a kinesin or a KLP situated at either the kinetochore or the chromosome-bound vesicles or both. It is important to note that the localization experiments were performed on the same chromosome samples used in our assays; therefore we infer that the kin2/HD antibody can bind its antigen in an unfixed or "native" state.

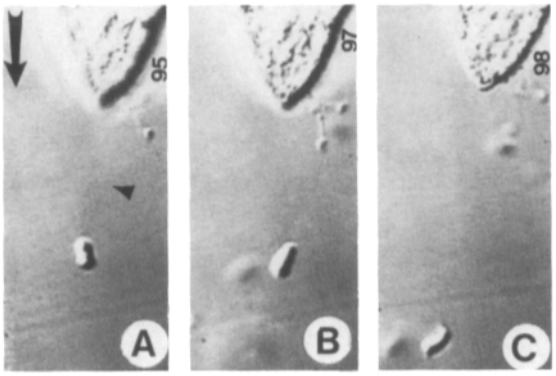

Figure 3. The kin2/HD antibody inhibits depolymerization-dependent chromosome motion. In this sequence the chromosome remains bound to a MT bundle (*arrowhead*) for over 90 s after perfusion of tubulin-free buffer. Other unbound MTs initiated from this same pellicle have already depolymerized. As the MTs associated with this chromosome shorten (in *B*), the chromosome shifts slightly in its position, but then is unable to move, and is released from the shortening MTs and is washed away in the flow (*C*). Time is in seconds.

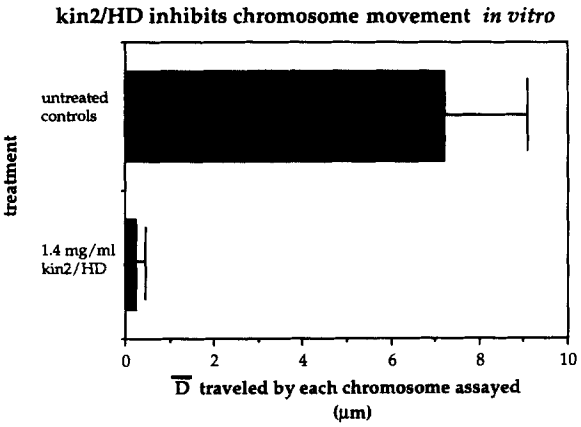

Figure 4. Histogram demonstrating that 1.4 mg ml^{-1} affinity-purified kin2/HD inhibits depolymerization-dependent chromosome motion. Controls using untreated chromosomes were alternated with experimental assays using kin2/HD-treated chromosomes. Flow-through immune sera from an affinity column and flow-through proteins off a protein A column were also used as control conditions. Chromosomes treated with these flow-through samples also failed to affect the frequency or rate of in vitro chromosome motion.

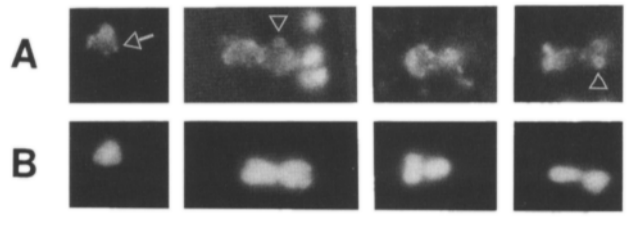

Figure 5. Immunofluorescent detection of kin2/HD on isolated CHO chromosomes. *A* shows that the kin2/HD antibody localizes to kinesin or KLP's on both kinetochores (*arrow*) and vesicles (*arrowheads*). *B* is the corresponding image of DNA using the dye, DAPI.

MgAMP-PNP Affects Depolymerization-dependent Chromosome Motions

AMP-PNP, a non-hydrolyzable analogue of ATP, is a potent inhibitor of the mechanochemistry of kinesin (Lasek and Brady, 1985) and some KLPs (Yen et al., 1992; Nislow et al., 1992). Micromolar concentrations of MgAMP-PNP inhibit the release of kinesin from MTs, locking kinesin into a tight-binding state (Vale et al., 1985; Lasek and Brady, 1985). We therefore asked whether MgAMP-PNP would affect chromosome motility in our assay (Table I). Even in the absence of ATP, 1–2 mM MgAMP-PNP does not detectably alter chromosome or vesicle motility. Incubations in 5 mM MgAMP-PNP gave variable results that ranged from slowing chromosome movement by a factor of 2 to inhibiting motion entirely. 10 mM MgAMP-PNP completely blocked motions of chromosomes to the pellicle. In all assays with MgAMP-PNP, MTs attached to chromosomes appeared to be both more stable and more bundled than those in untreated controls, yet motility was affected only by concentrations ≥5 mM. In all assays, MgAMP-PNP does not stabilize the free MTs in solution, so the slowing and inhibition effects do not appear to be due to a general increase in stability of MTs.

