Localization of Kinesin in Cultured Cells

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Abstract. Kinesin was isolated from bovine brain and used to elicit polyclonal antibodies in rabbits. The specificities of the resulting antibodies were evaluated by immunoblotting. Antibodies purified from these sera by their affinity for brain kinesin react with a polypeptide of ~120 kD in extracts from bovine brain, PtK_1 cells, and mouse neuroblastoma cells. They bind to a pair of polypeptides of ~120 kD present in crude kinesin prepared from *Xenopus* eggs and with a single polypeptide of ~115 kD in extracts from *Drosophila* embryos. Antibodies raised against kinesin prepared from fruit fly embryos (by W. M. Saxton, Indiana University, Bloomington, IN) and from neural tissues of the squid (by M. P. Sheetz, Washington University, St. Louis, MO) cross react with the mammalian, the fly, and the frog polypeptides. Kinesin antigen was localized in cultured cells by indirect immunofluorescence. PtK_1 cells in interphase showed dim background staining of cytoplasmic membranous components and bright staining of a small, fibrous, juxtanuclear structure. Double staining with antibodies to microtubules showed that the fibrous object was usually located near the centrosome. On the basis of shape, size, and location, we identify the kinesin-positive structure as a primary cilium. PtK_1 cells in mitosis are stained at their poles during all stages of division. The structure stained is approximately spherical, but wisps of faint fluorescence also extend into the body of the spindle. Antibodies to squid or fruit fly kinesin produce identical patterns in PtK_1 cells. Controls with preimmune and preabsorbed sera show that the centrosome staining is not due simply to the common tendency of rabbit antisera to stain this structure. Similar centrosome and spindle pole staining was visible when antibodies to bovine brain or squid kinesin were applied to the A6 cell line (kidney epithelial cells from *Xenopus laevis*). Some possible functions of kinesin localized at the spindle poles are discussed.

KINESIN is a protein that binds to microtubules in the absence of ATP or in the presence of 5′-adenylyl imidodiphosphate (AMP-PNP),[1] a nonhydrolyzable ATP analogue (35). It shows a microtubule-activated MgATPase activity (5, 16). Kinesin can bind to either glass or latex spheres, and in the presence of MgATP, it moves these objects over the surfaces of microtubules. The structural polarity of the microtubule defines the direction of this movement (38), and all kinesins so far studied move the attached objects toward the plus end of the microtubule (37). The velocity of kinesin-supported movement depends on the concentration of ATP and shows an apparent K_m of 50–120 μM (5, 24).

Kinesin is found in brain tissue, where it is thought to contribute to orthograde axonal transport (37). It is also present in sea urchin eggs and embryos, where it becomes concentrated in the mitotic spindle (30), and preliminary reports suggest that the protein is widely distributed among organisms and cultured cells (23, 26). Kinesin remains in mitotic spindles during their isolation from sea urchin embryos, even after the microtubules have been extracted (18), so one can conclude that the protein adheres to these spindles by bonds in addition to those it forms with tubulin. The role of kinesin in mitosis, or in any aspect of cell behavior, is still unknown. It appears likely that kinesin serves as a motor to move granules and vesicles over microtubule surfaces, but it may also mediate the interaction between microtubules and other structural components of cytoplasm, thereby moving microtubules and perhaps chromosomes through the cell (38).

To gain some insight into the possible roles of kinesin in vivo, we have studied its localization in several cultured cells where the flatness of the preparation facilitates microscopy. We have raised two antibodies to mammalian kinesin from bovine brain and have obtained antibodies to both squid and fruit fly kinesin through the generosity of colleagues. The evidence obtained with all these antibodies is consistent. A small amount of kinesin appears to be associated with a cytoplasmic, membranous reticulum, and there is a concentration of the antigen on centrosomal structures during all stages of the cell cycle. The morphology of the centrosomal staining changes from interphase to mitosis. In interphase PtK_1 cells, one sees a short, rodlike fiber, which we inter-

1. *Abbreviations used in this paper*: AMP-PNP, 5′-adenylyl imidodiphosphate; MT, microtubule.

pret as the primary cilium; a general staining of the spindle pole and associated material appears in mitosis.

Materials and Methods

General

Column chromotography was performed using routine procedures as recommended by the manufacturers (Bio-Rad Laboratories, Richmond, CA; Whatman, Inc., Clinton, NJ; and Pharmacia, Inc., Piscataway, NJ). Protein concentrations were estimated by the method of Bradford (4), with bovine gamma globulin as standard. SDS-PAGE was performed as described by Laemmli (17) and the resulting gels were stained with Coomassie Blue or according to the silver staining procedure of Merrill et al. (20). Molecular mass standards for SDS-PAGE were myosin, B-galactosidase, phosphorylase B, BSA, ovalbumin, and carbonic anhydrase. Immunoblots were prepared and probed as described in detail by Goldstein et al. (10).

Preparation of Proteins

Kinesin was prepared using 500 g of white matter from fresh bovine brain tissue. Initial steps were carried out as described by Vale et al. (35). Brains were homogenized in buffer (50 mM Pipes, 50 mM Hepes, 2 mM $MgCl_2$, 1 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsufonal fluoride, 10 μg/ml leupeptin, 10 μg/ml tosylarginine methylester, 0.5 mM ATP, pH 7.0) and centrifuged to produce a high speed supernatant. GTP and taxol were added to the supernatant, and it was warmed to allow microtubules (MTs) to polymerize. MTs containing the majority of the microtubule-associated proteins were removed by centrifugation. Succeeding steps were carried out by a modification of the method of Vale et al. (35). Apyrase (1 U/ml) was added to the MT-depleted supernatant and incubated for 10 min at 23°C to convert endogenous ATP and ADP to AMP. AMP-PNP (1 μM), taxol (10 μM), and 100 μg/ml of microtubules prepared from phosphocellulose-purified tubulin (40) polymerized for 15 min at 25°C in the presence of 10 μM taxol were then added to the extract and incubated for 30 min. The MTs and associated kinesin were sedimented at 37,000 *g* for 20 min at 20°C. Crude bovine kinesin was released from the taxol-MTs by homogenization of the pellets and extraction in a total of 10 ml of 10 mM Mg-ATP + 0.1 M NaCl for 20 min at 0°C, followed by centrifugation at 37,000 *g* for 20 min. The supernatant proteins were separated by gel filtration on a 2 × 30 cm column of Bio Gel A-5M (Bio-Rad Laboratories) that had been equilibrated with homogenization buffer. Fractions containing peak concentrations of proteins that induced microtubule or axonemal gliding over glass were pooled and carried through a second cycle of MT binding and release. The pooled proteins were mixed with taxol-polymerized phosphocellulose-purified tubulin in the presence of 1 U/ml apyrase and 1 μM AMP-PNP, then centrifuged as above. The pellet was again resuspended in the presence of ATP to release kinesin and centrifuged to pellet the MTs. The resulting supernatant is called MT-affinity-purified kinesin. It was frozen and stored at −70°C. The purity of the kinesin preparation was analyzed on 5–11% gradient SDS-PAGE stained with Coomassie Blue. To identify the 120-kD polypeptide of kinesin in the preparations, and to assess its purity at different stages of the procedure, the relevant protein mixtures were run on SDS-PAGE, blotted to nitrocellulose, and probed with antibodies raised against kinesin from squid axoplasm (Fig. 1).

Crude kinesin was prepared from PtK_1 and mouse neuroblastoma tissue culture cells as described above for bovine brain, except that the extraction buffer was 100 mM Pipes, 1 mM $MgCl_2$, 0.1 mM EDTA, 2 mM EGTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsufonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml tosylarginine methylester, and 0.5 mM ATP, pH 6.9. Whole cells were prepared for immunoblotting by washing them in extraction buffer, homogenizing them with a Potter homogenizer (Thomas Instruments, Philadelphia, PA) for 45 s at 0–4°C, and then pipetting the homogenate rapidly into electrophoresis sample buffer at 100°C.

