Kinesin is Associated with a Nonmicrotubule Component of Sea Urchin Mitotic Spindles

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PNAS 1987;84;2771-2775
doi:10.1073/pnas.84.9.2771

This information is current as of April 2007.

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Notes:
Kinesin is associated with a nonmicrotubule component of sea urchin mitotic spindles

(model of mitosis/immunolocalizations)

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Communicated by Keith R. Porter, December 29, 1986 (received for review August 12, 1986)

ABSTRACT

Sea urchin embryos in second division have been lysed into microtubule-stabilizing buffers to yield mitotic cytoskeletons (MCSs) that consist of two mitotic spindles surrounded by a cortical array of filaments. Microtubules have been completely extracted from MCSs by incubation at 0°C with Ca2+-containing buffer. An antibody to the microtubule translocator kinesin stains the spindles in MCSs and in MCSs treated with 5 mM ATP and also stains spindle-remnants of the MCSs after the microtubules have been extracted. We conclude that kinesin binds to a nonmicrotubule component in the mitotic spindle. Based on these results, we present several models of kinesin function in the spindle.

Kinesin, a mechanochemical protein that mediates ATP-dependent motility of microtubules (MTs) in vitro (1, 2) is a good candidate for the motor active in vesicle movement during fast axonal transport (3, 4, 32). Scholey et al. (5) have reported that kinesin localizes in mitotic spindles of sea urchin embryos as determined by antibodies to kinesin in sea urchin kinesin. This result raises the possibility that kinesin might be involved in some aspect of chromosome movement.

Mitosis includes a complex series of movements, any one of which might be mediated by kinesin. For example, the movement of chromosomes away from the spindle pole during prometaphase (6) could result from kinesin bound to the kinetochore working on MT surfaces by a mechanism analogous to that used in axon transport of MTs in vitro (2; M. E. Porter, D. L. Stemple, M. P. Sheetz, R. D. Vale, J.R.M., and J.M.S., unpublished data). Kinesin might act in the overlap between half-spindles to generate shearing forces between adjacent MTs (7, 8). Alternatively, kinesin may not act to move chromosomes at all but may move vesicles within the spindle (9–14).

To better understand the role of kinesin in mitosis, we need to identify the spindle component(s) with which the protein interacts to generate motile forces. For example, kinesin might cross-link MTs to vesicles or kinetochores to generate force for vesicle or chromosome translocation along MTs (6, 15). It might cross-link MTs to MTs to generate force for MT-sliding in anaphase B, or it might cross-link MTs to a structural matrix to generate shear forces between MTs and the matrix.

We therefore used antibodies to kinesin (5) to probe the location of this protein within spindles of sea urchin embryos. We found that spindles that have been depleted of MTs by extraction with Ca2+-containing buffer at 0°C contain no residual tubulin as determined by immunoblotting or immunocytochemistry. However, when these MT-depleted spindle remnants and the Ca2+ buffer extracts were examined with anti-kinesin antibodies by immunoblotting and immu

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Materials and Methods

Animals. Sea urchins Strongylocentrotus franciscanus and Lytechinus pictus were collected from the Santa Barbara Channel by John Holcomb and Douglas Trim of the California Urchin Divers' Association.

Development. Gametes were collected by intracoelomic injection of 0.52 M KCl. L. pictus eggs were fertilized and allowed to develop as described by Salmon (16). S. franciscanus eggs were fertilized in the presence of 10 mM p-aminobenzoic acid, and fertilization membranes were removed by seven passes through 150-μm Nitex filters. The eggs were allowed to develop to second division in Ca2+-free sea water at 15°C with aeration and stirring.