Immunoblot Analysis of Mitotic Chromosomes with kin2/HD and CENP-E Antibodies

Isolated chromosomes and MAPs were fractionated by SDS-PAGE, blotted, and probed with kin2/HD, to determine how many chromosomal proteins might be affected by the kin2/HD antibody. The blots of chromosomes revealed a single polypeptide of ~250 kD (Fig. 6, lane *3*), while blots of MAPs revealed a component of the same M_r as well as one with the apparent molecular mass of kinesin heavy chain. Rodionov et al. (1993*a*) also detected this high molecular mass band when probing chromosomal proteins with kin2/HD. By stripping and reprobing these blots with other, more specific anti-KLP antibodies, we learned that the high molecular mass polypeptide is likely to be CENP-E. For example, a mAb to CENP-E binds a component of the same electrophoretic mobility (compare Fig. 6, lanes *3* and *5*). CENP-E is a 312-kD kinetochore-associated KLP that consists of a conventional kinesin-like motor domain and an exceptionally long tail domain that migrates at ~250 kD on SDS-PAGE (Yen et al., 1992). A pAb raised against a portion of CENP-E, pAb 1.6 also recognizes a band of the same electrophoretic mobility (Fig. 6, lane *7*), as do the other CENP-E pAbs tested in our assays (not shown). The CENP-E antibodies recognize different components in the MAP preparations, raising the possibility that this enzyme is altered upon chromosome binding.

Table I. Treatments with MgAMP-PNP Affect Depolymerization-dependent Chromosome Movements

Treatment	Mean rate
	μm/min
Untreated	31 ± 6 (n = 3)
1-2 mM AMP-PNP	28 ± 8 (n = 4)
5 mM AMP-PNP	16 ± 1 (n = 3)
10 mM AMP-PNP	0 (n = 3)

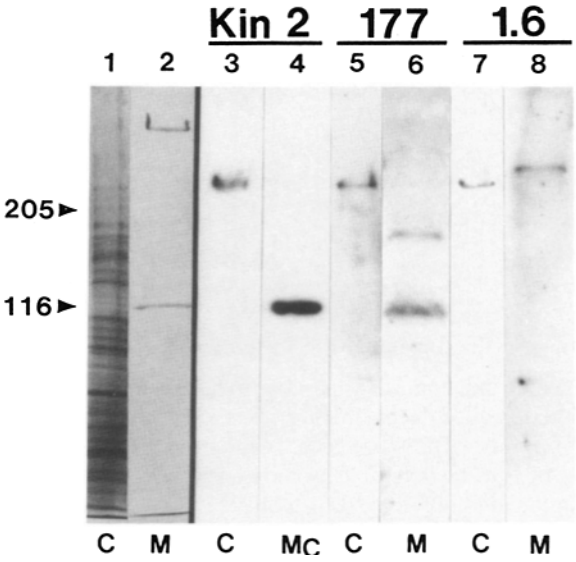

Figure 6. Kin2/HD primarily recognizes a peptide of ~250 kD on CHO chromosomes. Gels of chromosomal proteins (lane *1*) and ATP-extracted MT-associated proteins (MAPs) from HeLa cells (lane *2*) were probed with kin2/HD (lanes *3* and *4*), the CENP-E mAb 177 (lanes *5* and *6*), and the CENP-E pAb-1.6 (lanes *7* and *8*). Chromosome strips are marked as *C* and MAP strips are marked as *M*. Kin2/HD in lane *3* recognizes a 250-kD polypeptide recognized by both CENP-E antibodies in lanes *5* and *7*. Molecular mass is labeled on the left (in kD).

We also probed these immunoblots with antibodies that recognize MCAK, a 90-kD KLP, that localizes to kinetochores (Wordeman and Mitchison, 1994). Both a mAb and pAbs to this polypeptide recognize their antigen on the blots (not shown), but not the high molecular mass polypeptide bound by kin2/HD and CENP-E antibodies.