Kinesin was prepared from eggs of *Xenopus laevis* (a generous gift of M. W. Klymkowsky, University of Colorado), using the procedure that Scholey et al. (30) used for sea urchin eggs. *Drosophila* kinesin was prepared by W. M. Saxton (27). The presence of kinesin in each of these preparations was confirmed by the recognition of a polypeptide of ~120 kD on immunoblots by antibodies to squid and/or bovine kinesin.

Motility Assay

Kinesin, prepared as described above, was tested for its ability to induce the translocation of salt-extracted axonemes over glass. Fractions eluted from the Bio Gel A-5M column were allowed to adsorb to coverslips. Sea urchin sperm axonemes depleted of dynein arms (24) were added, together with 1 mM ATP. The coverslips were inverted onto glass slides and sealed. The gliding of axonemes was observed by means of videoenhanced, differential interference contrast optics on a Zeiss Photo microscope II equipped with a 100× oil immersion lens and a Dage Newvicon video camera.

Preparation of Antibodies

The MT-affinity-purified bovine brain kinesin used as immunogen was prepared as described above and subjected to electrophoresis in a 5–11% acrylamide gradient on a preparative slab gel, 1.5 mm thick. The gel was stained with Coomassie Blue and destained. The 120-kD kinesin band was excised, equilibrated in PBS, macerated, and emulsified with an equal volume of Freund's complete adjuvant.

Two 6–8-wk-old female New Zealand white rabbits were bled from their ear veins to collect preimmune sera to serve as controls. The rabbits were then injected subcutaneously at several sites on the back with 100–500 μg of the 120-kD polypeptide of kinesin. They were boosted six times within a period of 5 mo with electrophoretically purified kinesin emulsified with incomplete Freund's adjuvant. The rabbits were test bled at regular intervals and the sera were analyzed by immunofluorescence microscopy on PtK_1 cells and by immunoblotting against crude kinesin transferred to nitrocellulose, using the method of Goldstein et al. (10). Antisera that reacted with the 120-kD polypeptide at a dilution of 1:1,000 were present after the second boost. At the seventh bleed, contaminating antibodies were detected. The rabbits were then anesthetized and exsanguinated by cardiac puncture.

Purification of Antibodies

IgG was prepared from preimmune and immune sera by three different methods: (*a*) by precipitation with 40–50% $(NH_4)_2SO_4$, pH 7.4; (*b*) by ion exchange chromatography on DEAE cellulose (DE-52; Whatman), in which IgG was eluted with a 5–300 mM gradient of phosphate buffer, pH 8.0 (14); (*c*) by affinity chromotography on protein A–Sepharose CL-4B (Pharmacia, Inc.) for which IgG was eluted with 0.1 M citrate-phosphate buffer, pH 2.9, and quickly brought to pH 7.4 by the addition of 1 M Tris, pH 9.0 (13). Purified IgG was dialyzed against PBS, and either stored at 4°C with 0.02% axide or further purified.

Antibodies of defined specificity were purified from the immune IgG fractions either by the blot-affinity purification procedure of Olmsted (32) or by affinity purification on a column of kinesin immobilized on Sepharose. In the former method, the antibody was eluted from the nitrocellulose paper with 3 M potassium thiocyanate at pH 7.5, followed by dialysis against PBS. In the latter method, ~300 μg of MT-affinity purified kinesin was reacted with 1 g of washed, cyanogen bromide-Sepharose 4B, according to manufacturer's (Pharmacia, Inc.) instructions. After conjugation, the column was equilibrated with PBS, and the IgG fraction from 10 ml of immune serum was run into the affinity matrix, allowed to equilibrate, and then eluted with 0.2 M glycine, pH 2.8. Eluted fractions were quickly neutralized with 1 M Tris, pH 9, pooled, dialyzed against PBS, and concentrated by ultrafiltration (Amicon model M3 or Centricon model 30; Amicon Corp., Danvers, MA).

The specificity of purified antibodies was tested by immunoblotting against whole cell extracts from cultured cells or crude kinesin from PtK_1 cells, bovine brain, and eggs of either *Drosophila* or *Xenopus*. Preimmune IgGs were tested as controls.

Immunofluorescence Microscopy

Kidney cells from the rat kangaroo (strain PtK_1) and neuroblastoma cells from the mouse (strain HL-1) were grown in Ham's F-12 (Gibco, Grand Island, NY) supplemented with 10% FCS. Kidney epithelial cells from *Xenopus laevis* (strain A6) were grown in 85% Leibovitz's L-15 medium supplemented with 10% newborn calf serum. Cells were subcultured for immunofluorescence microscopy on 12 mm coverslips. The preparations were fixed and permeabilized directly in anhydrous methanol at −20°C for 10 min, or in 2% paraformaldehyde, 0.1% glutaraldehyde in Pipes buffer. After rehydration in PBS and preincubation in 10% FCS in PBS, the coverslips were incubated for 90 min at 37°C in various dilutions of rabbit antibodies to bovine, squid, or *Drosophila* kinesins. Preimmune IgG fractions of the antibodies and mouse anti-sea urchin beta tubulin (29) were used as controls. After three 10-min washes in PBS, the cells were incubated for 90 min at 37°C in a dilution of 1:100–1:500 of affinity-purified, fluorescein-

