Monoclonal Antibodies to Kinesin Heavy and Light Chains Stain Vesicle-like Structures, but not Microtubules, in Cultured Cells

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Abstract. Kinesin, a microtubule-activated ATPase and putative motor protein for the transport of membrane-bounded organelles along microtubules, was purified from bovine brain and used as an immunogen for the production of murine monoclonal antibodies. Hybridoma lines that secreted five distinct antikinesin IgGs were cloned. Three of the antibodies reacted on immunoblots with the 124-kD heavy chain of kinesin, while the other two antibodies recognized the 64-kD light chain. When used for immunofluorescence microscopy, the antibodies stained punctate, cytoplasmic structures in a variety of cultured mammalian cell types. Consistent with the identification of these structures as membrane-bounded organelles was the observation that cells which had been extracted with Triton X-100 before fixation contained little or no immunoreactive material. Staining of microtubules in the interphase cytoplasm or mitotic spindle was never observed, nor were associated structures, such as centrosomes and primary cilia, labeled by any of the antibodies. Nevertheless, in double-labeling experiments using antibodies to kinesin and tubulin, kinesin-containing particles were most abundant in regions where microtubules were most highly concentrated and the particles often appeared to be aligned on microtubules. These results constitute the first direct evidence for the association of kinesin with membrane-bounded organelles, and suggest a molecular mechanism for organelle motility based on transient interactions of organelle-bound kinesin with the microtubule surface.

One widespread function of microtubules (MTs)[1] is to serve as tracks along which membrane-bounded organelles travel throughout the cytoplasm. This phenomenon has been inferred by indirect studies of numerous cell types (as reviewed by Schliwa, 1984) and observed directly using video-enhanced light microscopic methods in cells as diverse as invertebrate neurons (Allen et al., 1982; Brady et al., 1982, 1983, 1985), vertebrate mesenchymal cells (Hayden and Allen, 1984), and amoebas (Koonce and Schliwa, 1985). The classes of organelles that apparently move along MTs are equally impressive in their diversity and include endosomes, lysosomes, mitochondria, and various types of secretory vesicles (Brady et al., 1985; Herman and Albertini, 1984; Martz et al., 1984; Matteoni and Kreis, 1987). A particularly well studied class of these MT-based movements is fast axonal transport. Investigations of this form of motility in neuronal cell models has demonstrated a requirement for ATP (Adams, 1982; Brady et al., 1982; Forman et al., 1983), suggesting that one or more ATPases capable of interacting with both organelles and MTs serve as the motors.

Among the leading candidates for such a motor molecule is the protein, kinesin. This hypothesis is based on several factors. First, kinesin binds stably to MTs in vitro in the presence of 5'-adenylylimidodiphosphate (AMP-PNP), but not ATP (Brady, 1985; Vale et al., 1985*a*; Scholey et al., 1985). This unusual property also applies to membrane-bounded organelles in the squid giant axon (Lasek and Brady, 1985), the observation of which directly led to the discovery of kinesin. Secondly, kinesin isolated or purified from a number of sources has been shown to possess a MT-stimulated ATPase activity (Brady, 1985; Kuznetsov and Gelfand, 1986; Cohn et al., 1987; Saxton et al., 1988; Wagner et al., 1989). Finally, kinesin is able to perform work in vitro. Purified MTs or isolated axonemes are able to glide along glass coverslips coated with kinesin in an ATP-dependent manner (Vale et al., 1985*a*; Scholey et al., 1985; Porter et al., 1987). The gliding proceeds unidirectionally towards the "minus," or slow-growing ends of the MTs, prompting suggestions that kinesin is responsible for organelle translocation in the opposite direction, corresponding to anterograde fast axonal transport (Vale et al., 1985*b*). A comparable assay has been used to determine that a different protein, the dynein-like MAP1C (MT-associated protein 1C) is capable of moving MTs in the opposite direction and, therefore, was suggested to be a motor protein for retrograde organelle motility (Paschal and

1. *Abbreviations used in this paper*: AMP-PNP, 5'-adenylylimidodiphosphate; DIC, differential interference contrast; MDBK, Madin-Darby bovine kidney; MTs, microtubules.

Vallee, 1987). Other dynein-like proteins with similar mechanochemical properties have been reported in protists (Euteneuer et al., 1988) and a nematode (Lye et al., 1987). Despite the well-documented abilities of kinesin and cytoplasmic dyneins to generate forces which can act upon MTs in vitro, the precise roles played by these proteins in organelle movement remain to be determined.

The difficulty in establishing that these proteins move organelles along MTs in vivo has been that direct evidence for interactions between organelles, and either kinesin or cytoplasmic dyneins has been lacking. This report describes the first such evidence. Using multiple monoclonal antibodies made against both the heavy and light chains of bovine brain kinesin, we have demonstrated by immunofluorescence microscopy that kinesin is localized on particulate, vesicle-like organelles in numerous cell types. These particles were found to be distributed throughout the cytoplasm in a manner expected for motile membrane-bounded organelles, and differed in appearance and location from other membrane-associated structures, such as the plasma membrane, Golgi stacks, and nuclear envelope. The immunoreactive structures were not observed in cells that had been extracted before fixation with the nonionic detergent, Triton X-100, further supporting their identification as membrane-bounded organelles. Although we did not observe MTs to be stained by any of our antibodies in either interphase or mitotic cells, kinesin-containing particles often appeared to be closely associated with MTs in cells that were stained with antibodies to both kinesin and tubulin. The results presented here suggest that kinesin molecules act as ATP-dependent, organelle transport motors by residing on the organelle surface and binding transiently to the MT.

Materials and Methods

Purification of Kinesin

Kinesin was purified from bovine brain cytosol by a procedure that is described in full detail elsewhere (Wagner et al., 1989) and was derived from our previously published protocol (Bloom et al., 1988). The procedure involved the following sequential steps: MT assembly in the presence of AMP-PNP, gel-filtration and ion-exchange chromatography, and sucrose density-gradient ultracentrifugation. Three major changes have been made since our original method was developed. First, IME buffer (15 mM imidizole, pH 7.0, 2 mM $MgCl_2$, 1 mM EGTA) was used for all chromatographic steps and for the sucrose gradient. Next, an *S*-Sepharose cation exchange column (Pharmacia Fine Chemicals, Piscataway, NJ) was added after the gel-filtration step. Finally, the kinesin peak from the cation column was concentrated on a hydroxylapatite column (Calbiochem-Behring Corp., La Jolla, CA) and dialyzed into IME before loading on a 5–20% sucrose gradient in IME. SDS-PAGE was performed as described previously (Bloom et al., 1988). Molecular mass markers were obtained from Sigma Chemical Co. (St. Louis, MO). Protein assays were performed using the method of Bradford (1976).