Using More Specific Antibodies to Test the Involvement of Particular KLPs in Depolymerization-driven Motion

Since kin2/HD is known to recognize several KLPs in addition to kinesin, we performed motility experiments with more specific antibodies that would recognize individual KLPs, hoping to identify the specific chromosomal antigen(s) whose activity is (are) affected by kin2/HD. The frequency and velocity of chromosome motility was unaffected by the antibodies to the 90-kD kinetochore-associated KLP, MCAK (not shown). A CENP-E specific monoclonal antibody, that recognized a portion of the rod domain showed no effect on motility (Table II), even though this antibody slows mitotic progression in vivo after microinjection (Yen et al., 1991). However, three polyclonal antibodies raised against specific domains of bacterially synthesized CENP-E did affect chromosome motion in our assay. The locations of the portions of CENP-E used to raise these pAbs are illustrated schematically in Fig. 7. All the CENP-E antibodies recognized their antigens in CHO kinetochores (as seen in Fig. 8 *a*), but there was no corresponding staining of chromosome-bound vesicles. Antibodies that recognized either the motor (Not) or a portion of the central rod (HX) of CENP-E reduced the velocity of moving chromosomes nearly threefold (Table II). A third antibody (1.6), raised against a portion of

Table II. Effect of CENP-E Antibodies on Depolymerization-driven Chromosome Movement

Treatment	Region on CENP-E	Mean rate
		(μm/min)
Control	n/a	36.2 (SD 8.5, n = 9)
mAb 177	rod	41.1 (SD 2.8, n = 5)
HX	rod	15.6 (SD 3.3, n = 5)
Not	motor domain	12.2 (SD 4.7, n = 5)
1.6	"neck" domain	0.0 (n = 7)

the molecule that comprises part of the motor and part of the stalk (the so called "neck" region), blocked motion entirely. The specificity of this antibody on immunoblots is documented in Fig. 6, lane 7. Chromosomes incubated in this neck region antibody for over 1 h on ice did not move in our assay. The effect caused by pAb-1.6 resembled that seen with kin2/HD. Control (untreated) chromosomes assayed during alternating experiments (on the same day) behaved normally (see Table II).

CENP-E Localizes to Mammalian Kinetochores throughout Anaphase A

Initial work on the cellular localization of CENP-E suggested that it began to leave kinetochores at anaphase onset, the time when chromosome motion is most obviously associated with MT depolymerization (Yen et al., 1991). In light of our observations that CENP-E may be involved in disassembly-dependent chromosome motion, we reinvestigated CENP-E localization in U2-OS cells, a human osteosarcoma line in which cells in anaphase A are seen more frequently than in the previously studied HeLa cells. With this cell type, as with PtK cells (not shown), it is evident that CENP-E stays on kinetochores until sometime late in anaphase A (Fig. 8 *b*), by which time the disassembly of kinetochore-associated MTs is over.

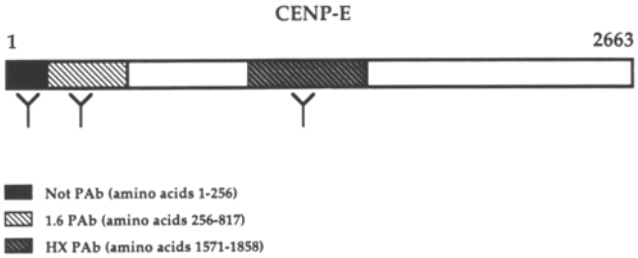

Figure 7. Schematic diagram showing the regions of the CENP-E molecule that were used for antibody production in rabbits (*Not*, *1.6*, and *HX*).

Discussion

We have used an immunological approach to examine the role of various chromosomal proteins in depolymerization-dependent chromosome movement in vitro. Our results implicate CENP-E in coupling chromosomes to depolymerizing MTs.