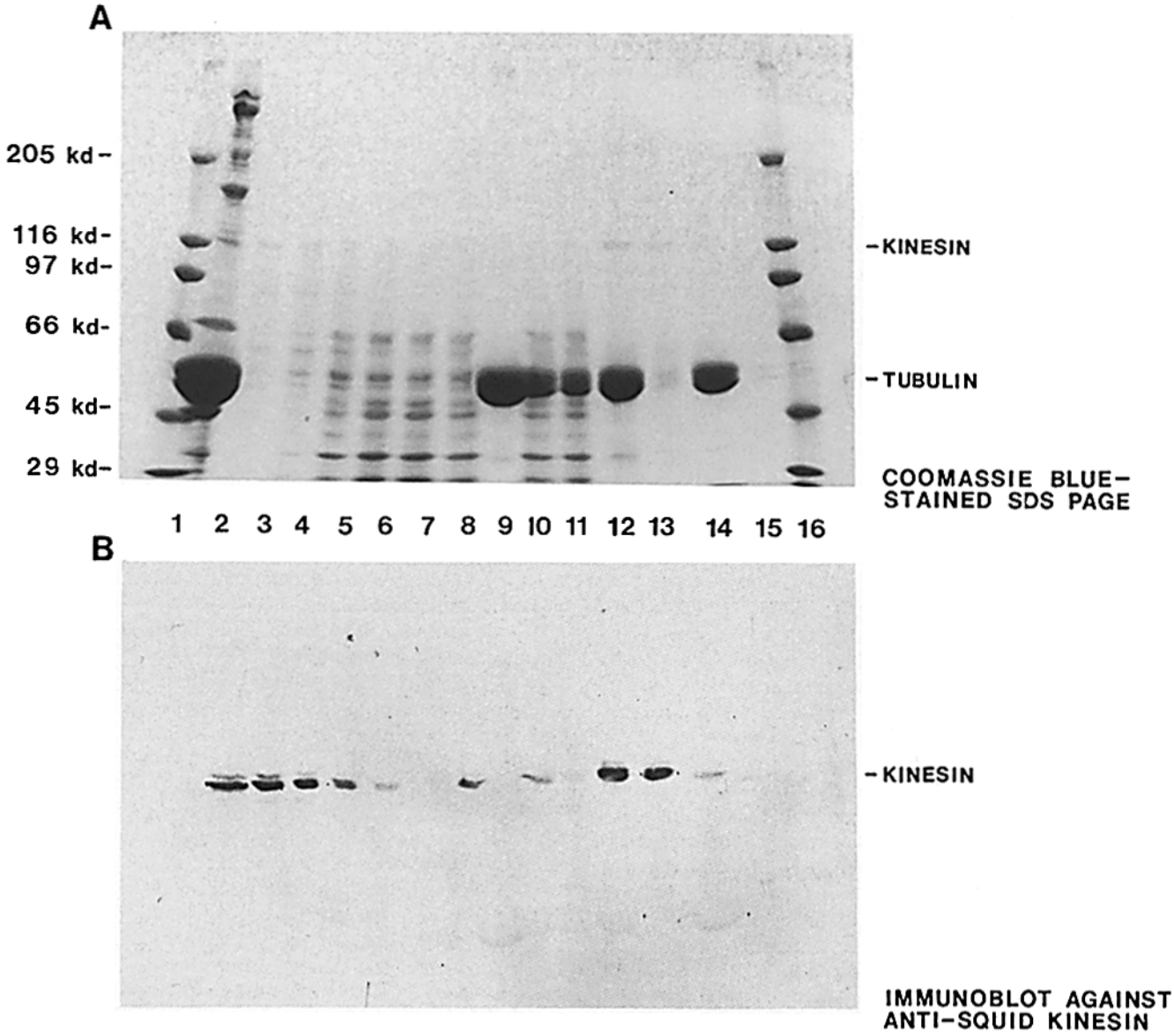


Figure 1. Preparation of bovine brain kinesin. (*A*) Slab SDS-PAGE showing stages in the preparation of bovine kinesin. Lanes *1* and *16* are molecular mass markers. Lane *2* is the pellet of MTs and associated proteins, including kinesin, formed from the MT-depleted brain extract upon the addition of apyrase, AMP-PNP, tubulin, and taxol as described in Materials and Methods. Kinesin was released from the MTs and chromatographed on a gel filtration column. The fractions that induced axoneme movement over glass are shown in lanes *3–7*. These were pooled (lane *8*). Taxol-stabilized MTs (lane *9*) were mixed with the pooled kinesin fractions, apyrase, and AMP-PNP (lane *10*), and spun. Lane *11* shows the supernatant; lane *12* the pellet. The kinesin was extracted and the MTs again sedimented; lane *13* is the kinesin-containing supernatant; and lane *14* is the pellet. The latter was extracted a second time, and spun, yielding little additional kinesin in the supernatant, as shown in lane *15*. (*B*) Nitrocellulose immunoblot of the gel in *A* incubated with anti-squid kinesin antiserum, a gift from M. Sheetz, diluted to 1:500. After incubation in peroxidase-conjugated goat anti-rabbit IgG (1:1,000 dilution; Bio-Rad Laboratories) the blot was developed in 4-chloro-1-naphthol. The immunoblot provides evidence that our procedure purifies kinesin.

or rhodamine-labeled goat anti-rabbit or goat anti-mouse immunoglobulins (Zymed, South San Francisco, CA). After three 10-min washes in PBS and one wash in distilled H_2O, the coverslips were mounted in Gelvatol (Monsanto, Indian Orchard, MA) with *n*-propyl galate (9). Localization of kinesin was observed on a Zeiss Photomicroscope II equipped with epi-fluorescence optics, using a 63× planapo oil immersion objective. Images were recorded in Ilford XP1-400 film.

Results

Characterization of the Antibodies

The presence of kinesin in preparations from bovine brain was established by several criteria. A polypeptide of ~120 kD was seen by SDS-PAGE (Fig. 1 *A*). This mammalian protein crossreacted with antibodies to squid kinesin generously provided by Dr. Michael P. Sheetz of Washington University, St. Louis, MO (Fig. 1 *B*). Note that this antiserum, used without affinity purification, also recognized a faint band just above kinesin in the preparations containing larger amounts of kinesin (Fig. 1, *A* and *B*, lanes *3*, *4*, *12*, and *13*). It also gave a faint staining of the tubulin band (Fig. 1, lanes *9–12*, and *14*). The elution behavior on Bio Gel A-5M of the native protein containing the 120-kD, anti-kinesin-positive poly-

peptide corresponded to a spherical molecule with a molecular mass of several hundred kilodaltons. The fractions, containing peak amounts of the 120-kD polypeptide, supported an ATP-dependent translocation of axonemes over glass coverslips (data not shown).

The bovine brain 120-kD polypeptide used as immunogen was further purified by a second step of MT binding and release, (see lanes *12–14* in Fig. 1), then by preparative SDS-PAGE, as described in Materials and Methods. The resulting antisera were fractionated by chromatography. The fractions containing IgG included antibodies that recognized polypeptides of ~120 kD in immunoblots against crude preparations of kinesin from mouse neuroblastoma or PtK_1 cells and from bovine brain (Fig. 2). A single polypeptide was also recognized, albeit more weakly, in a purer preparation of kinesin from embryos of *Drosophila*, an isolate which was shown by W. M. Saxton to translocate MTs in vitro (26). Comparable concentrations of the preimmune sera from the rabbits immunized with bovine kinesin did not react detectably with any component of the preparations (data not shown). Neither the crude antisera nor the purified antibodies recognized any polypeptides in immunoblots against whole cell extracts suggesting that the level of kinesin in these cells is too low to be detected without some prior enrichment of the antigen.

Affinity-purified antibodies against squid kinesin also recognized polypeptides of ~120 kD in preparations of kinesin made from cultured cells and eggs of *Xenopus*. (Fig. 3). The immunoblot against crude *Xenopus* egg kinesin suggested that this protein comprises a doublet of polypeptides (Fig. 3). This observation was confirmed by immunoblots with affinity-purified antibodies to bovine brain kinesin. Fig. 3 *C* shows the patterns of polypeptides obtained from *Xenopus* egg extracts when kinesin was prepared by MT affinity in the presence or absence of AMP-PNP. Fig. 3 *D* is the immunoblot of an identical gel, showing the polypeptides that react with affinity-purified anti-bovine kinesin. A doublet of bands binding anti-kinesin at ~115 and 120 kD is seen in the lane showing MTs prepared with AMP-PNP. The facts that the two polypeptides are equally prevalent, that they both bind MTs in an AMP-PNP-dependent manner, and that both bands bind both affinity-purified anti-kinesins, suggest that *Xenopus* eggs contain two kinds of kinesin that differ slightly in their molecular masses.

Localization of Kinesin Antigen in Cultured Cells

All of our antibody preparations were used for indirect immunofluorescence to localize the corresponding epitopes in fixed, cultured cells. The staining of PtK_1 cells obtained with the DEAE-purified IgG fraction from the anti-bovine brain kinesin is shown in Fig. 4. Interphase cells show strong staining of a small, juxtanuclear rodlike fiber (Fig. 4 *A*). All PtK_1 cells examined showed this component, and multinuclear or polyploid cells in the cultures usually contained more than one such structure. On the basis of their size, shape, and position, we interpret the kinesin-positive fibers as primary cilia, the nonmotile axonemes that frequently grow from the mother centriole of interphase cells in culture (1, 15, 39). The interphase cytoplasm also shows a faint fibrous and reticular staining with the anti-bovine kinesin antibodies. Though not dramatic, this staining is above the background level seen in the preimmune serum controls (Fig. 4 *F*). There is also a persistent staining of the interphase nucleoli.

Mitotic cells show a different pattern of kinesin staining. Some general fibrous staining of the dividing cells is evident, and from prometaphase through anaphase there is bright staining of an approximately spherical object at each spindle pole (Fig 4, *B–D*). Similar staining is seen in prophase and telophase as well. The preimmune sera showed no similar pattern of staining (Fig. 4 *F*), even though spindle pole staining is a feature common to many rabbit preimmune sera (6). Some of the brightly staining prometaphase and metaphase cells showed a faint and wispy staining of fibers extending from the poles into the body of the spindle (Fig. 4 *C*), but in PtK_1 cells we never saw the general spindle staining previously described in embryos of sea urchins (30). Occasional cells showed punctate staining at the chromosomes, which may have been due to binding of the antibody to the kinetochore, but this was not a consistent feature of our preparations.