Extracted Mitotic Cytoskeletons (MCSs). Embryos in second division were washed with isotonic buffer (560 mM NaCl/30 mM KCl/5 mM Tris, pH 8.0) and then were lysed into a MT-stabilizing buffer consisting of 100 mM Pipes, 1 mM magnesium acetate, 5 mM EGTA, 1 mM dithiothreitol, 1% Nonidet P-40, 10% glycerol, and a protease-inhibitor cocktail (0.1 mM phenylmethylsulfonyl fluoride and 1 mg of Nα-p-tosyl-L-arginine methyl ester, 1 μg of peptstatin A, 1 μg of leupeptin, 2 μg of aprotinin, and 0.1 mg of soybean trypsin inhibitor per ml) to yield MCSs. The embryos were pelleted, washed with glycerol-free MT-stabilizing buffer, and resuspended in the same glycerol-free MT-stabilizing buffer. MTs were extracted at 0°C from the cytoskeletons by addition of 1 M CaCl2 to make a 6 mM final concentration of total Ca2+ and a minimum of 1 mM free Ca2+ (Ca2+ buffer). The extraction process was confirmed by loss of spindle birefringence monitored with a Leitz Dialux microscope equipped with Polaroid HN22 linear polarizers. The Ca2+ buffer-solubilized proteins were separated from the insoluble cytoskeletons by centrifugation in an Eppendorf Microfuge for 10 sec. The binding of kinesin to spindles was examined by incubating MCSs for 10 min at room temperature in MT-stabilizing buffer containing 10 μM taxol, followed by a 10-min incubation in the kinesin-extraction buffer previously described (5) with ATP and taxol added at a final concentration of 5 mM and 10 μM, respectively.

Immunolocalizations of Kinesin and Tubulin. Lysed embryos were immobilized on polylysine-coated coverslips (0.1 mg/mL). The embryos were washed with either 10% glycerol or 10% glycerol/0.05% Triton X-100 and then immunostained as described previously (5). The antibodies used were a polyclonal antibody against sea urchin kinesin (5, 32) and a monoclonal antibody against sea urchin tubulin (14, 15).

Abbreviations: MT, microtubule; MCS, mitotic cytoskeleton; p[NH]ppA, 5'-adenylyl imidodiphosphate.

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FIG. 1. Extraction of tubulin from MCSs by Ca\textsuperscript{2+} buffer at 0°C. The birefringent MCSs were extracted in the presence of 1 mM free Ca\textsuperscript{2+} at 0°C, and the insoluble cytoskeletons were separated from the calcium-solubilized proteins by centrifugation as described. (A) Ca\textsuperscript{2+} buffer-insoluble cytoskeletons. Proteins from extracted MCSs were separated by two-dimensional gel electrophoresis and silver-stained. No stain was observed in the area of the gel in which tubulin is normally detected, shown by parentheses. (B) Ca\textsuperscript{2+} buffer-solubilized proteins. The supernatant from Ca\textsuperscript{2+} buffer extraction is enriched for tubulin.

of polylysine per ml) and then fixed by the method of Balczon and Schatten (17) with 90% methanol and 50 mM EGTA at −20°C. Fixed MCSs were washed with phosphate-buffered saline P\textsubscript{i}/NaCl (0.15 M NaCl/10 mM sodium phosphate, pH 7.4) and stained with rabbit antiserum to kinesin (5) or with a monoclonal antibody to β tubulin provided by M. Klymkowsky (Univ. of Colorado, Boulder). Antibody localization was visualized by the peroxidase–antiperoxidase method developed by Neighbors et al. for localization of monoclonal antibodies to sea urchin egg 80- and 78-kDa MT-associated proteins (B. Neighbors, P. Grissom, J.R.M., and J.M.S., unpublished data). Briefly, methanol-fixed embryos were washed into P\textsubscript{i}/NaCl (three times for 10 min each) and incubated in primary antiserum (2 hr at 37°C). Embryos were then incubated (1 hr at 37°C) in peroxidase-conjugated goat anti-rabbit antiserum diluted 1:20, washed with P\textsubscript{i}/NaCl (3 times for 10 min each), incubated (1 hr at 37°C) with peroxidase-conjugated goat anti-peroxidase antiserum diluted 1:40, and washed with Tris-buffered saline (three times for 10 min each); the bound peroxidases were stained with 4-chloro-1-naphthol (0.5 mg/ml). Bright-field images were recorded with a Zeiss photomicroscope III on technical pan film developed in Kodak HC110 at dilution B for 6 min.

Electrophoresis. Proteins were separated on polyacrylamide gels as described by Laemmli (18) or O’Farrell et al. (19) and electrophoretically blotted to nitrocellulose as described by Towbin et al. (20). Antibodies were labeled on blots with 125I-labeled protein A isolated from Staphylococcus aureus and then detected by autoradiography.