This report does not support a role for cytoplasmic dynein in depolymerization-dependent motility of chromosomes. Although the anti-dynein antibody used has been shown to bind kinetochores and to inhibit ATP-dependent dynein motor activity in vitro (Vaisberg et al., 1993), it does not affect MT-depolymerization–dependent chromosome movement. This result is consistent with the observation that UV-VO_4 cleavage of dynein fails to block chromosome motion in our assay. Neither of these negative results, however, rules out a role for dynein in disassembly-dependent chromosome movement. UV-cleaved cytoplasmic dynein retains the ability to bind and cosediment with MTs, even though its ATP-

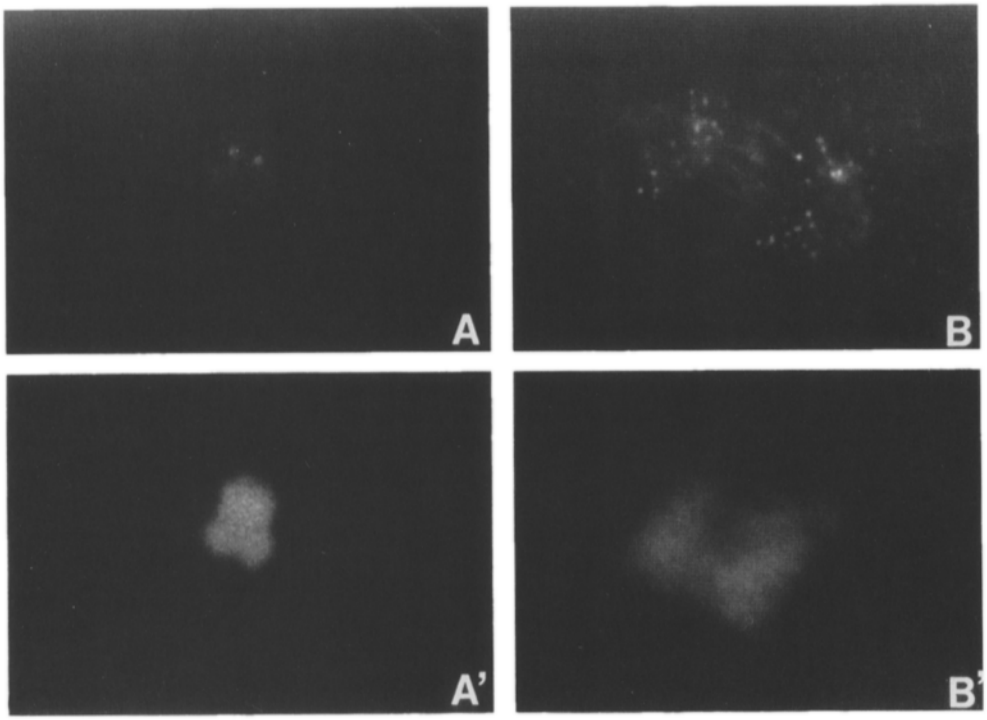

Figure 8. CENP-E staining on chromosomes in vitro and in vivo. (*A*) CENP-E is localized to kinetochores of isolated CHO chromosomes. The image is representative of kinetochore staining observed with all CENP-E antibodies used in this study (mAb 177 and pAbs-1.6, Not, and HX). This particular chromosome is stained with mAb 177 (in *A*) and DAPI (in *A'*). (*B*) CENP-E is localized to kinetochores throughout anaphase A in cells. Staining U2-OS human osteosarcoma cells with CENP-E antisera (*HX*) revealed that CENP-E remains kinetochore-bound throughout anaphase A. As reported earlier (Yen et al., 1991), CENP-E loses its kinetochore localization toward the later stages of mitosis and is then diffusely localized to spindle fibers. *B'* is the same cell stained with DAPI to visualize the DNA.

dependent motility is inhibited (Pfarr, 1990). Thus, if dynein is involved in coupling chromosomes to pellicle-initiated MTs, the cleavage reaction may not affect the binding required for disassembly-dependent motility. Moreover, the DD1 antibodies used here do not affect dynein's binding to MTs (Vaisberg et al., 1993), so this antibody may not inactivate the functions of dynein that are important for the motility we observe. A more appropriate reagent for assessing dynein's role in disassembly-dependent motility might be an antibody that recognizes the entire dynein motor domain or, more specifically, its MT-binding site. More direct tests of dynein's contribution to this kind of movement using dynein-coated spheres show that purified cytoplasmic dynein alone does not support depolymerization-driven motility in vitro (Lombillo et al., 1995). A role for dynein in depolymerization-dependent chromosome motility thus remains to be determined.