Production and Purification of Antibodies

Monoclonal antibodies to kinesin were produced by a modification of the method of Brodsky (1985). Kinesin, which had been purified through the hydroxylapatite step (>50% pure kinesin), was injected into the footpads of five BALB/c mice on six occasions separated by 3 d each. Ribi adjuvant (monophosphoryl lipid A + trehalase dimycolate emulsion; Ribi ImmunoChem Research, Inc., Hamilton, MT) was used for the first two injections for each mouse and subsequent boosts were given in the absence of adjuvant. The mice were bled from their tails on day 16 after the initial injections, and all five sera were found to contain antibodies against the kinesin heavy (124-kD) and light (64-kD) chains (Bloom et al., 1988) as judged by immunoblotting (Bloom et al., 1984) of partially purified kinesin. The next day, the mice were killed and popliteal lymph node lymphocytes were fused with NS-1 myeloma cells. Fusion was accomplished using a solution of 37% polyethylene glycol and 5% DMSO, and a ratio of two lymphocytes per myeloma cell. Wells with colonies growing actively in HAT (hypoxanthine-aminopterin-thymidine) medium were screened by immunoblotting for the presence of kinesin-specific antibodies from 7–14 d after fusion. Hybridoma cultures that secreted antibodies to 124- or 64-kD polypeptides were cloned two or three times by limiting dilution.

The isotypes of the resulting antibodies were determined by double immunodiffusion in agar using conditioned tissue-culture media and rabbit antibodies specific for mouse antibody isotypes (ICN Laboratories Inc., Costa Mesa, CA). Antibodies were isolated from ascites fluids as pure IgG isotypes using a fast performance liquid chromatography protein A-Superose column (Pharmacia Fine Chemicals) according to methods supplied by the manufacturer. Briefly, ascites fluids were diluted 1:1 with 1.5 M glycine, pH 8.9, 3.0 M NaCl (column buffer), and clarified by passage over a Sephadex G25 column (Pharmacia Fine Chemicals) equilibrated in half strength column buffer. The sample was then passed through the protein A-Superose column using column buffer as the mobile phase. Isotype-specific antibodies were then eluted by changing the mobile phase to 0.1 M sodium citrate and varying the pH. IgG1, IgG2a, and IgG2b eluted at pH 6.0, 5.0, and 4.0, respectively. Purified antibodies were dialyzed into PBS (10 mM sodium phosphate, pH 7.4, 0.15 M NaCl).

Tissue Culture and Immunofluorescence Microscopy

Stable lines of hybridoma cells were grown in RPMI 1640 medium, PtK_1 and Hepa 1-6-J cells were cultured in MEM supplemented with 1 mM sodium pyruvate, and Madin-Darby bovine kidney (MDBK) cells were maintained in F-12 nutrient mixture. Gentamycin and 10% calf serum (HyClone Laboratories, Logan, UT) were included in all media. Rat primary brain cultures were prepared and maintained according to the method of Bloom and Vallee (1983). All tissue culture reagents, except calf serum, were obtained from Sigma Chemical Co.

Immunofluorescence microscopy was performed as described previously (Bloom et al., 1984) with some modifications. Fixation and permeabilization of cells was accomplished either by immersing coverslips in methanol at −20°C for 5 min, or placing them in a 3.7% solution of formaldehyde in PBS followed by a 5-min incubation in 0.5% Triton X-100 in PBS. For the experiment documented in Fig. 7, the coverslips were incubated for 5 min at 37°C in a solution of 0.5% Triton X-100 in a MT-stabilizing buffer (0.1 M Pipes, pH 7.0, 1 mM $MgCl_2$, 1 mM EGTA) before fixation with methanol. For single immunofluorescence (Figs. 4, 5, and 7), three antibody layers were used. Purified preparations of antikinesin, normal mouse IgG, or an irrelevant monoclonal at 10 μg/ml served as primary antibodies, TRITC-labeled goat anti-mouse IgG (Fisher Scientific Co., Pittsburgh, PA) was used as a secondary antibody at 10 μg/ml, and TRITC-labeled rabbit anti-goat IgG (Jackson Immunoresearch Laboratories, Inc., Avondale, PA) was used as a tertiary antibody at 1 μg/ml. For double-immunofluorescence microscopy of kinesin and tubulin (Fig. 6), the primary antibodies were a rabbit antiserum to tyrosinated alpha tubulin (Gundersen et al., 1985) and H1 antikinesin. The secondary antibodies in this case were TRITC-labeled goat anti-mouse IgG at 20 μg/ml and FITC-labeled sheep anti-rabbit IgG at 0.25 μg/ml (Jackson Immunoresearch Laboratories, Inc.). No tertiary antibodies were used in this experiment and the fluorescently labeled antibodies used throughout this study were obtained as affinity-purified products. In all experiments, the coverslips were washed between antibody incubations with PBS containing 0.3 M NaCl and 0.5% Triton X-100. Photomicrographs were taken on a Carl Zeiss Inc. (Thornwood, NY) IM-35 microscope through 63× or 100× planapochromatic objectives using TMAX 400 film (Eastman Kodak Co., Rochester, NY), which was processed with TMAX developer at EI 800.

Results

Immunochemical Characterization of Antikinesin Antibodies

Five monoclonal antikinesins representing a variety of IgG subisotypes were produced. As illustrated in Fig. 1 *A*, three of the antibodies (H1, H2, and H3) react specifically with the kinesin heavy chain (124 kD) on immunoblots of purified