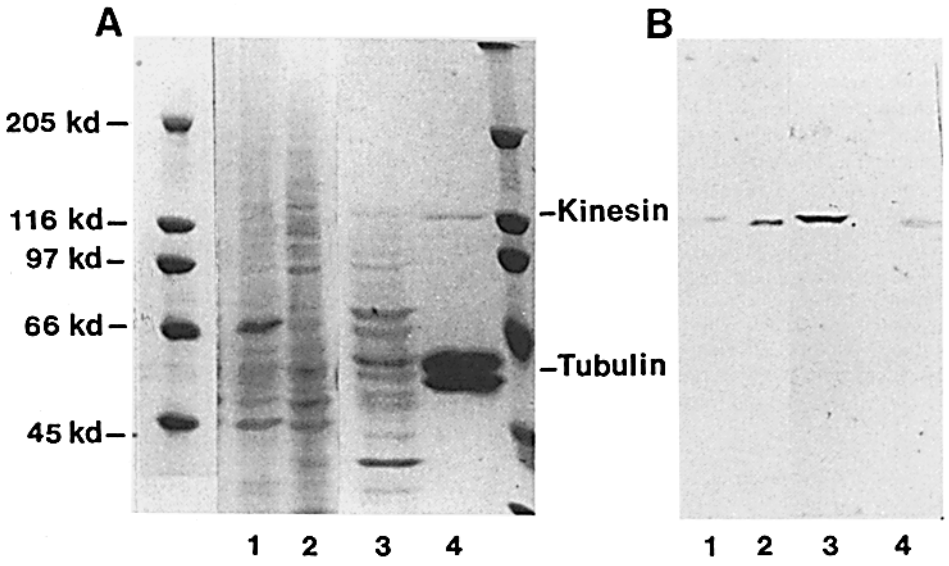
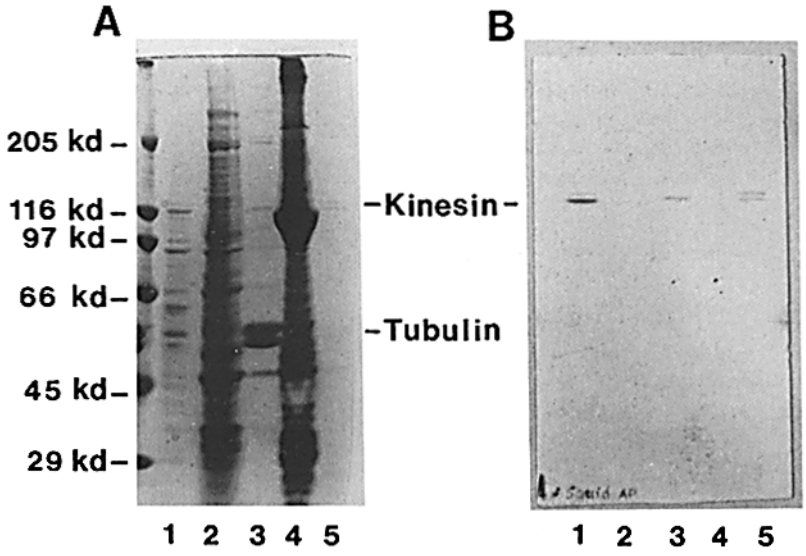


Figure 2. Immunoblots against anti-bovine kinesin. Crude kinesin was prepared from cultures of mouse neuroblastoma and PtK_1 cells as described in Materials and Methods. Partially purified kinesin from eggs of *Drosophila melanogaster* was a gift from W. M. Saxton. (*A*) Coomassie Blue-stained 5–11% gradient SDS-PAGE of crude kinesin prepared from mouse neuroblastoma (lane *1*), PtK_1 cells (lane *2*), bovine brain (lane *3*), and *Drosophila* eggs (lane *4*). (*B*) Immunoblot of an identical gel transferred to nitrocellulose and incubated with DEAE-purified IgG from anti-bovine brain kinesin and developed with peroxidase-labeled goat anti-rabbit IgG.

As a control for the possibility that fixation with cold methanol might not preserve the cellular distribution of kinesin antigen, several preparations were fixed with paraformaldehyde/glutaraldehyde. The immunofluorescent localization of kinesin in these preparations was essentially the same as that in the methanol-fixed material, except that the background staining was brighter and the kinesin staining was fainter (Fig. 4 *E*).

Although the IgG fraction from both antisera appeared on immunoblots to be monospecific for the 120-kD polypeptide of kinesin, we affinity-purified antibodies specific for kinesin by column (Fig. 5, *A* and *B*) and blot (Fig. 5, *C* and *D*) affinity purification techniques, as described in Materials and Methods. The extent of general nuclear and cytoplasmic staining was thereby reduced, but the staining of the reticular material in the cytoplasm was still above background, particularly with the blot affinity-purified antibodies (Fig. 5 *C*), suggesting that this weak antibody binding is really due to kinesin. The stainings of the juxtanuclear fiber of interphase and of the spindle poles in mitosis were indistinguishable in the preparations stained with affinity-purified anti-bovine kinesin (Fig. 5) and with DEAE-purified anti-bovine kinesin IgG (Fig. 4).

As a further control for antibody specificity, we absorbed preparations of anti-bovine kinesin IgG with bovine kinesin purified by two cycles of microtubule affinity. (The purity of the antigen used is shown in Fig. 1 *A*, lane *13*.) Unabsorbed antibody stained the kinesin band on an immunoblot strongly at 1:1,000 dilution. Immunoblot staining with the absorbed serum was undetectable after dilution by 1:50. The unabsorbed serum at 1:100 gave the characteristic immunofluorescence images shown in Fig. 4. The absorbed serum at the



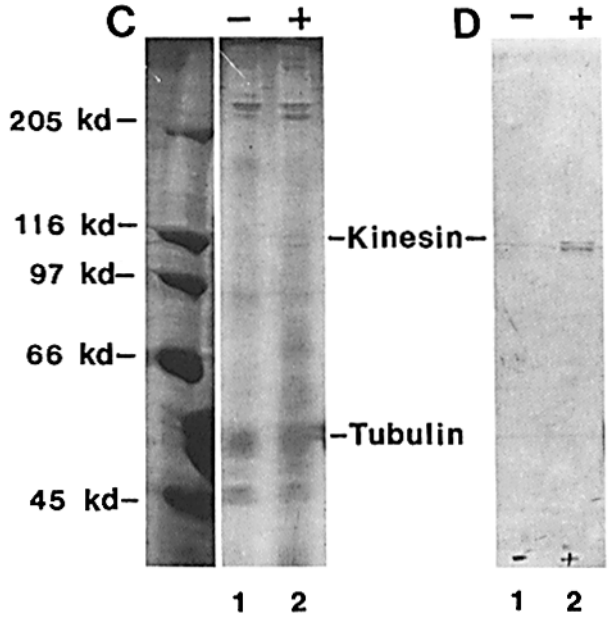


Figure 3. Cross-reactivity of anti-kinesin against different kinesin polypeptides prepared as described in Materials and Methods. (*A*) Coomassie Blue-stained 5–11% gradient gel of crude bovine kinesin (lane *1*), whole PtK_1 cell homogenate (lane *2*), crude PtK_1 kinesin (lane *3*), whole egg extract from *Xenopus laevis* (lane *4*), and crude *Xenopus laevis* kinesin (lane *5*). (*B*) Immunoblot of an identical gel transferred to nitrocellulose and incubated with column affinity-purified anti-squid kinesin and developed with perixodase-labeled goat anti-rabbit IgG. A faint band is seen above kinesin in the bovine preparation and the *Xenopus* kinesin contains approximately equal amounts of two reacting components. *C* and *D* confirm that *Xenopus* kinesin appears to comprise two electrophoretic components of similar molecular mass. (*C*) Silver-stained 5–11% gradient gel samples of crude *Xenopus* kinesin prepared in the absence of AMP-PNP (lane *1*) or in the presence of AMP-PNP (lane 2). (*D*) Immunoblot transfer of a gel identical to *C* stained with anti-bovine kinesin. A doublet staining with this antibody is evident.

