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<sub>2</sub> 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<sub>2</sub> 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<sub>1</sub> 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<sub>1</sub> 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.

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 chromatography 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 staining procedures of Merril 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). Bovine brain homogenates were incubated at 6°C for 10 min with 100 mM Pipes, pH 7.0, and quickly brought to pH 7.4 by the addition of 1 M Tris, pH 9.0. Samples were shaken for 20 min. Kinesin was purified using 500 g of white matter from fresh bovine brain as described in detail by Vale et al. (35).

**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 Axiovert microscope II equipped with a 100X 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 with homogenization buffer. Fractions containing peak concentrations of the pellets and extraction in a total of 10 mM Mg-ATP were pooled and carried through a second cycle of MT binding and release. The purity of the kinesin preparation was analyzed on 5-11% gels. The purity of the kinesin preparation was analyzed on 5-11% gels. The protein mixtures were run on SDS-PAGE, blotted to nitrocellulose, and probed with antibodies raised against kinesin from squid axoplasm (Fig. 1).

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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.

**Immunofluorescence Microscopy**

Kidney cells from the rat kangaroo (strain PtK2) 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 kinesin. Preimmune IgG fractions were tested as controls.

The specificity of purified antibodies was tested by immunoblotting against whole cell extracts from cultured cells or crude kinesin from PtK2 cells, bovine brain, and eggs of either Drosophila or Xenopus. Preimmune IgGs were tested as controls.
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-l-naphthol. The immunoblot provides evidence that our procedure purifies kinesin.

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 J2–J4 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 PtK1 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 PtK1 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 PtK1 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 PtK1 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.
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, partic-

ularly 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 cell homogenate (lane 2), crude Ptk 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 peroxidase-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.
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\textsubscript{1} cells stained with affinity-purified anti-bovine kinesin. The PtK\textsubscript{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 \(\mu\)m.

Preferably...
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 cilia in A6 interphase cells, although this organelle is not so common in frog cells as in PtK₁. 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 PtK₁ 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 cilia, 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 cilia 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 cilia are moved along it in a fashion reminiscent of the rapid bidirectional
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 PtK1 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

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.
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 whisks 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, 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. Schap 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 on 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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