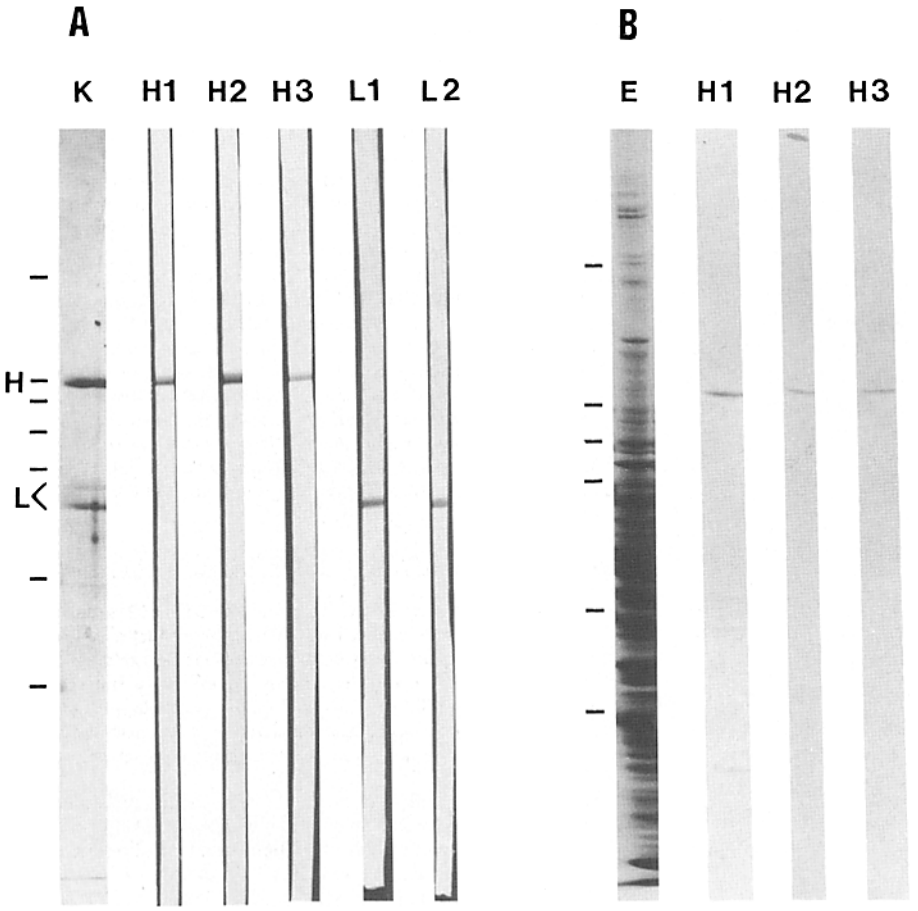

Figure 1. Subunit specificities of monoclonal antikinesins as determined by immunoblotting. (*A*) Purified kinesin was resolved into subunits by SDS-PAGE, a portion of the curtain gel was stained for total protein with Coomassie blue (*K*), and the remainder of the gel was transferred to nitrocellulose. Strips of nitrocellulose were then incubated with 10 μg/ml of purified monoclonal antibody followed by peroxidase-conjugated goat anti-mouse IgG. Note that the H1, H2, and H3 antibodies specifically recognize the 124-kD heavy chain of kinesin (*H*), and that the L1 and L2 antibodies react solely with the 64-kD light chain (*L*). Horizontal lines to the immediate left of the first lane (*A* and *B*) indicate the positions of molecular mass markers. These include (from top to bottom): rabbit skeletal muscle myosin, 205 kD; *E. coli* beta galactosidase, 116 kD; rabbit muscle phosphorylase B, 97.4 kD; bovine serum albumin, 66 kD; chicken ovalbumin, 45 kD; and bovine erythrocyte carbonic anhydrase, 29 kD. (*B*) A cytosolic extract of bovine brain was used as starting material for the purification of kinesin. Cytosolic proteins were resolved by SDS-PAGE and visualized by staining of the gel with Coomassie blue (*E*). The kinesin heavy chain was detected in parallel immunoblots using the H1, H2, and H3 antibodies.

kinesin, while the other two (L1 and L2) recognize only the light chains (64 kD). The three heavy chain–specific antibodies are also capable of detecting 124 kD in cytosolic extracts of bovine brain, as shown in Fig. 1 *B*. A unique property of H1 demonstrated here is that it reacts with a few minor polypeptides, whose electrophoretic mobilities are >124 kD and which presumably represent proteolytic fragments of the kinesin heavy chain. On occasion, the heavy chain–specific antibodies labeled a high molecular mass polypeptide that inconsistently contaminated kinesin throughout its purification, and which appeared to be an aggregate of kinesin subunits (not shown). As described below and summarized in Table I, each of the other antibodies also exhibits its own particular set of immunochemical properties, indicating that the set of five antibodies recognizes five distinct kinesin epitopes.

Among the most noteworthy features of the antibodies are the abilities of H2 and L1 to recognize minor isoforms of the kinesin heavy and light chains, respectively. This can be seen in Fig. 2, where SDS-PAGE and immunoblotting were used to analyze the major purification steps for bovine brain kinesin. The lanes, each of which was loaded with 5 μg of total protein, correspond to the ATP wash of kinesin-enriched MTs, followed by pooled peak fractions from gel-filtration and cation-exchange chromatography, and sucrose density-gradient ultracentrifugation. The major and minor forms of the kinesin heavy and light chains can be seen to coenrich at each step as judged by Coomassie blue staining and immu-

Table I. Properties of Monoclonal Antibodies to Kinesin

Antibody name	Subunit specificity	Isotype	Distinctive features
H1	Heavy chain (124 kD)	IgG1	Reacts with the major but not minor form of 124 kD and its proteolytic fragments
H2	Heavy chain	IgG2b	Reacts with both the major and minor forms of 124 kD
H3	Heavy chain	IgG1	Reacts only with the major form of 124 kD
L1	Light chain (64 kD)	IgG1	Reacts with both the major and minor forms of 64 kD
L2	Light chain	IgG2a	Reacts only with 64 kD