Results of our experiments with the kin2/HD antibody strongly support a role for a KLP in disassembly-dependent motility. This antibody is known to inhibit kinesin-driven vesicle dispersion in melanophores (Rodionov et al., 1991) and the extension of vimentin networks in vivo (Gyoeva and Gelfand, 1991). In vitro, kin2/HD blocks the binding of MTs to kinesin-coated glass (if MTs are added last) or inhibits motility of glass-bound MTs (if antibodies are added last) (Rodionov et al, 1991). Kin2/HD does not noticeably inhibit the binding of isolated chromosomes to MTs in our assay, although this reaction is difficult to quantify. Our assays with kin2/HD do, however, reveal that this antibody inhibits the ability of a chromosome to remain associated with the end of a MT as it shortens, which in turn causes the chromosome to fall off the MTs with which it is associated.

Given the broad range of reactivity of kin2/HD, we sought to determine which particular KLP might be affected by the kin2/HD antibody, by using more specific probes against kinetochore-associated KLPs. Wordeman and Mitchison (1994) have recently identified a 90-kD KLP that localizes specifically to kinetochores of isolated CHO chromosomes, called MCAK. Antibodies raised against MCAK showed no effect on depolymerization-dependent motion. It is not known if these antibodies are able to block the function of their antigen (Wordeman, L., personal communication), but the observation that kin2/HD does not recognize MCAK on immunoblots of chromosomal proteins suggests that this antigen is not a target of kin2/HD on chromosomes.

CENP-E is another KLP associated with the kinetochore during several stages of mitosis (Yen et al., 1992). The kin2/HD antibody strongly reacts with a chromosomal protein whose mobility is identical to CENP-E on immunoblots. Rodionov et al. (1993*a*) have also observed a strong cross reaction of kin2/HD with a 250-kD polypeptide. Kin2/HD typically recognizes several polypeptides in cell extracts and in isolated spindles, the most prominent polypeptide being conventional kinesin migrating at 120 kD (Rodionov et al., 1993*a*). Kinesin is associated with many vesicles (Schroer et al., 1988), so it is interesting that our immunoblots do not reveal a 120-kD band from the vesicles that are present in the chromosome preparation. These results suggest that the primary target affected by the kin2/HD antibody on chromosomes is the kinetochore-associated CENP-E.

Antibodies raised against bacterially expressed segments of CENP-E also affect chromosome movement in our assay. Though chromosome motility was not altered by an anti-CENP-E mAb, pAbs raised against either the motor domain or the tail of CENP-E slowed motility nearly threefold. The mechanistic implications of this "slowing phenotype" are not yet clear. One possibility is that CENP-E is indeed a coupling molecule, but it is only partially inactivated by these antibodies. Perhaps the compromised antigen can still bind near the end of the MT, but the resulting complex decreases the overall MT disassembly rate. In contrast, the antibodies raised against the "neck" region of CENP-E (part of the motor, part of the stalk) inhibited motion completely, as we observed with the kin2/HD antibody. Further studies on the function of this neck region of CENP-E should prove interesting. The neck of CENP-E could conceivably act like a hinge in an analogous manner to such a domain in the myosin molecule. Thus, bound 1.6 antibodies might change the molecular flexibility of this region and thereby alter some property that enables CENP-E to "ride" along a shortening MT lattice.

The ATP-dependent motility characteristics of purified CENP-E are not yet known. Neither its direction of movement along MTs nor its velocity have yet been observed. However, the directionality of a motor enzyme during its ATP-dependent motion might not be relevant here, since in our assays, the ATP concentration is much too low to allow motors to work by their conventional mechanism. Under our conditions, the disassembly-dependent motility may override a motor's ATP-dependent mechanochemical polarity. Further experiments will be needed to distinguish these multiple activities of kinetochore-associated motors.

Although our results with AMP-PNP are consistent with a role for a kinesin in disassembly-dependent motility, the effective concentrations are high relative to those required to block motility of true kinesin, especially since our experiments are performed in the absence of competing ATP. NCD, another KLP, displays mechanochemistry very similar to kinesin (Lockhart and Cross, 1994), but other KLPs show a variety of responses to AMP-PNP: CENP-E binds to and cosediments with stable MTs in the presence of 1 mM MgAMP-PNP (Yen et al., 1992; Liao et al, 1994) and MKLP-1 will bind tightly to MTs only at $\geq$2.5 mM AMP-PNP (Nislow et al., 1992). Given these observations, if kinesin or a KLP is involved in this form of chromosome movement, it may well possess a different sensitivity to AMP-PNP than that described for kinesin or known KLPs. On the other hand, depolymerization-dependent motility may simply be less sensitive to AMP-PNP than conventional motor activity.