Taxol-Stabilized MTs. Taxol-stabilized MTs with kinesin bound were prepared essentially as described (5) with the following minor modifications. Unfertilized sea urchin eggs were homogenized in MT-stabilizing buffer minus Nonidet P-40 by using a loose-fitting pestle. The homogenate was clarified by centrifugation at 150,000 \times g for 60 min, and taxol was added to the supernatant to a final concentration of 20 μM (taxol buffer). 5'-Adenylyl imidodiphosphate (p[NH\textsubscript{2}]\textsubscript{2}ppA) was added to the taxol buffer-stabilized MTs to a final concentration of 1 mM (p[NH\textsubscript{2}]ppA buffer). MTs were pelleted by centrifugation at 100,000 \times g for 30 min. To confirm that S. franciscanus eggs contain kinesin, we prepared kinesin from MTs in p[NH\textsubscript{2}]ppA buffer by addition of ATP, which releases the kinesin, followed by Bio-Gel A-5m chromatography as described (5); with video microscopy we...

FIG. 2. (A) Monoclonal antibody to β tubulin stains spindles in MCSs as detected by peroxidase-antiperoxidase stain. (B) After Ca\textsuperscript{2+} buffer extraction, no stain is observed. (×95.)

FIG. 3. A polyclonal antibody to 134-kDa sea urchin kinesin stains proteins in taxol buffer-stabilized MTs and extracted MCSs but not in the tubulin-rich Ca\textsuperscript{2+} buffer extract. A silver-stained 10% polyacrylamide gel (lanes 1, 2, and 3) and its replica blot (lanes 1', 2', and 3') stained with the antibody to sea urchin kinesin show stain in a 134-kDa band in taxol buffer-stabilized MTs (lanes 1 and 1') and in extracted MCSs (lanes 3 and 3') but not in the Ca\textsuperscript{2+} buffer extract (lanes 2 and 2'). Antibodies were labeled on the blot with 125I-labeled protein A and then detected by autoradiography.
assayed fractions for their ability to translocate MTs over glass coverslips. As with other species of sea urchins (5), the motility-inducing activity copurifies with a 134-kDa polypeptide.

RESULTS

The preparation of MCSs from lysed sea urchin embryos has been described (16). We solubilized tubulin and associated proteins from such MCSs by extraction with Ca$_{2+}$ buffer at 0°C, and the tubulin-rich fraction was separated from the insoluble extracted MCSs by centrifugation. These two fractions were examined for the presence of kinesin and tubulin by gel electrophoresis, immunoblotting, and immunoperoxidase localizations of the antigens in fixed cells.

Behavior of Tubulin During Extraction from MCSs. We examined the efficiency of the Ca$_{2+}$ buffer extraction at 0°C of tubulin from MCSs in three ways. Our results indicate that tubulin is completely extracted from the MCSs by incubation with Ca$_{2+}$ buffer at 0°C. In a two-dimensional gel of proteins from extracted MCSs (Fig. 1A), silver staining was not observed in the region of the gel where tubulin is normally detected (marked by parentheses). A two-dimensional gel of proteins in the Ca$_{2+}$ buffer extract shows that tubulin was the most prominent protein in this extract (Fig. 1B). Tubulin was localized in spindles of MCSs (Fig. 2A) but not in Ca$_{2+}$ buffer-extracted MCSs (Fig. 2B) as demonstrated when these structures were stained with a monoclonal antibody to $\beta$ tubulin. The completeness of the extraction is also revealed in Fig. 3, lanes 2 and 3, in which a silver-stained polyacrylamide gel shows that tubulin (small arrow) was depleted from the extracted MCSs and enriched in the extract.

Behavior of Kinesin During Extraction of MCSs. To follow the behavior of spindle kinesin during microtubule extraction, we probed the tubulin-rich and tubulin-depleted fractions from MCSs with an antibody to kinesin. Proteins from extracted MCSs, the 0°C Ca$_{2+}$ buffer extract from MCSs, and taxol-stabilized MTs from unfertilized eggs prepared in the presence of p[NH]ppA to enhance kinesin binding were separated by NaDodSO$_4$/polyacrylamide gel electrophoresis. Fig. 3 shows a silver-stained gel containing these protein fractions (lanes 1, 2, and 3) and the blot of a replica gel (lanes 1', 2', and 3') that was stained with an anti-kinesin antibody raised against the sea urchin 134-kDa polypeptide (5). Kinesin is present in the preparation of taxol-stabilized MTs and in the tubulin-depleted MCSs but not in the tubulin-rich Ca$_{2+}$ buffer extract.