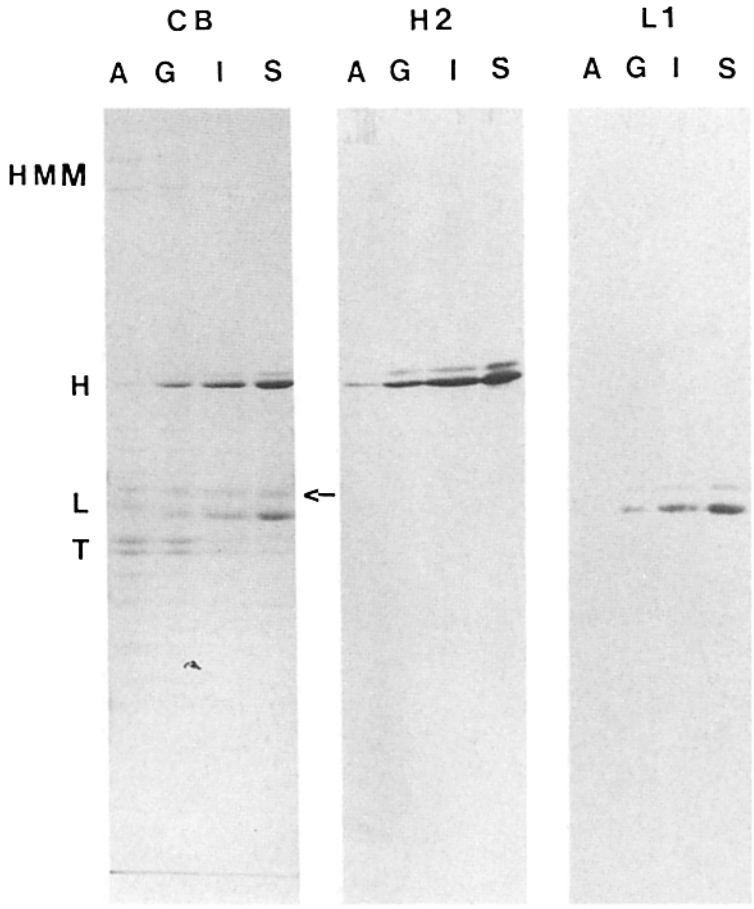

Figure 2. Analysis of the purification steps for kinesin from bovine brain by SDS-PAGE and immunoblotting. Four major enrichment steps were used to purify kinesin from cytosolic extracts of bovine brain. Successively, these included an ATP wash of kinesin-containing MTs (*A*) that had been isolated from cytosol, and peak kinesin-containing fractions from gel-filtration (*G*) and ion-exchange (*I*) chromatography, and sucrose density-gradient ultracentrifugation (*S*). A Coomassie blue–stained SDS–polyacrylamide gel (*CB*), and equivalent immunoblots stained with the H2 and L1 antibodies are shown. 5 μg total protein was loaded in each lane. Note that H2 reacts with both the major and minor heavy chain, and that L1 similarly recognizes both forms of the light chain. The positions of high molecular mass MT-associated proteins (*HMM*), tubulin (*T*), and the heavy (*H*) and light (*L*) chains of kinesin are indicated to the left.

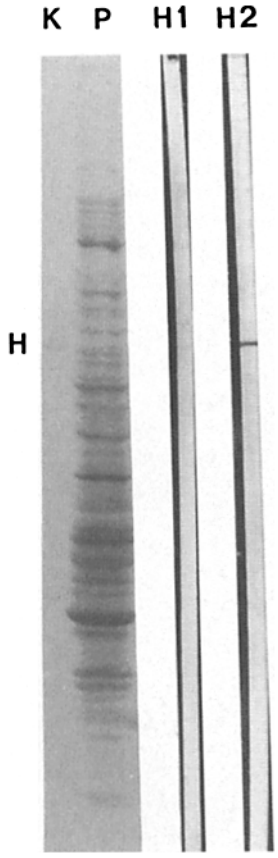

Figure 3. Detection of kinesin in cultured cells by immunoblotting. A crude preparation of MTs was isolated from Hepa-1-6-J mouse hepatoma cells using taxol (Vallee, 1982) and AMP-PNP, and the proteins were resolved by SDS-PAGE and transferred by electroblotting to nitrocellulose. A portion of the blot stained with Ponceau S is shown on the left. Lanes *K* and *P*, respectively, illustrate purified bovine brain kinesin and the kinesin-enriched MT pellet from the hepatoma cells. Immunoblots of the hepatoma cell MTs were stained using the H1 and H2 antibodies are also shown. The position of the kinesin heavy chain (*H*) is indicated to the left.

noblotting with H2 and L1. The greater sensitivity of H2 than L1 on these blots reflects our finding that higher molar levels of kinesin light chains than heavy chains are usually required for detection by immunoblotting. The minor heavy chain was usually, but not always, resolved on gels and blots (as in Fig. 1), and was recognized by H2 alone. Based on this unique property of H2 and the apparent fragments of 124 kD recognized exclusively by H1 (see Fig. 1 *B*), we conclude that the three heavy chain–specific antibodies recognize three distinct epitopes on 124 kD. Similarly, the failure of L2 to react with the minor light chain of kinesin distinguishes that antibody from L1.

The monoclonal antibodies cross-react with kinesin from a variety of sources other than bovine brain. All of the antibodies recognize appropriate kinesin subunits in MTs isolated from rat brain (not shown) and the H2 monoclonal antibody reacts with the heavy chain of squid kinesin (Brady, S. T., K. K. Pfister, and G. S. Bloom, manuscript submitted for publication). Fig. 3 demonstrates that H1 and H2 react on immunoblots with a kinesin-enriched, cytosolic fraction obtained from Hepa-1-6-J cells, a cultured mouse hepatoma line that was used for some of the immunofluorescence experiments documented in Fig. 5. The 64-kD kinesin light chain was also detected by immunoblotting in hepatoma cell samples, but was very weakly stained (not shown). This observation is consistent with our finding that the kinesin light chains generally have a higher threshold concentration of detectability by immunoblotting than the heavy chains. Results similar to those shown in Fig. 3 were obtained using analo-