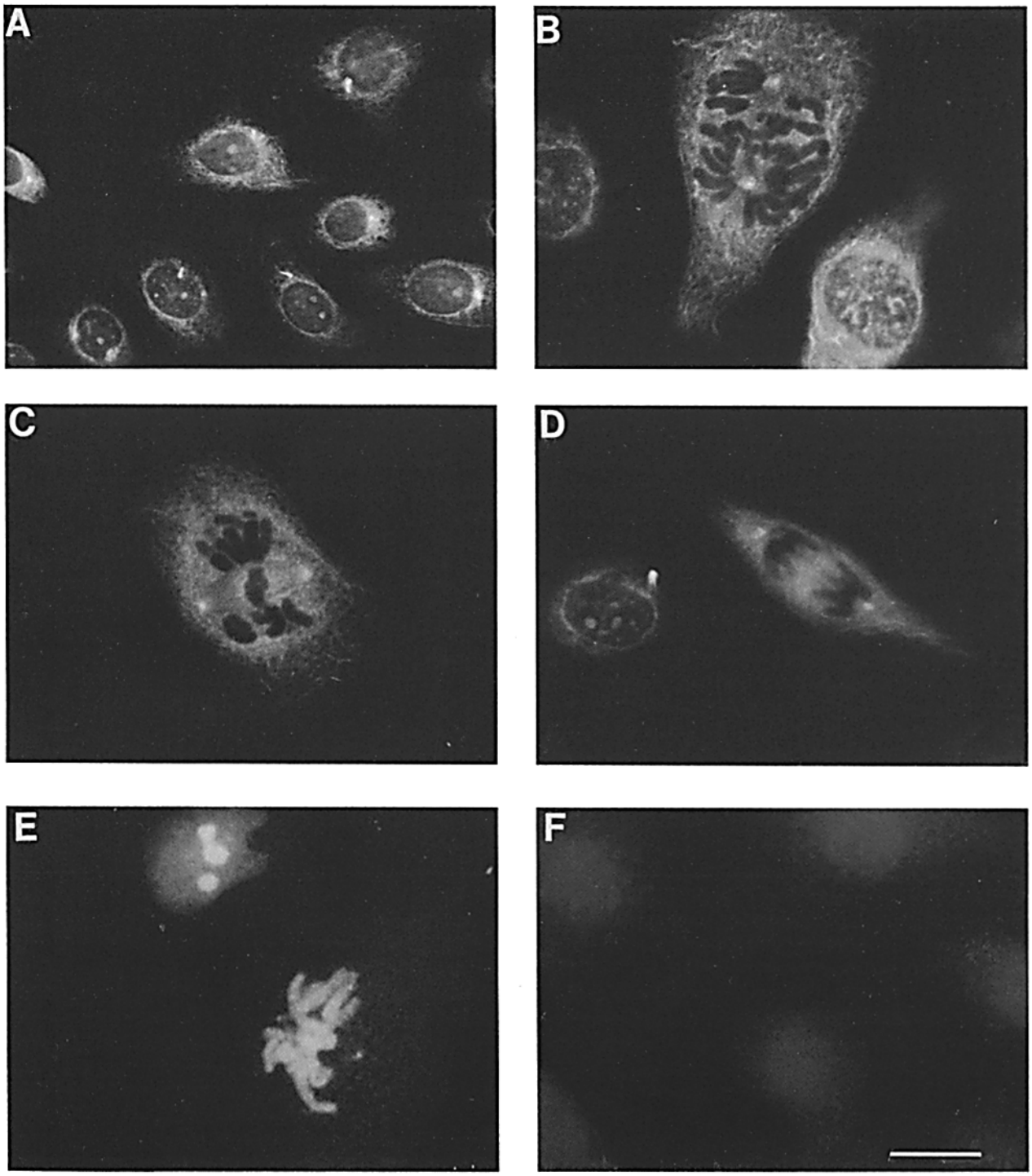


Figure 4. Immunofluorescence of PtK_1 cells stained with DEAE-purified antibodies to bovine kinesin. The images shown are PtK_1 cells fixed in −20°C methanol except for *E*, which was fixed in paraformaldehyde-glutaraldehyde and stained with Hoescht 33258 (Calbiochem-Behring Corp., La Jolla, CA) to reveal the chromosomes. For *A–E*, cells were incubated with anti-bovine kinesin followed by incubation with fluorescein-labeled goat anti-rabbit IgG. (*A–E*) *F* shows PtK_1 cells fixed and stained under equivalent conditions, using the same concentration of DEAE-purified preimmune IgG. Bar, 10 μm.

same dilution gave no visible staining of centrosomes (data not shown).

The pattern of staining in PtK_1 cells was related to the morphology of the cytoskeleton by the double staining of some preparations with rabbit anti-bovine kinesin and mouse anti-sea urchin tubulin. These preparations revealed that the juxtanuclear fiber stained in interphase was usually placed at a focal emanation of cytoplasmic MTs. This position is consistent with the kinesin-positive fibers being located at the centrosome, the location one expects for a pri-