The physiological significance of motility driven by MT dynamics is not yet clear. Most spindle MTs are highly dynamic during mitosis (Salmon et al., 1984; Saxton et al., 1984). Until anaphase, even the connections between chromosomes and MTs are labile (Gorbsky and Borisy, 1989), and kinetochore-associated MTs are continually changing length (for review see Bajer and Mole-Bajer, 1972). During anaphase A the MTs connecting kinetochores to spindle poles shorten, primarily depolymerizing at their kinetochores (Mitchison et al., 1986; Gorbsky et al., 1987, 1988; Michison and Salmon, 1992). It is therefore conceivable that kinetochore components like CENP-E could interact with dynamic MTs to contribute to chromosome motion during mitosis.

The movements studied here occur in the absence of ATP,

but it is unlikely that cellular ATP levels vary appreciably during mitosis. Furthermore, disassembly-dependent motion in our system is usually achieved by lowering the concentration of tubulin, but the cellular tubulin concentration is unlikely to drop dramatically during mitosis. While these properties of cells might imply that the motions we have studied are not relevant in vivo, disassembly-dependent movements can occur in the presence of ATP, both in principle and in fact (Lombillo et al., 1995). We have omitted nucleotide triphosphates in the experiments reported here to minimize the chances that the motions observed were contaminated by ATP-dependent events. In a similar vein, MT dynamics could be altered by changes less dramatic than subunit dilution, e.g., by posttranslational modification of tubulin or of MT-associated proteins, so physiological changes might promote an analogous MT disassembly to power motion in cells. Indeed, chromosome motions seen in our system are faster than all but the earliest prometaphase movements of chromosomes in vivo. If MT dynamics play a role in cellular chromosome movement, the factors that modulate tubulin polymerization in vivo are probably subtle departures from the steady state.

Efforts to interpret the biological significance of the data so far available must consider two important caveats: (*1*) we are working outside the cell and (*2*) we are following the behavior of kinetochores, which are biochemically complex structures. Kinetochores contain at least 2 KLPs, at least 1 cytoplasmic dynein, MT-associated structural proteins, and potentially modifying enzymes such as kinases and phosphatases. Given this complexity, the antibody effects described here could be indirect. For example, an antibody specific for a single protein might inactivate not only its target antigen but also other proteins that lie nearby. So, in conjunction with the "native" assay used here, we have recently developed an assay that uses only defined components (Lombillo et al., 1995). Accordingly, we have tested directly whether purified molecules bound to latex microspheres support motion on disassembling MTs and observed that kinesin isolated from HeLa cells will support minus end-directed, depolymerization-driven motion of microspheres (Lombillo et al., 1995).

The immunological study reported here strongly suggests that a KLP at the kinetochore is involved in depolymerization-dependent motility of chromosomes. Given the results with both the kin2/HD and the CENP-E (1.6) antibodies, CENP-E is likely to be part of the machinery that couples MT dynamics to chromosome motion.

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References

Bajer, A. S., and J. Mole-Bajer. 1972. Spindle dynamics and chromosome movements. *Int. Rev. Cytol. Suppl.* 3:1–271.

Bajer, A. S., C. Cypher, J. Mole-Bajer, and H. M. Howard. 1982. Taxol induced anaphase reversal: evidence that elongating microtubules can exert a pushing force in living cells. *Proc. Natl. Acad. Sci. USA.* 79:6569–6573.

Chandra, R., S. A. Endow, and E. D. Salmon. 1993. An N-terminal truncation of the ncd protein supports diffusional movement of MTs in motility assays. *J. Cell Sci.* 104:899–906.

Coue, M., V. A. Lombillo, and J. R. McIntosh. 1991. Microtubule depolymerization promotes particle and chromosome movement in vitro. *J. Cell Biol.* 112:1165–1175.

Endow, S. A., S. J. Kang, L. L. Satterwhite, M. D. Rose, V. P. Skeen, and E. D. Salmon. 1994. Yeast Kar3 is a minus-end microtubule motor protein that destabilizes microtubules preferentially at the minus ends. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:2708–2713.