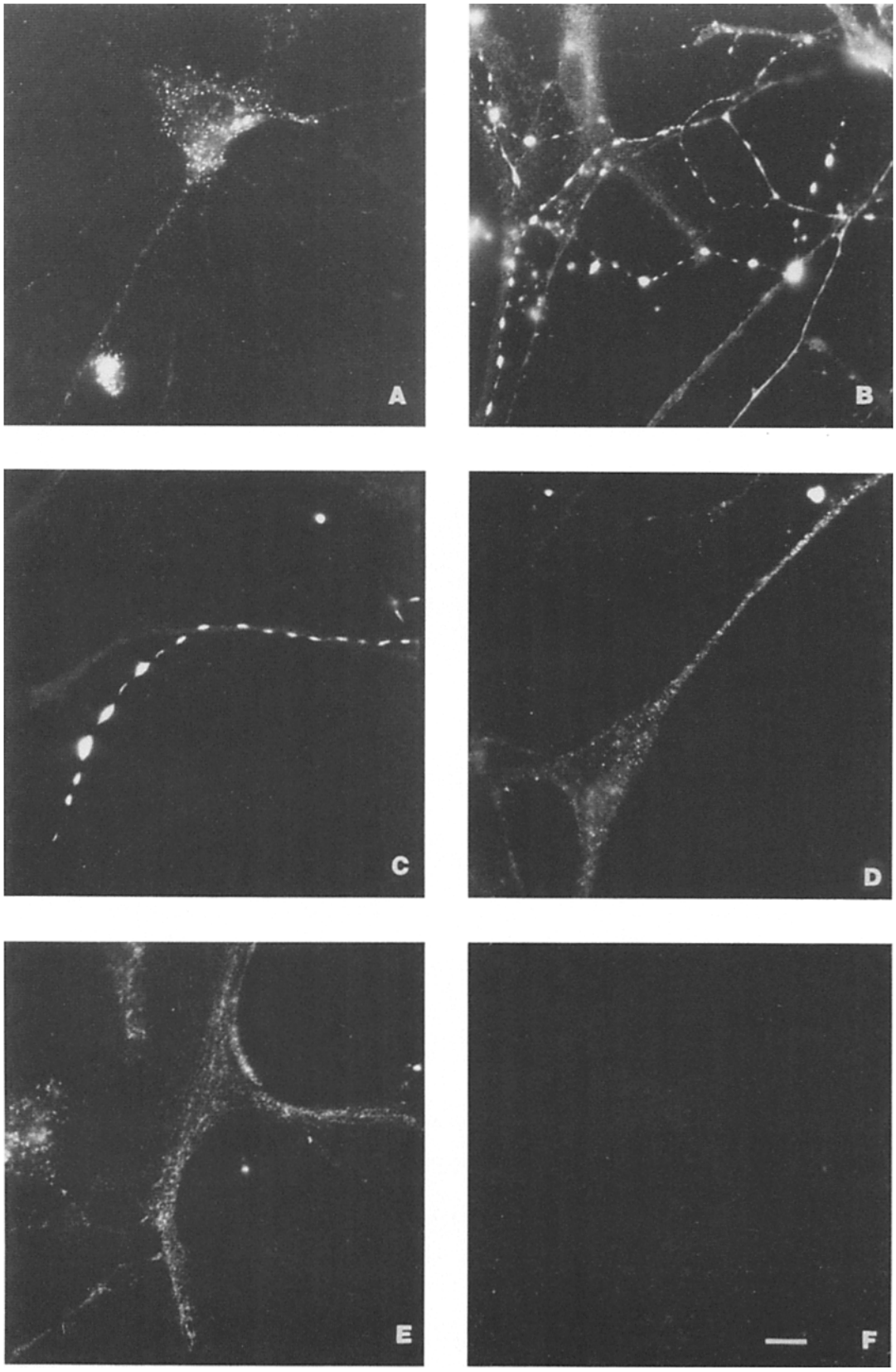

Figure 4. Localization of kinesin in primary rat brain cells. Cells were cultured and stained for immunofluorescence microscopy as described in Materials and Methods. Shown here are cells stained with H1 (*A*), H2 (*B*), H3 (*C* and *D*), L2 (*E*), and normal mouse IgG (*F*). Note the staining of cytoplasmic, vesicle-like particles in glial cells (*A*, *D*, and *E*), and in the varicosities of axonal processes (*B* and *C*). Such varicosities are known to contain an abundance of membrane-bounded organelles (Hollenbeck and Bray, 1987; Sasaki-Sherrington et al., 1984). Staining was not observed in the control (*F*). Bar, 10 μm.

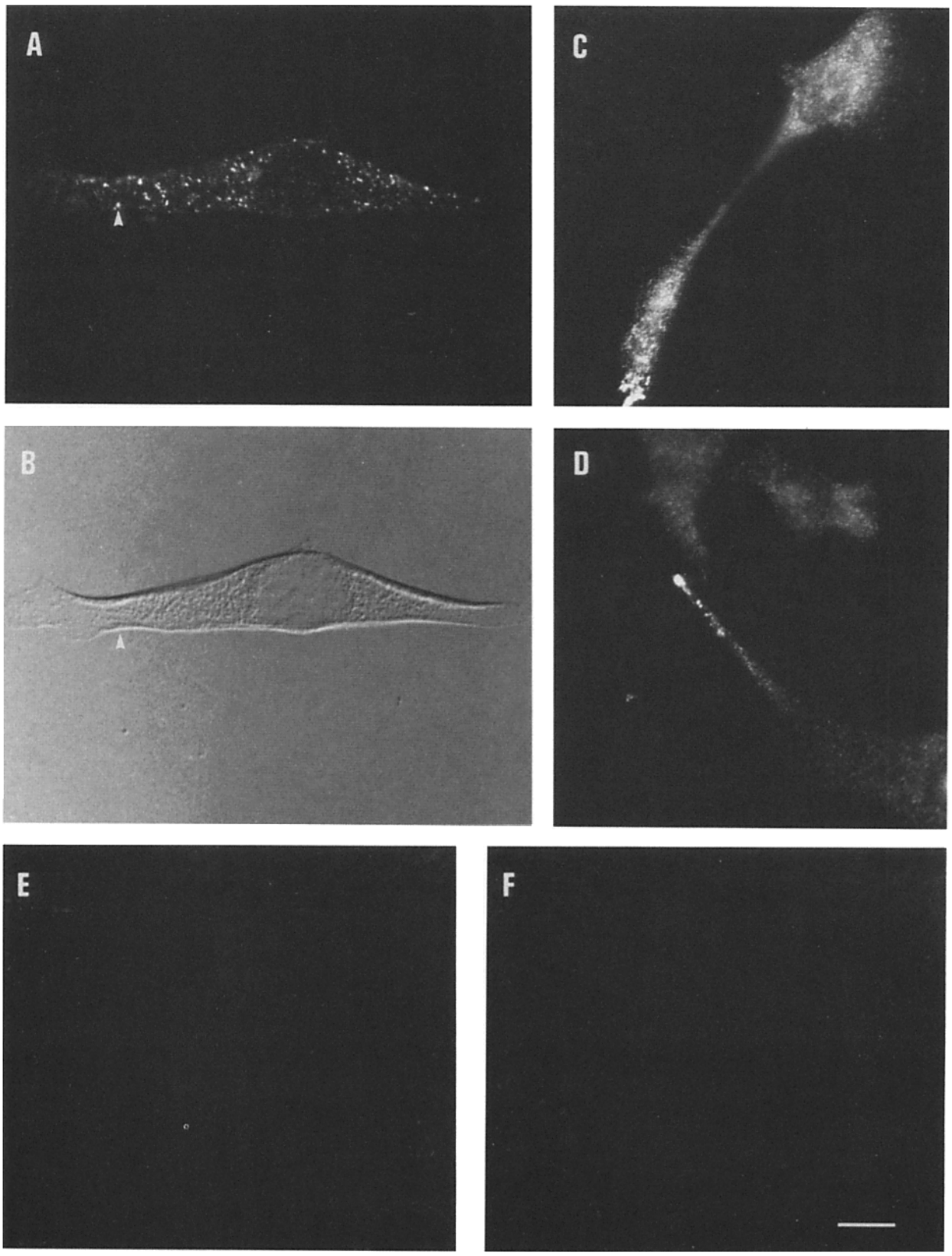

Figure 5. Localization of kinesin in cultured, nonneural cells. Comparative immunofluorescent (*A*) and DIC (*B*) views of an MDBK (bovine kidney epithelial) cell stained with H1. Note that fluorescent spots often appear to correspond in position to refractile particles that may represent membrane-bounded organelles. An example of this is indicated by the arrowheads. Punctate, vesicle-like structures were stained by H2 (*C*) and L2 (*D*) in Hepa-1-6-J (mouse hepatoma) cells. No staining was observed when PtK_1 (rat kangaroo kidney) cells were incubated with H1 that had been preadsorbed with purified kinesin (*E*), or with an irrelevant monoclonal antibody (*F*). Bar, 10 μm.