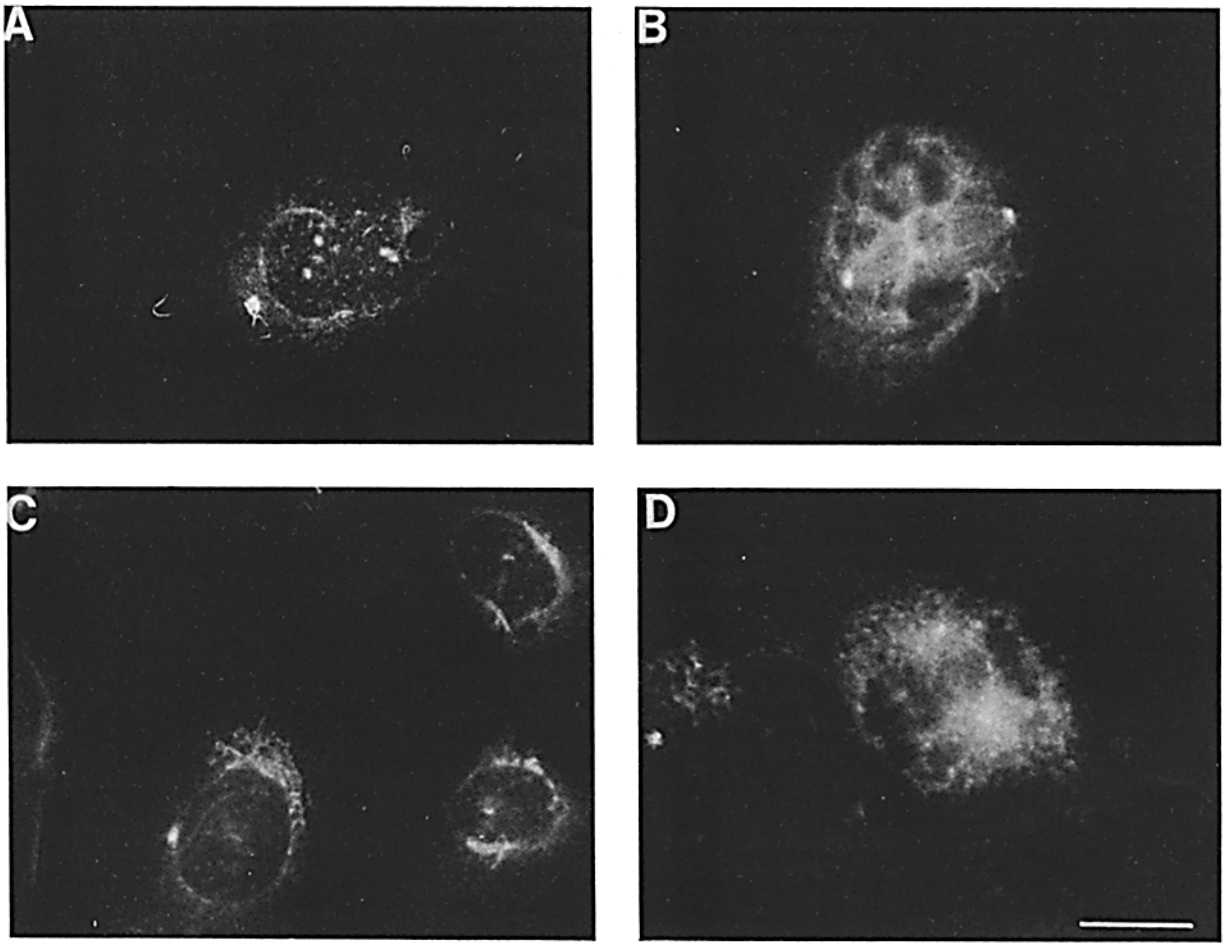


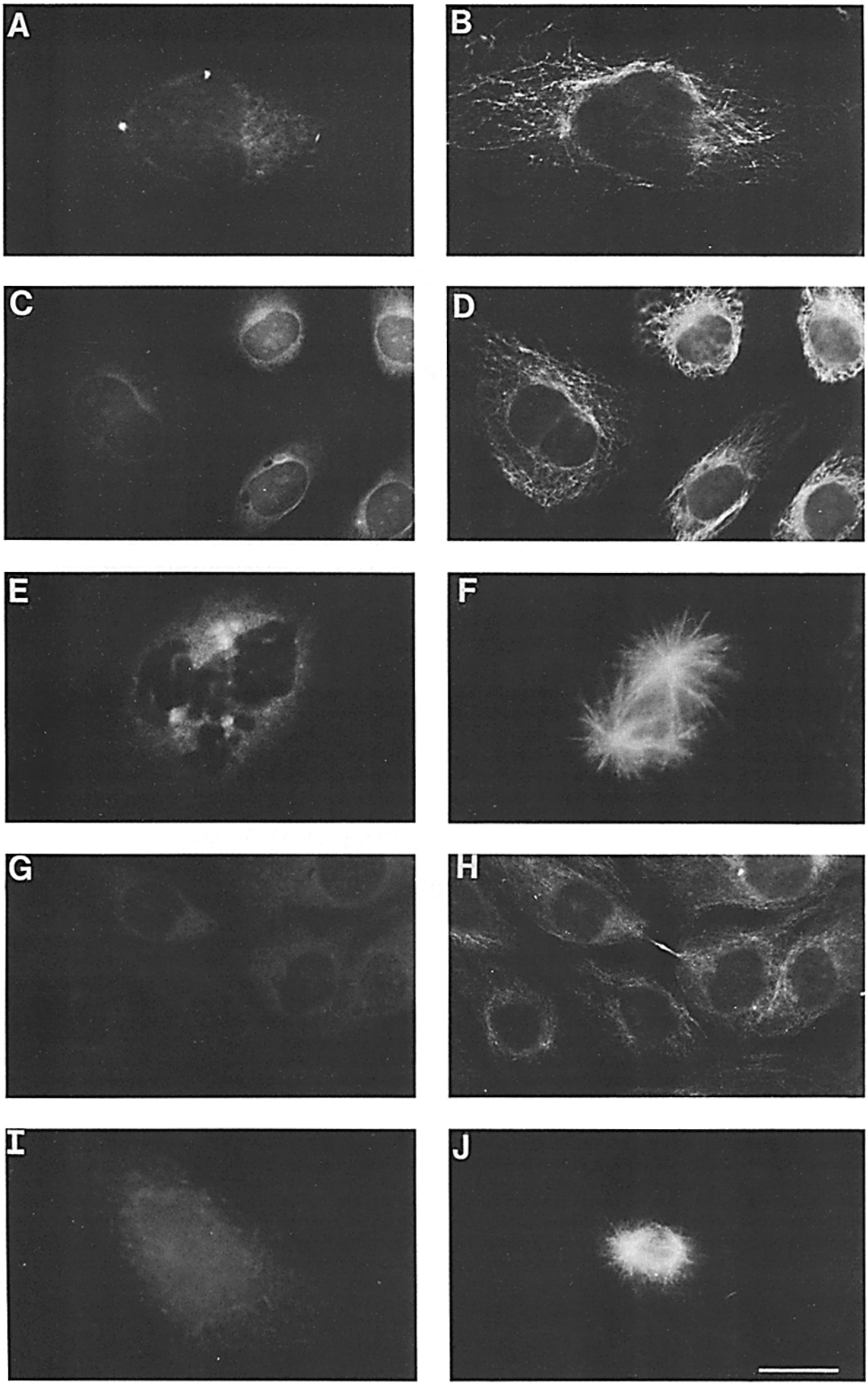
Figure 5. Immunofluorescence of PtK_1 cells stained with affinity-purified anti-bovine kinesin. The PtK_1 cells were fixed and stained as in Fig. 4. (*A* and *B*) Immunofluorescence localization of kinesin using column affinity-purified anti-kinesin, and (*C* and *D*) localization using blot affinity-purified anti-kinesin. Bar, 10 μm.

mary cilium (Fig. 6, *A* and *B* for anti-bovine kinesin and anti-tubulin; Fig. 6, *C* and *D* for anti-squid kinesin and anti-tubulin). Fig. 6, *E* and *F* compare the kinesin and tubulin distributions in a multipolar mitosis, showing that kinesin is localized at all the poles. Fig. 6, *G* and *H*, and *I* and *J* compare the preimmune serum staining from a rabbit immunized with kinesin versus the same cells stained with anti-tubulin to confirm the absence of spindle pole or centrosome staining with these antibodies.

Previous studies of kinesin localization by immunocytochemistry have demonstrated a concentration of this protein in whole mitotic spindles of sea urchin eggs (18, 30). It was therefore puzzling that while our antibodies showed strong staining of the spindle poles, we saw only weak staining of the spindle as a whole. We tried to localize kinesin in mammalian cells with affinity-purified antibodies to sea urchin kinesin, but found only nonspecific staining. (Grissom, P., and J. M. Scholey, unpublished observations.) The reciprocal experiment with anti-bovine kinesin applied to sea urchin embryos gave comparable results. Apparently these anti-kinesins are rather poorly cross-reacting. The anti-squid kinesin, on the other hand, is unusually cross-reactive, and we have used this antibody to compare kinesin localization in the cells of these two species. Fig. 7, *C* and *D* show PtK_1 cells stained with affinity-purified anti-squid kinesin, and the images look strikingly similar to those obtained with anti-bovine kinesin (Figs. 4 and 5). Anti-squid kinesin from the same rabbit was used by Scholey et al. to check the localization of kinesin in sea urchin embryos, and they confirmed the whole spindle localization they had previously demonstrated with anti-sea urchin kinesin (30). Since both localizations are confirmed with the same, broadly cross-reacting antibody, we conclude that both localizations are likely to be correct, even though they are different.

As a further check for the localization described here, we have examined the distribution of kinesin in mammalian cells, using antibodies to *Drosophila* kinesin (Fig. 7, *A* and *B*). Again, the pattern resembles the one seen with anti-bovine kinesin. Similar images have been obtained with anti-bovine kinesin prepared as described above in two mice (data not shown). While one could argue that the contaminants in the preparations of squid neural kinesin might be similar to those in bovine neural kinesin, the antibodies to *Drosophila* kinesin used for these micrographs were prepared against kinesin isolated from fruit fly embryos. It seems unlikely that the same sorts of contaminants would plague all three preparations.