Localization of the Kinesin-Containing Component of the MCS. It has been reported (5) that both antisera and monoclonal antibodies to the 134-kDa subunit of sea urchin egg kinesin [affinity-purified by using electrophoretically purified egg kinesin 134-kDa subunit (21)] or with electrophoretically purified squid kinesin 110-kDa subunit] stained sea urchin egg mitotic spindles. We subsequently found that antisera raised to squid kinesin (a gift of R. Vale and M. P. Sheetz) affinity-purified on blots of electrophoretically purified sea urchin egg kinesin 134-kDa subunit also specifically stained spindles (J.M.S., unpublished data). These results support the hypothesis that kinesin is located in spindles.

To determine the location of kinesin within the MCS, we stained fixed second-division embryos with antibodies to kinesin. Sea urchin embryos in second division were lysed into MT-stabilizing buffer or Ca$_{2+}$-containing MT-destabilizing buffer. Polarization optical images of the MCSs show that all detectable spindle birefringence (Fig. 4A) was extracted from the MCSs lysed in the presence of Ca$_{2+}$ (Fig. 4D). Spindles stained with antikinesin show a fibrous distribution for kinesin consistent with an association with MTs in the spindles of sea urchin embryos, confirming the results of Scholey et al. (5). It is shown at high magnification in Fig. 4C that, in the presence of MTs, the kinesin antigen displays a fibrous staining pattern in half-spindles and asters of the metaphase spindle.

However, when tubulin was extracted from MCS, the kinesin antibody stained a spindle-shaped structure. The staining of this structure was densest in the areas between the kinetochores and the poles in both metaphase (4F) and anaphase (4E) spindles. In contrast to the fibrous staining observed in unextracted spindles, the staining in the absence of MTs was amorphous. The metaphase spindle shortened upon Ca$_{2+}$ buffer extraction of MTs, as reported by Salmon and Segall (22).

Kinesin bound to MTs is released by millimolar concentrations of ATP (1, 5). To determine whether binding of kinesin in spindles is also sensitive to ATP, we treated MCSs from L. pictus with 5 mM ATP in taxol buffer. The buffer used was similar to that used in the preparation of kinesin from partially purified MTs isolated in [p[NH]ppA buffer. Spindles stained with the sea urchin kinesin antibody in control and ATP-treated MCSs were indistinguishable. Thus,
the association of kinesin to the spindle in vitro does not appear to be ATP sensitive. We examined the accuracy of our identification and localizations of kinesin. Kinesin could be identified on electrophoretic immunoblots stained with whole antisera by its characteristic migration at 134 kDa (5). Localizations in fixed embryos were specific for kinesin because anti-134-kDa kinesin (5), anti-squid kinesin (from R. Vale and M. P. Sheetz), and blot affinity-purified anti-134-kDa kinesin gave similar staining patterns. As further evidence of specificity, we demonstrated that sea urchin kinesin prepared by Bio-Gel A-5m chromatography translocated MTs in vitro. After preincubation of the antisera with this active isolated sea urchin kinesin (2 hr at 37°C), spindle-staining was abolished, whereas preincubation of the antisera with phosphocellulose-purified bovine brain tubulin (23) had no effect on staining.

**DISCUSSION**

Our results suggest that kinesin is associated with a non-MT component in the spindle. The biochemical evidence (two-dimensional gels, immunoblots, and immunolocalizations) confirm that extraction with Ca2+ buffer at 0°C removes tubulin from MCSs, but kinesin remains behind. Our cytochemical evidence shows that the insoluble kinesin is localized in the remnant of the mitotic spindle and that the kinesin is not released from spindles by addition of 5 mM ATP to buffer. However, the fibrous character of the anti-kinesin staining of the spindle suggests that kinesin might be associated with MTs in addition to another spindle component. We have modeled some potential roles for kinesin in various aspects of spindle function (Fig. 5). Certain of these models are favored by the results reported here.