Gibbons, B. H., and I. R. Gibbons. 1987. VO_4-sensitized cleavage of dynein heavy chains by 365 nm irradiation of demembranated sperm flagella and its effects on flagellar motility. *J. Biol. Chem.* 262:8354–8359.

Gliksman, N. R., and E. D. Salmon. 1993. Microtubule associated motility in cytoplasmic extracts of sea urchin eggs. *Cell Motil. Cytoskeleton.* 24:167–178.

Gorbsky, G. J., and G. G. Borisy. 1989. Microtubules of the kinetochore fiber turn over in metaphase but not in anaphase. *J. Cell Biol.* 109:653–662.

Gorbsky, G. J., P. J. Sammak, and G. G. Borisy. 1987. Chromosomes move poleward in anaphase along stationary microtubules that coordinately disassemble from their kinetochore ends. *J. Cell Biol.* 104:9–18.

Gorbsky, J. G., P. J. Sammak, and G. G. Borisy. 1988. Microtubule dynamics and chromosome motion visualized in living anaphase cells. *J. Cell Biol.* 106:1185–1192.

Gyoeva, F. K., and V. I. Gelfand. 1991. Coalignment of vimentin intermediate filaments with microtubules depends on kinesin. *Nature (Lond.).* 353: 445–448.

Heidemann, S. R., and J. R. McIntosh. 1981. Visualization of the structural polarity of microtubules. *Nature (Lond.).* 286:517–519.

Inoue, S. 1981. Cell division and the mitotic spindle. *J. Cell Biol.* 91:131s–147s.

Koshland, D. E., T. J. Mitchison, and M. W. Kirschner. 1988. Polewards chromosome movement driven by microtubule depolymerization *in vitro*. *Nature (Lond.).* 331:499–504.

Lasek, R. J., and S. T. Brady. 1985. Attachment of transported vesicles to microtubules in axoplasm is facilitated by AMP-PNP. *Nature (Lond.).* 316:645–647.

Liao, H., G. Li, and T. J. Yen. 1994. Mitotic regulation of microtubule cross-linking by CENP-E kinetochore protein. *Science (Wash. DC).* 265:394–398.

Lockhart, A., and R. A. Cross. 1994. Origins of reversed directionality in the ncd molecular motor. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:751–757.

Lombillo, V. A., M. Coue, and J. R. McIntosh. 1993. *In vitro* motility assays using microtubules tethered to *Tetrahymena* pellicles. *Methods Cell Biol.* 39:149–165.

Lombillo, V.A., R. J. Stewart, and J. R. McIntosh. 1995. Kinesin supports minus end-directed, depolymerization-dependent motion of microspheres. In press.

McDonald, H. B., R. J. Stewart, and L. S. B. Goldstein. 1990. The kinesin-like ncd protein of *Drosophila* is a minus end-directed microtubule motor. *Cell.* 63:1159–1165.

Mitchison, T. J., and E. D. Salmon. 1992. Poleward kinetochore fiber movement occurs during both metaphase and anaphase-A in newt lung cell mitosis. *J. Cell Biol.* 119:569–582.

Mitchison, T. J., L. Evans, E. Schulze, and M. W. Kirschner. 1986. Sites of microtubule assembly and disassembly in the mitotic spindle. *Cell.* 45: 515–527.

Miyamoto, H., and H. Hotani. 1988. Polymerization of microtubules in liposomes produces morphological changes of shape. *Proc. Tanaguchi Internat. Symp.* 14:220–242.

Nislow, C., V. A. Lombillo, R. Kuriyama, and J. R. McIntosh. 1992. A plus-end-directed motor enzyme that moves antiparallel microtubules in vitro localizes to the interzone of mitotic spindles. *Nature (Lond.).* 359:543–547.

Pfarr, C. M. 1990. Characterization of cytoplasmic dynein from HeLa cells. PhD. Thesis, MCD Biology, University of Colorado, Boulder. pp. 56–58.

Pfarr, C. M., M. Coue, P. M. Grissom, T. S. Hays, M. E. Porter, and J. R. McIntosh. 1990. Cytoplasmic dynein is localized to kinetochores during mitosis. *Nature (Lond.).* 345:263–265.