We were interested to learn whether the pattern of kinesin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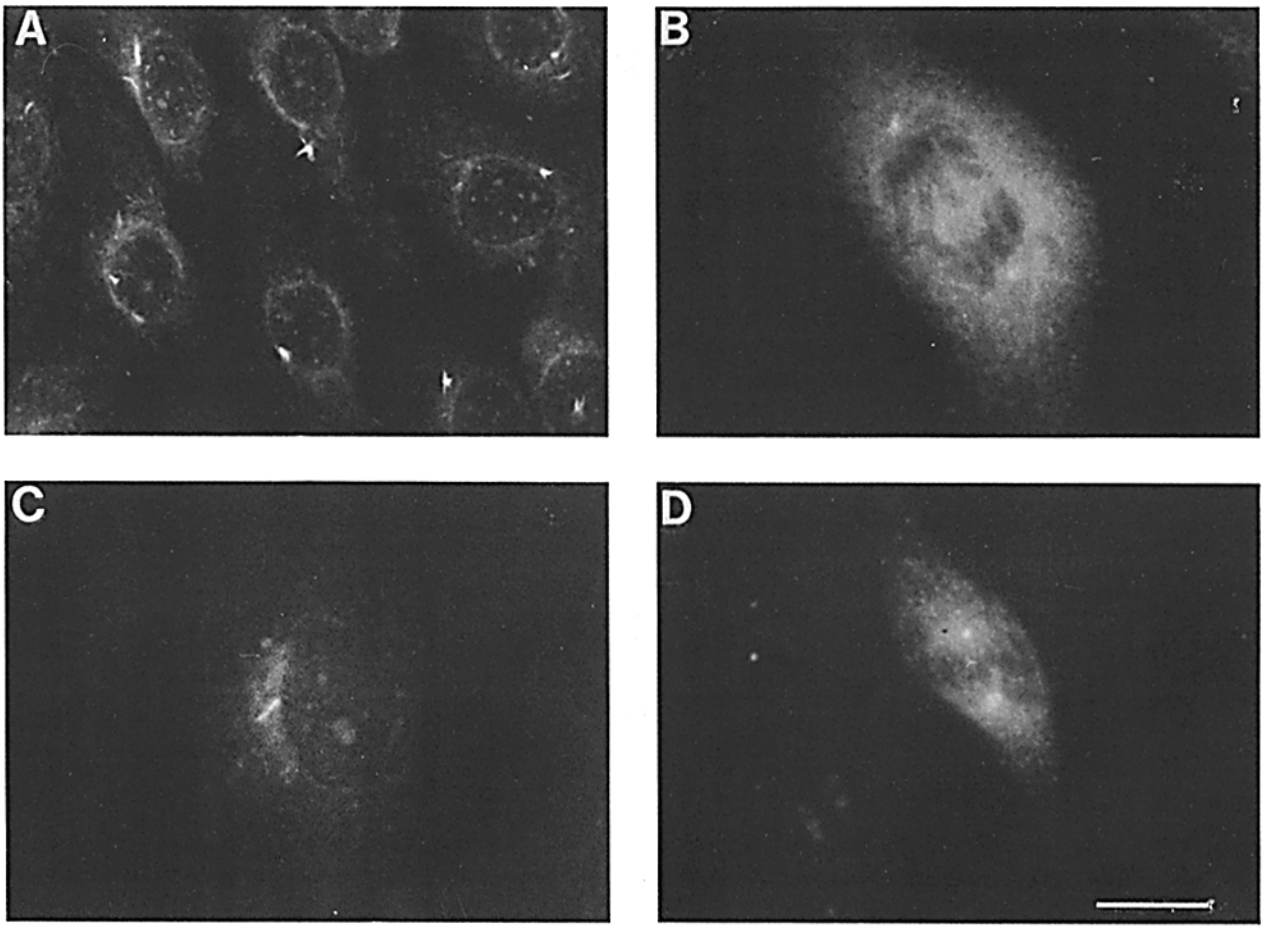

Figure 7. Immunofluorescence of PtK_1 cells stained with other anti-kinesins. Cells were fixed and stained as in Fig. 4 with anti-*Drosophila melanogaster* kinesin whole serum and anti-squid kinesin, column affinity-purified IgG. (*A* and *B*) Fluorescein anti-*Drosophila* kinesin localization in PtK_1 cells. (*C* and *D*) Fluorescein anti-squid kinesin localization in PtK_1 cells. Bar, 10 μm.

staining seen in mammalian cells would also be found in other cultured cells. Fig. 8, *A* and *B* show interphase and mitotic A6 cells stained with affinity-purified anti-bovine kinesin; Fig. 8, *C* and *D* show analogous pictures for the same cell type stained with affinity-purified anti-squid kinesin. We conclude that there is staining of the primary cilium in A6 interphase cells, although this organelle is not so common in frog cells as in PtK_1. The centrosome is usually stained as a small dot, and there is background cytoplasmic staining rather like that seen in the mammalian cells. The mitotic cells are small and round, and hence difficult to photograph, but the spindle poles are always staining more brightly than the rest of the dividing cells. The general spindle staining in the A6 cells is somewhat stronger than that seen in PtK_1 cells. These results show that the anti-kinesin distribution seen in mammalian cells is also found in another vertebrate.

Discussion

We have prepared monospecific antibodies to mammalian brain kinesin and used them to localize the corresponding antigen in fixed mammalian cells. Interphase cells show a weak reaction with a membranous reticulum in the cytoplasm. Strong anti-kinesin staining is observed on a small, juxtanuclear structure that resides at the focus of MTs characteristic of the centrosome. The shape of this object and its location are consistent with its being a primary cilium, the diminutive nine-plus-two array that often grows from the mother centriole in cultured cells. Staining by anti-kinesin is also seen during mitosis, but at this stage the antibody reveals an approximately spherical object at each spindle pole and traces of fibrous staining extending into the regions between chromosomes and poles.

Staining of the primary cilium was unexpected because preliminary experiments with immunoblots have failed to reveal a reaction between antibodies to sea urchin kinesin and axonemes from sea urchin sperm tails (Scholey, J. M., and M. E. Porter, unpublished observations). Apparently kinesin is not an essential part of an axoneme. It is noteworthy, however, that Roth and co-workers (25a) have recently shown that varicosities and granules in a primary cilium are moved along it in a fashion reminiscent of the rapid bidirectional

Figure 6. Double-label immunofluorescence of PtK_1 cells stained with anti-kinesins or preimmune serum and anti-tubulin. (*A* and *E*) Fluorescein anti-bovine kinesin; (*B* and *F*) rhodamine anti-tubulin; (*C*) fluorescein anti-squid kinesin; (*D*) rhodamine anti-tubulin; (*G* and *I*) fluorescein preimmune serum; (*H* and *J*) rhodamine anti-tubulin. Bar, 10 μm.