**Stability of MCSs.** MCSs from *S. franciscanus* are stable structures when prepared by high dilution of second-division embryos into EGTA lysis buffers. The cortices that surround the birefringent spindles stabilize them against disruption by the mechanical forces generated during rigorous washing procedures. These washing procedures allow us to examine a subset of spindle proteins selected for their strong association with the mitotic cytoskeleton.
Identification of Kinesin. We believe the antibody is monospecific for kinesin because antisera to squid and sea urchin kinesin and a blot affinity-purified antibody all stain spindles in similar locations. Also, preadsorption of the antiserum with isolated sea urchin kinesin abolished spindle staining, while a similar preincubation with tubulin had no effect on spindle staining.

Unique Binding Characteristics of Spindle Kinesin. When sea urchin egg extract supernatants are treated with apyrase to deplete ATP levels or with p[NH]ppA, kinesin binds and cosediments with MTs, whereas ATP dissociates kinesin from MTs (5). In contrast, kinesin was observed to localize in the spindle independent of the presence or absence of p[NH]ppA (5), but whether this was due to (i) ATP depletion during preparation of spindles and lysed cells, (ii) because kinesin binds spindle MTs and isolated MTs differently, or (iii) because kinesin is bound to a second component in the spindle was not known. Our observations that kinesin remains in the spindle after incubation with ATP or cold Ca\(^{2+}\) buffer favor the interpretation that kinesin is bound to a second component. Kinesin is not coextracted with MTs and, thus, is not bound solely to MTs, and it is not extracted after addition of ATP—a treatment known to dissociate kinesin and MTs in vitro.

There is a possibility that the tight association between spindles and kinesin observed here is an artifact of the preparative techniques. For example, vesicles with bound kinesin might be trapped by spindle structures during lysis. The specific concentration of kinesin in spindles of MECs suggests that our observations may be relevant to the living cell, but experiments that address the issue of function are needed.

Hypothetical Roles for Kinesin in Mitosis. The simplest explanation for the presence of kinesin in spindles is that it functions in vesicle transport; one likely function of kinesin in axons (Fig. 5, scheme A). Saltatory movements of vesicles in spindles have been demonstrated in embryos of marine organisms (10–13, 25). Fuge (14) notes that spindles from marine organisms contain unusually large quantities of vesicles. Based on in vitro observations of kinesin function (1–3), we would expect that kinesin in the spindle would be involved in transport of vesicles towards the plus ends of MTs, which are known to be distal to the spindle poles (26). Vesicles would not necessarily accumulate in these areas because there also could be a retrograde (pole-directed) motor active in the spindle.

Our data demonstrate that spindles that have been extracted and washed extensively with nonionic detergents, a treatment that extracts lipids from membranes, still stain with antikinesin antibody. It is possible that kinesin binds to membrane proteins of the spindle vesicles, and these proteins do not coextract with membrane lipids but are immobilized on cytoskeletal components analogous to the band III–spectrin interaction observed in erythrocyte ghosts (27).

Kinesin might be more directly involved in chromosome movement as depicted in Fig. 5, models B–D. Model B in Fig. 5 depicts the results of a hypothetical interaction of kinesin with a fibrous corona and interpolar MTs. An association of this form would create forces acting away from the spindle pole (6) and cause the chromosome to move towards the metaphase plate. Fig. 5, model C and Inset give details of how kinesin might act to extend an elastic or contractile matrix along MTs. Such a matrix or microtrabecular lattice has been proposed to be active in anaphase A (6, 28, 29). Kinesin might generate shear forces between MTs (Fig. 5, model D), which are thought to be important in spindle elongation (8, 9, 30, 31). Vale et al. (24) have demonstrated that kinesin can cause adjacent MTs to shear. The distribution of kinesin observed here is consistent with an involvement of kinesin in vesicle translocation or as a motor in a microtrabecular lattice. However, these results do not eliminate the possibility that kinesin might act specifically at kinetochores or in spindle elongation. The observations presented here spawn many new questions about the role of kinesin in mitosis.

We are very grateful for the enthusiastic help we received from Jon Holcomb and Douglas Trim of the California Urchin Divers’ Association. This work was supported by National Research Service Award GM09657 from the National Institute of General Medical Sciences to R.J.L., by Public Health Service Grant NS 13560 to L.W., by American Cancer Society Grant BC-498 to J.R.M., by a Medical Research Council traveling postdoctoral fellowship, an American Cancer Society grant BC 530, and by a British–American exchange fellowship from the British Heart Foundation and the American Heart Association to J.M.S.