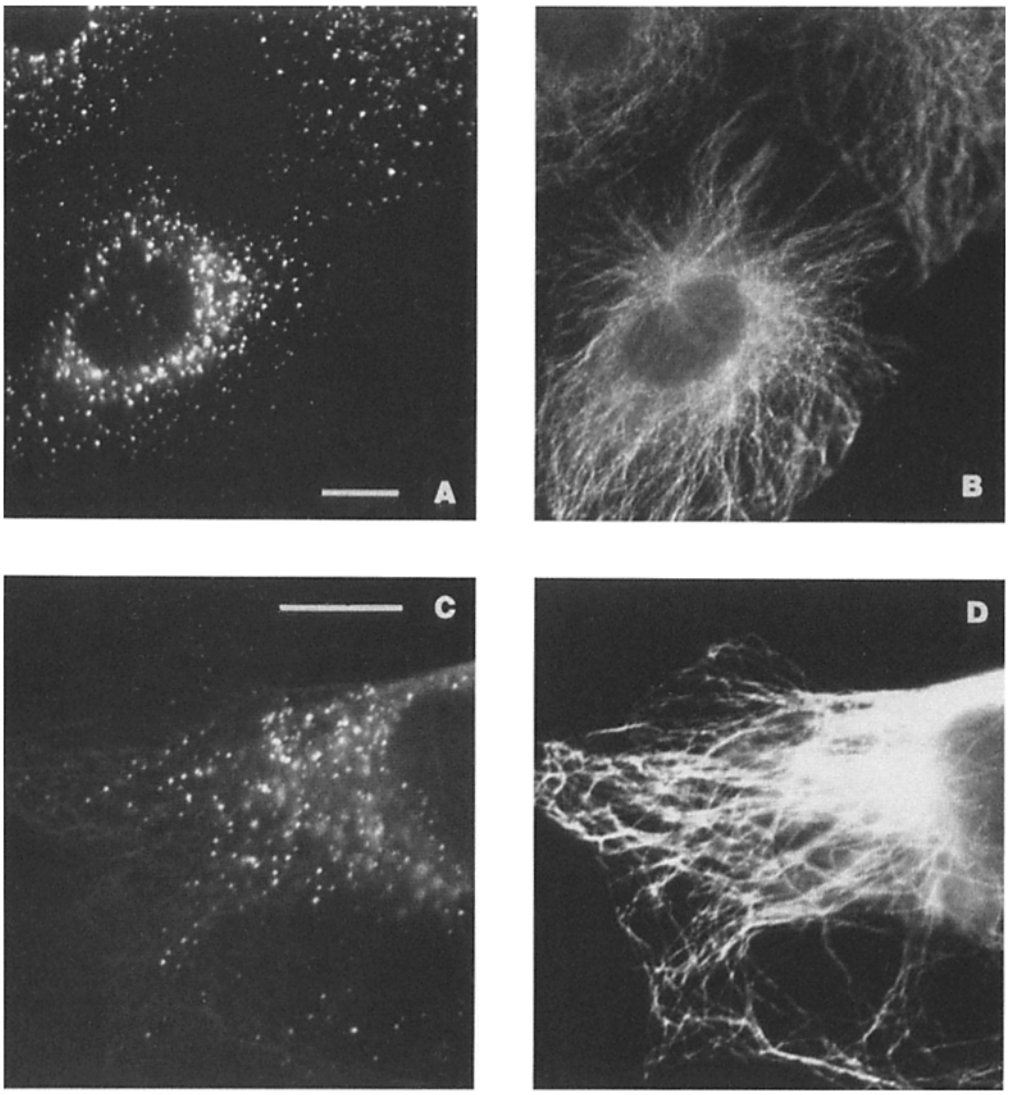

Figure 6. Double immunofluorescence microscopy of PtK_1 cells using antibodies to kinesin and tubulin. Staining was performed as described in Materials and Methods using the H1 antibody to the kinesin heavy chain and a polyclonal rabbit antibody to tyrosinated alpha tubulin (Gunderson et al., 1985). TRITC-labeled goat anti-mouse IgG and FITC-labeled goat anti-rabbit IgG were used as secondary antibodies. In *A* and *B*, the cells were stained with an extremely low dilution (1:5,000) of antitubulin (*B*) to eliminate spurious cross-reactivities by the secondary antibodies with inappropriate primary antibodies. Note that kinesin-containing particles (*A*) are most abundant in the perinuclear region, where MTs are also most heavily concentrated. In *C* and *D*, the antitubulin was used at a higher concentration (1:500), resulting in weak staining of MTs by the TRITC-labeled goat anti-mouse IgG. (*C*) Faintly visible MTs, many of which seem to define tracks on which brightly stained kinesin-containing particles are located, can be seen in the rhodamine channel. (*D*) The MTs are more readily visible in the fluorescein channel. Bars, 10 μm.

gous fractions isolated from cultured PtK_1 (rat kangaroo kidney) and CHO-K1 (Chinese hamster ovary) cells (not shown). The PtK_1 strain was also used extensively throughout this study for immunofluorescence microscopy (see Figs. 5–7).

To determine whether the antibodies were capable of reacting with MT-bound kinesin, immunofluorescence microscopy was performed. Taxol-stabilized, MT-associated protein-free MTs were mixed in solution with purified kinesin and, to promote kinesin binding, AMP-PNP was added. Aliquots of these MTs were then adsorbed to coverslips, and fixed and stained with the anitkinesins. All five antibodies yielded bright staining of MTs that was virtually indistinguishable from that observed in samples stained with antitubulin. When MTs lacking kinesin were used for parallel experiments, they were stained by antitubulin, but not by any of the antibodies to kinesin (data not shown).