Rattner, J. B., M. J. Rao, M. J. Fritzler, D. W. Valencia, and T. J. Yen. 1993. CENP-F is a ca. 400 kDa kinetochore protein that exhibits a cell-cycle dependent localization. *Cell Motil. Cytoskeleton.* 26:214–226.

Rodionov, V. I., F. K. Gyoeva, and V. I. Gelfand. 1991. Kinesin is responsible for centrifugal movement of pigment granules in melanophores. *Proc. Natl. Acad. Sci. USA.* 88:4956–4960.

Rodionov, V. I., V. I. Gelfand, and G. G. Borisy. 1993*a*. Kinesin-like molecules involved in spindle formation. *J. Cell Sci.* 106:1179–1188.

Rodionov, V. I., F. K. Gyoeva, E. Tanaka, A. D. Bershadsky, J. M. Vasiliev, and V. I. Gelfand. 1993*b*. Microtubule-dependent control of cell shape and pseudopodial activity is inhibited by the antibody to kinesin motor domain. *J. Cell Biol.* 123:1811–1820.

Salmon, E. D., R. J. Leslie, W. M. Saxton, M. L. Karow, and J. R. McIntosh. 1984. Spindle microtubule dynamics in sea urchin embryos: analysis using a fluorescein-labeled tubulin and measurements of fluorescence redistribution after laser photobleaching. *J. Cell Biol.* 99:2165–2174.

Saxton, W. M., D. L. Stemple, R. J. Leslie, E. D. Salmon, M. Zavortink, and J. R. McIntosh. 1984. Tubulin dynamics in cultured mammalian cells. *J. Cell Biol.* 99:2175–2186.

Schroer, T. A., B. J. Schnapp, T. S. Reese, and M. P. Sheetz, 1988. The role of kinesin and other soluble factors in organelle movement along microtubules. *J. Cell Biol.* 107:1785–1792.

Sheldon, E., and P. Wadsworth. 1992. Microinjection of biotin-tubulin into anaphase cells induces transient elongation of kinetochore microtubules and reversal of chromosome to pole motion. *J. Cell Biol.* 116:1409–1420.

Steuer, E. R., L. Wordeman, T. A. Schroer, and M. P. Sheetz. 1990. Localization of cytoplasmic dynein to mitotic spindles and kinetochores. *Nature (Lond.).* 345:266–268.

Stewart, R. J., J. P. Thaler, and L. S. B. Goldstein. 1993. Direction of microtubule movement is an intrinsic property of the motor domains of kinesin heavy chain and *Drosophila* ncd protein. *Proc. Natl. Acad. Sci. USA.* 90:5029–4213.

Vaisberg, G., M. P. Koonce, and J. R. McIntosh. 1993. Cytoplasmic dynein plays a role in mammalian mitotic spindle formation. *J. Cell Biol.* 123: 849–858.

Vale, R. D., T. S. Reese, and M. P. Sheetz. 1985. Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell.* 42:39–50.

Vale, R. D., D. R. Soll, and I. R. Gibbons. 1989. One dimensional diffusion of microtubules bound to flagellar dynein. *Cell.* 59:915–925.

Walker, R. A., E. T. O'Brien, N. K. Pryer, M. F. Soboeiro, W. A. Voter, H. P. Erickson, and E. D. Salmon. 1988. Dynamic instability of individual microtubules analyzed by video light microscopy: rate constants and transition frequencies. *J. Cell Biol.* 107:1437–1448.

Wordeman, L. G., and T. J. Mitchison. 1994. Identification and characterization of MCAK, a kinesin-related protein that associates with centromeres during mitosis. *J. Cell Biol.* In press.

Wordeman, L. G., E. Steuer, M. P. Sheetz, and T. J. Mitchison. 1991. Distinct chemical subdomains within the kinetochore region of isolated CHO chromosomes. *J. Cell Biol.* 114:285–294.

Yen, T. J., D. A. Compton, D. Wise, R. P. Zinkowski, B. R. Brinkley, W. C. Earnshaw, and D. W. Cleveland. 1991. CENP-E, a novel centromere associated protein required for progression from metaphase to anaphase. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:1245–1254.

Yen, T. J., G. Li, B. T. Schaar, I. Szilak, and D. W. Cleveland. 1992. CENP-E is a putative kinetochore motor that accumulates just before mitosis. *Nature (Lond.).* 359:536–539.