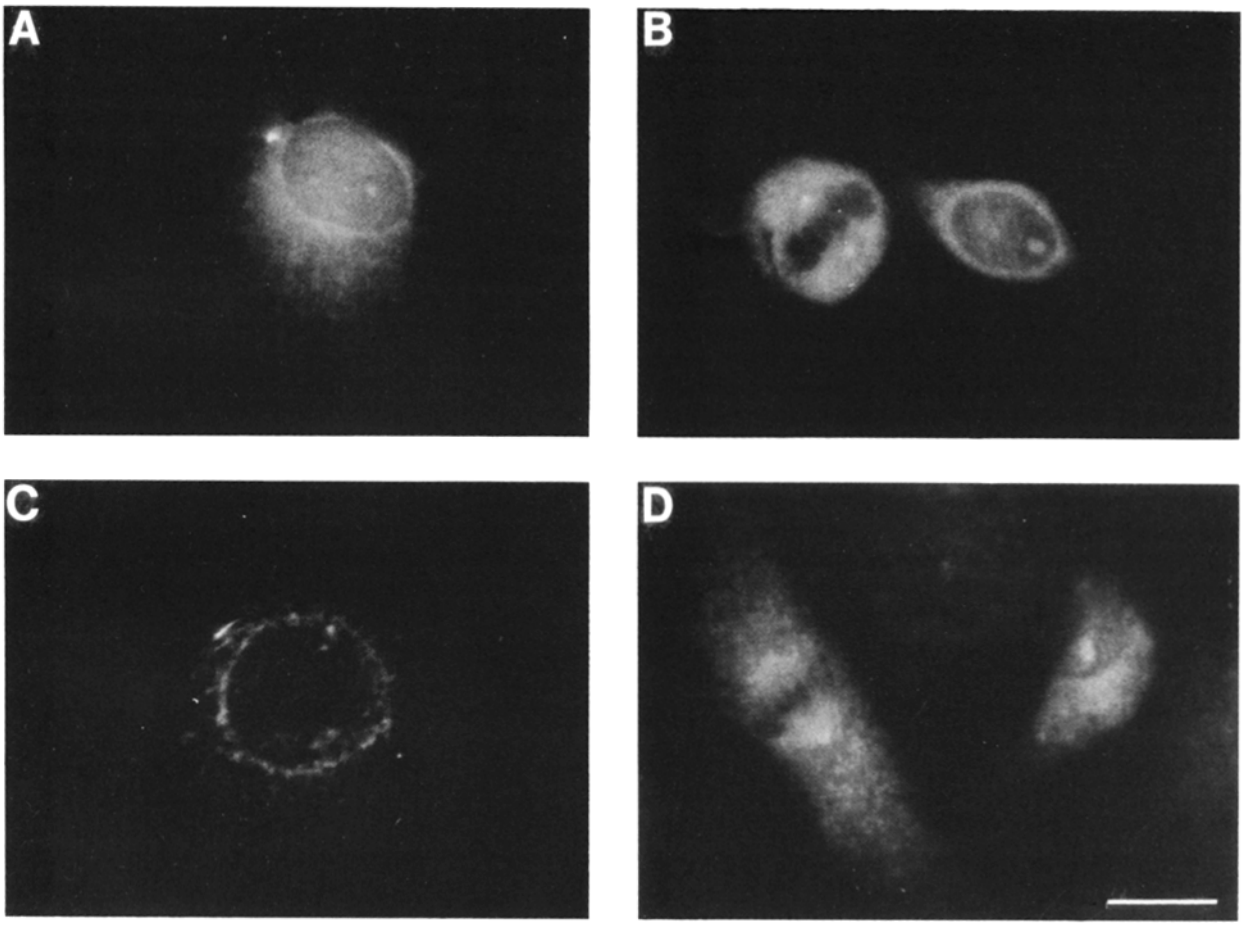

Figure 8. Immunofluorescence of A6 cells stained with affinity-purified anti-bovine kinesin and anti-squid kinesin. A6 cells derived from *Xenopus laevis* kidney epithelium were fixed and stained with anti-kinesin as in Fig. 4. (*A* and *B*) Fluorescein anti-bovine kinesin localization in A6 cells; (*C* and *D*) fluorescein anti-squid kinesin localization in A6 cells. Bar, 10 μm.

motions described for objects attached to the cilia of *Chlamydomonas* (3). We speculate that kinesin can attach to the outer doublet microtubules of an axoneme and interact with nearby material to effect motility. Any object that binds to kinesin could thus be conveyed toward the distal end of the axoneme. Since the motions associated with axonemes are bidirectional, there is probably a retrograde motility factor associated with these microtubules too.

The antibodies and fixations used here have been unable to detect kinesin associated with the interphase array of cytoplasmic microtubules. This was true also of cells lysed before fixation in MT-stabilizing buffers, with or without AMP-PNP (data not shown). The primary cilium appears, therefore, to be an exception, and kinesin is not generally associated with MTs in interphase cells.

The faint staining of membranous material in the interphase cytoplasm is consistent with the role proposed for kinesin in moving vesicles along MTs in axons (39). Indeed, it has recently been shown that some vesicles migrate in cultured cells in a fashion consistent with their being moved by kinesin and MTs (34).

The staining of the mitotic poles by our antibodies suggests that kinesin is concentrated at this important but enigmatic part of the spindle. Our observations on PtK_1 and A6 cells are much the same, suggesting that both are valid. The confirmation by anti-squid kinesin of both the pattern of spindle staining seen here and of the pattern reported for sea urchin spindles (30) suggests that both reports are correct and that the mitotic distribution of kinesin shows some variability. We speculate that the strong anti-kinesin staining pattern seen in sea urchins may result in part from the large number of vesicles characteristically found in the spindles of those species (25). This idea is consistent with the observation that spindle kinesin staining in sea urchin embryos is not extracted when the microtubules are removed from mitotic cytoskeleton by Ca^{++} (18). There may, however, be practical problems, such as the fixation of a labile spindle component, that contribute to the lack of general spindle staining observed in this study. While we have tried both methanol and aldehydes as fixatives, we may have failed to preserve a part of the spindle in cultured cells.

It is possible that the kinesin staining described here for cultured mitotic cells is also due to kinesin bound to vesicles located in the spindle, but we think this unlikely. Extensive fine structure studies of mammalian spindles have failed to reveal vesicles or granules accumulated at the poles. Special methods for fixation and staining have shown that mammalian spindles contain numerous elements derived from the endoplasmic reticulum distributed along the microtubule bundles that run from the chromosomes to the pole (12, 19, 22). Golgi

apparatus-derived vesicles are more uniformly distributed through the spindle (31). The only material visible in the electron microscope that correlates with the strong kinesin staining is the amorphous pericentriolar material visible as an osmiophilic matrix. It is possible that the whisps of staining that project into the body of the spindle correspond to the "collar material" seen by Pickett-Heaps and his co-workers in diatoms (33), but analogous structures are not yet documented in other cells.

Spindle pole staining has been a perennial problem for scientists trying to make monospecific antibodies in rabbits (6). The preimmune serum of a significant fraction of the rabbits used in laboratories contains antibodies against something that resides at the spindle poles. It appears, however, that the staining reported here is not the result of preimmune antibodies. IgG prepared from the preimmune sera of the two rabbits immunized with bovine brain kinesin showed no spindle staining at a concentration more than threefold greater than that necessary to give strong centrosome staining with the immune IgG. The anti-fruit fly kinesin preimmune serum behaved similarly. Antibodies purified by kinesin affinity from the anti-bovine and anti-squid kinesin sera both stained the spindle poles. The patterns of staining in both mitosis and interphase were essentially identical using antibodies from two rabbits immunized with bovine kinesin, one immunized with squid kinesin, and one immunized with fruit fly kinesin. The pattern has now been confirmed with sera from two mice immunized with mammalian kinesin (Neighbors, B. W., and M. M. Rozdzial, unpublished observations). The chances that all of these antisera contained contaminating antibodies against spindle pole components and that all these contaminating antibodies would purify on both columns and blots of kinesin seems remote.

Assuming that kinesin is concentrated at the poles of the mitotic spindle, it is interesting to ask what function it might serve there. Kinesin walks along MTs toward their plus ends (37), the ends distal to the spindle pole (7). It is therefore a bit surprising that the protein should be concentrated near the minus ends of the spindle microtubules. Since cells contain ample ATP to let kinesin move to the pole-distal MT ends, and yet the protein stays concentrated at the poles, we infer that it is held there by some restraining force that is strong enough to block the ATP-dependent movement of the motor over the MTs. Note that if kinesin were bound to the pericentriolar material but moved toward the plus ends of the spindle MTs only as far as it could before being restrained by its polar association, it would provide a strong but flexible connection between the MTs and the poles.

One might imagine that if kinesin is a regular feature of spindle poles, it would be contributing to the mechanical work done by spindles during mitosis. Schaap and Forer (28), Mitchison et al. (21), and Gorbsky et al. (11) have recently reported that when a chromosome moves poleward during anaphase, the spindle MTs disassemble at their kinetochore ends. If this idea is the whole truth, then the kinesin at the spindle poles is unlikely to contribute to anaphase chromosome motions. On the other hand, Mitchison et al. (21) have presented evidence that at metaphase the kinetochore microtubules migrate slowly toward the poles in a fashion reminiscent of the migration of areas of reduced birefringence (8) and of "particles or states" (2). These motions might readily be a result of the action of kinesin bound at the poles, as suggested above. As the motor moved toward the plus ends of the microtubules, yet retained its binding to the pericentriolar material of the pole, it would reel in the microtubules to the pole. If spindle microtubules were able to lose subunits at their minus ends, then a treadmilling effect would naturally result. Tests of this and other tempting speculations must await the availability of probes for kinesin function in vivo.

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