Localization of Kinesin in Cultured Cells

The cellular and subcellular distribution of kinesin in primary cultures of newborn rat brain cells was documented by immunofluorescence microscopy using all five antibodies, several examples of which are shown in Fig. 4. Labeling of punctate, vesicle-like structures confined to the cytoplasm was consistently observed with all these antibodies. Both glial cells (Fig. 4, *A*, *D*, and *E*) and neurons (Fig. 4, *B* and *C*) were labeled by the antibodies. The neuronal staining was

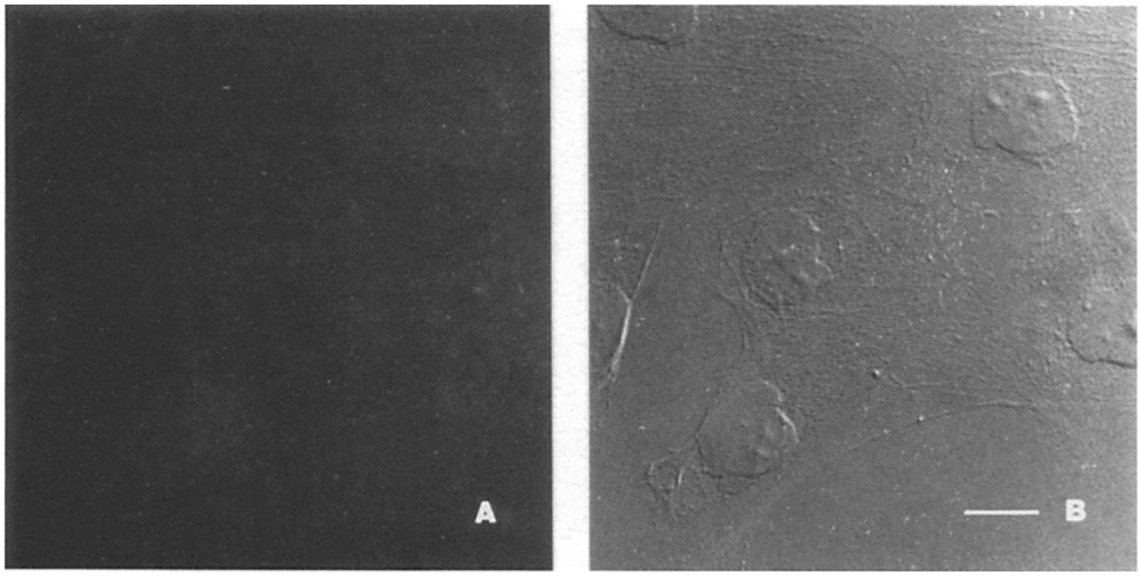

Figure 7. Kinesin is extracted from cells exposed to Triton X-100 before fixation. PtK_1 cells were extracted with 0.5% Triton X-100 in a MT-stabilizing buffer before methanol fixation, as described in Materials and Methods. The cells were then processed for immunofluorescence using antibody H2. Corresponding immunofluorescence (*A*) and DIC (*B*) images are shown. All immunoreactivity was removed by the detergent, suggesting that kinesin was associated with membrane-bounded organelles. Bar, 10 μm.

particularly prominent in organelle-rich varicosities commonly found in axons that are regenerating in culture (Hollenbeck and Bray, 1987; Sasaki-Sherrington et al., 1984). A through-focus series of these varicosities indicated that the immunoreactive material was of a granular nature, consistent with the presence of closely packed organelles, such as those seen in electron micrographs of neuronal varicosities (Sasaki-Sherrington et al., 1984). The structures stained most conspicuously by H1 appeared to be larger and less numerous than those labeled most commonly by the other antibodies, but were otherwise similar. The functional significance of this difference is unclear at present, but may reflect differential accessibility of kinesin epitopes on distinct classes of organelles. Consistent with this interpretation is our observation that structures with morphologies suggestive of mitochondria were stained more prominently by L2 than by other antibodies. Despite the subtle differences among the antibodies in their patterns, as well as intensities of staining, the kinesin was consistently localized on punctate, cytoplasmic structures.

Three continuous strains or lines of tissue-culture cells, PtK_1, Hepa 1-6-J, and MDBK, were also examined by immunofluorescence microscopy (Figs. 5 and 6). In these cells, monoclonal antikinesins stained vesicle-like structures that were very similar in appearance to those labeled in primary brain cultures. When fluorescence and differential interference contrast (DIC) images of individual cells were compared, immunoreactive structures frequently appeared to correspond in position to particles that were visible in DIC (Fig. 5, *A* and *B*). We did not observe staining of MTs or associated structures, such as primary cilia or centrosomes, with any of the antibodies in any of the cell types we examined during either interphase or mitosis (not shown), even though staining with antibodies to tubulin indicated excellent preservation of MTs (see Fig. 6). Results of antikinesin staining obtained using formaldehyde or methanol fixation were indistinguishable from one another.

Double-labeling experiments with H1 and a polyclonal rabbit antibody to tyrosinated alpha tubulin (Gundersen et al., 1985) demonstrated that kinesin-containing, vesicle-like structures are most abundant in regions where MTs are maximally concentrated (Fig. 6, *A* and *B*). In fact, superimposition of tubulin and kinesin patterns in individual cells revealed that kinesin-containing structures frequently reside in close proximity to MTs (Fig. 6, *C* and *D*), as predicted by the hypothesis that kinesin is a motor protein for translocating membrane-bounded organelles along MTs.

In all of the cell types that we examined, the appearance of the immunoreactive structures was highly suggestive of vesicle-like, membrane-bounded organelles. To obtain further evidence that this was indeed the case, unfixed cultured cells were lysed in a MT-stabilizing buffer containing the nonionic detergent, Triton X-100, which is known to dissolve virtually all cytoplasmic, membrane-bounded organelles (Brown et al., 1976). After exposure to the detergent, the cells were fixed and stained as usual. As can be seen in Fig. 7 for the H2 antibody, nearly all of the immunoreactive material that could be detected in unextracted cells (see Figs. 4–6) was removed by the detergent treatment. Identical results were obtained in parallel experiments using the other four antibodies (not shown). Judging from their appearance as numerous, punctate objects in the cytoplasm and their ability to be dissolved by Triton X-100, we suggest that the structures stained by the antikinesins are membrane-bounded organelles. This hypothesis receives further support from two additional lines of investigation. First, kinesin can be detected by immunoblotting of synaptic vesicles isolated from rat and bovine cerebral cortex (unpublished results). Second, the H2 antibody reacts with the heavy chain of squid kinesin, stains membrane-bounded organelles in squid axons by im-

munofluorescence, and inhibits the transport of those organelles along MTs in isolated axoplasm (Brady, S. T., K. K. Pfister, and G. S. Bloom, manuscript submitted for publication).

Discussion

Earlier studies of kinesin had indicated that the protein exhibits several properties expected of a motor protein for the transport of membrane-bounded organelles along MTs. Kinesin has been shown to bind MTs (Brady, 1985; Vale et al., 1985*a*; Scholey et al., 1985), possess MT-stimulated ATPase activity (Brady, 1985; Kuznetsov and Gelfand, 1986; Cohn et al., 1987; Wagner et al., 1989), and be capable of generating forces that can act upon MTs (Vale et al., 1985*a*,*b*; Porter et al., 1987). Nevertheless, a role for kinesin in organelle motility has remained largely speculative because of the lack of direct evidence that kinesin associates with membrane-bounded organelles. Such direct evidence could take several forms, including immunocytochemical detection of kinesin on organelles of the type that move along MTs, inhibition of such movements by antibodies to kinesin, and copurification of kinesin with membrane-bounded organelles.

Using a library of five monoclonal antibodies made against highly purified bovine brain kinesin, we obtained direct immunocytochemical evidence that kinesin is, indeed, associated with cytoplasmic structures whose properties are consistent with their identification as membrane-bounded organelles. All of the antibodies stained punctate structures in the cytoplasm of a variety of cultured mammalian cell types. These structures could be dissolved by treatment of the cells with Triton X-100 before fixation and staining, suggesting their association with membranous structures. Based on the morphologies and location of the immunoreactive structures, they did not appear to include such membranous compartments as the plasma membrane, nuclear envelope, and Golgi stacks. Significantly, none of the antibodies stained either interphase or mitotic spindle MTs in any of the cell types examined, but kinesin-containing, vesicle-like structures were most concentrated in regions where MTs were also most plentiful. Thus, the results obtained with each antibody represent an independent test of the specificity of kinesin localization. Based on their appearance, sensitivity to detergent, and abundance in MT-enriched domains of cytoplasm, it is likely that the kinesin-containing structures correspond to membrane-bounded organelles.

The exact identities of the organelles that contain kinesin are not currently known. Included among them could be structures as distinct as synaptic vesicles, endosomes, lysosomes, and secretory vesicles, for example. Staining of apparent mitochondria was evident using the L2 antibody, particularly in primary brain cells. Further studies using techniques such as immunoelectron microscopy will be required to identify the specific classes of organelles that contain kinesin.

The failure of the antikinesins to stain MTs in cells did not reflect any innate inability of those monoclonals to label MT-bound kinesin by immunofluorescence. This was indicated by the observation that all five antibodies yielded bright, immunofluorescent staining of isolated, kinesin-containing MTs (not shown). Hence, kinesin is a protein capable of binding stably to MTs under appropriate in vitro conditions, as in the presence of AMP-PNP, but appears to be associated principally with membrane-bounded organelles in the cell. In that regard, kinesin resembles a novel squid protein, vesikin, that binds to MTs in vitro and seems to be localized on vesicles in squid axoplasm (Sloboda and Gilbert, 1988).

Kinesin is clearly present in cytosolic extracts (see Fig. 1 *B*), raising the question of why a soluble pool of the protein was not detected by immunofluorescence microscopy. At least two explanations could potentially account for this. First, homogenization of cells or tissues might have had the effect of artifactually solubilizing much of the kinesin that was associated with membrane-bounded organelles in vivo. Alternatively, a significant fraction of the kinesin in cells could exist in the cytoplasm without being bound to any structures and at a concentration below the threshold of detectability by immunofluorescence microscopy. Such a situation exists for both actin and tubulin, as antibodies to those proteins respectively stain microfilaments and MTs by immunofluorescence, but generally do not reveal the sizeable unpolymerized pools of immunoreactive protein (Lazarides, 1982; Osborn and Weber, 1982). Regardless of which explanation, if either, may be correct, the fact that all five monoclonal antibodies to kinesin yielded similar immunofluorescence results constitutes compelling evidence that kinesin is more highly concentrated on vesicle-like structures than at any other location in the cell. Further studies that take into account the effects of homogenization conditions will be required to determine the relative pool sizes of organelle-bound and soluble kinesin.

The results presented here are fundamentally different from those described in the few prior reports on the intracellular localization of kinesin, all of which involved the use of polyclonal antibodies to kinesin heavy chains. Primary cilia, faintly visible cytoplasmic fibers, reticular structures, centrosomes, and spindles were stained by such antibodies in PtK_1 and *Xenopus* epithelial cells (Neighbors et al., 1988), while mitotic spindles were prominently labeled in sea urchin zygotes and embryos (Scholey et al., 1985; Leslie et al., 1987). The differences between our results and those described for the sea urchin may simply reflect the relative abundance of membrane-bounded organelles found in the mitotic spindles of sea urchin and mammalian cells. Such organelles are sparse in mammalian spindles (Brinkley and Cartwright, 1971; McIntosh and Landis, 1971), but are copious in the spindles of sea urchin zygotes (Harris, 1975), where they undergo impressive translocations during mitosis (Rebhun, 1972). The kinesin found in sea urchin spindles may be associated with these organelles, as has been suggested in an earlier study (Leslie et al., 1987). A variety of other explanations could account for the differences between our results and those described previously for sea urchin and vertebrate cells. Included among these, for example, are variations in the methods used for producing and purifying antibodies.

Our use of a library of monoclonal antibodies, each of which was purified as a specific IgG isotype and all of which were raised against a highly purified antigen, should effectively eliminate concerns about their specificities. As shown in Figs. 4–6, monoclonals to the kinesin heavy or light chains yielded immunofluorescent staining patterns that were very similar to one another. Since each of these five antibodies reacts with a different kinesin epitope, the specificity of the staining pattern appears unambiguous. It is reasonable to

suppose, therefore, that the antibodies accurately revealed the sites of maximal kinesin concentration in cultured mammalian cells. We suspect, moreover, that those sites represent membrane-bounded organelles.

At least two types of mechanistic models for the movement of membrane-bounded organelles along MTs have been seriously considered in recent years. One of these stipulates that motor molecules are distributed uniformly along MTs to which they are firmly bound. In that case, an organelle would move along a MT by being passed along a procession of adjacent motor molecules (Allen et al., 1985) and the model predicts that MTs should be decorated continuously along their lengths with kinesin. The staining of membrane-bounded organelles, but not MTs, by our antibodies to kinesin effectively rules out this model. The alternative paradigm specifies that the motor molecules are bound firmly to the organelles and interact transiently with the MT wall (Miller and Lasek, 1985; Langford et al., 1987). Obviously, this model requires kinesin to be localized on the organelles and is consistent with the immunofluorescence data reported here.

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