Immunochemical Analysis of Kinesin Light Chain Function

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The kinesin heterotetramer consists of two heavy and two light chains. Kinesin light chains have been proposed to act in binding motor protein to cargo, but evidence for this has been indirect. A library of monoclonal antibodies directed against conserved epitopes throughout the kinesin light chain sequence were used to map light chain functional architecture and to assess physiological functions of these domains. Immunocytochemistry with all antibodies showed a punctate pattern that was detergent soluble. A monoclonal antibody (KLC-All) made against a highly conserved epitope in the tandem repeat domain of light chains inhibited fast axonal transport in isolated axoplasm by decreasing both the number and velocity of vesicles moving, whereas an antibody against a conserved amino terminus epitope had no effect. KLC-All was equally effective at inhibiting both anterograde and retrograde transport. Neither antibody inhibited microtubule-binding or ATPase activity in vitro. KLC-All was unique among antibodies tested in releasing kinesin from purified membrane vesicles, suggesting a mechanism of action for inhibition of axonal transport. These results provide further evidence that conventional kinesin is a motor for fast axonal transport and demonstrate that kinesin light chains play an important role in kinesin interaction with membranes.

INTRODUCTION

The movement of membrane-bound organelles within cells requires microtubules and molecular motors. This motility is especially important in axons where vesicles must travel long distances from their sites of origin to their various destinations. Vesicles containing secretory products and other proteins necessary for axonal growth and maintenance travel anterogradely from the cell body to the axon terminal. In contrast, vesicles containing endocytosed materials must travel in the retrograde direction back to the cell body. The conventional view is that kinesin is the motor for anterograde transport and cytoplasmic dynein is the motor for retrograde transport but the exact roles of these motor proteins, as well as the roles of other molecular motors in these processes, are still not clear at this time (Brady and Sperry, 1995).

Studies on isolated squid axoplasm revealed the requirement for ATP and showed that a nonhydrolyzable analogue of ATP, 5'-adenylylimidodiphosphate (AMP-PNP), inhibited organelle transport by causing vesicles to become stably attached to microtubules (Lasek and Brady, 1984, 1985). This finding led to a means of purifying kinesin using a microtubule affinity step in the presence of AMP-PNP (Brady, 1985; Vale *et al.*, 1985a). Purified kinesin was found to be a microtubule-stimulated ATPase (Kuznetsov and Gelfand, 1986; Wagner *et al.*, 1989) that could induce gliding of microtubules in vitro (Porter *et al.*, 1987). Immunofluorescent staining of cells with antibodies against kinesin light or heavy chains revealed a punctate staining pattern enriched in cellular regions containing microtubules (Pfister *et al.*, 1989b).

These structures were detergent extractable, suggesting that the kinesin was associated with organelle membranes in vivo. Furthermore, kinesin copurified with a variety of organelles providing direct evidence for the association of kinesin with organelles (Leopold *et al.*, 1992). Direct evidence that kinesin is a motor for

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fast axonal transport was provided by the demonstration that a monoclonal antibody that recognized both mammalian and squid kinesin heavy chains inhibited fast axonal transport in isolated squid axoplasm (Brady *et al.*, 1990). Experiments with other antibodies (Hollenbeck and Swanson, 1990; Wright *et al.*, 1993), antisense RNA (Ferreira *et al.*, 1992; Amaratunga *et al.*, 1993; Feiguin *et al.*, 1994), and genetic approaches (Saxton *et al.*, 1991; Gho *et al.*, 1992) have all implicated kinesin in transport of at least some classes of membrane-bound organelles. However, relatively little has been known about the molecular basis for interaction of kinesin with membrane surfaces.

The kinesin holoenzyme is a heterotetramer consisting of two heavy and two light chains (Bloom *et al.*, 1988; Kuznetsov *et al.*, 1988). The kinesin heavy chains are responsible for the microtubule-binding and mechanochemical activities of kinesin (Yang *et al.*, 1990). Functions proposed for kinesin light chains include binding to vesicles and regulation of the heavy chains (Cyr *et al.*, 1991), but evidence for these functions has been indirect.

Rotary-shadowed electron microscopic (EM) studies demonstrated that kinesin exists as an 80-nm rod-shaped molecule with two globular heads and a fan-like tail connected by a long stalk (Hirokawa *et al.*, 1989). Antibodies against kinesin heavy chains localized to the globular heads, whereas antibodies against light chains localized to the fan-like tail (Hirokawa *et al.*, 1989). Light chains may also form part of the stalk (Cyr *et al.*, 1991). This fan-like tail is the portion of kinesin that interacts with membrane-bound organelles. The EM studies also showed a kink in the stalk where folding of kinesin could occur. Hydrodynamic studies suggest that purified kinesin in solution may indeed be folded (Bloom *et al.*, 1988; Hackney *et al.*, 1992; Stenoien and Brady, unpublished observations). Such folding of kinesin might serve to regulate kinesin ATPase by placing kinesin light chains in a position where they could interact with the globular head domains of the heavy chains. Consistent with this possibility, removal of light chains by limited proteolysis increases the specific activity of the kinesin ATPase *in vitro* (Kuznetsov *et al.*, 1989; Hackney *et al.*, 1991). However, none of these studies directly addressed the question of light chain function.

Structural analysis of polypeptides encoded by kinesin light chain cDNAs revealed a number of distinct domains within the light chains (Cyr *et al.*, 1991). Near the amino terminus is a region of 15 heptad repeats that are predicted to form an α helical coiled coil structure. This region may be involved in light chain dimerization or in binding to the kinesin heavy chains through interchain α helical coiled coils. The carboxyl termini are subject to alternative splicing and were proposed to act as targeting sequences. Finally, five imperfect copies of a 42-amino acid motif are tan-

demly repeated in a central domain between amino acids 238 and 488 in the rat kinesin polypeptide (Cyr *et al.*, 1991). Cloning of the light chains from other species (Beushausen *et al.*, 1993; Cabeza-Arvelaiz *et al.*, 1993; Gauger and Goldstein, 1993; Wedaman *et al.*, 1993; Fan and Amos, 1994) confirm this domain structure and demonstrate that the tandem repeat domain is very highly conserved.

Given the high degree of conservation between species, we chose to make a monoclonal antibody against an epitope in this tandem repeat region (KLC-All) for two reasons. First, an antibody against this region should be able to recognize the kinesin light chains from a wide variety of species, making it a useful tool. Second, the high degree of conservation for this region indicates that these sequences play an essential role in kinesin function; therefore, antibodies against the tandem repeat domain have the potential to interfere with light chain function. Probes that inhibit kinesin activity would illuminate the physiological roles of kinesin light chains. Concurrent with generation of the KLC-All antibody, a second monoclonal antibody (63–90) was generated against a conserved region located in the light chain amino terminus. In the present study, we show that monoclonal antibodies directed against these two conserved regions recognize both mammalian and squid kinesin light chains.

The KLC-All antibody inhibited fast axonal transport when perfused into squid axoplasm and displaces kinesin from purified membrane-bound vesicles. The characteristics for inhibition of axonal transport by KLC-All differed from those seen in previous studies with a kinesin heavy chain antibody, suggesting that the mechanisms of inhibition may be different. In contrast, the 63–90 antibody had no effect on either axonal transport or membrane association of kinesin. Neither antibody affected the ability of kinesin to bind microtubules or its microtubule-activated ATPase activity. These observations confirm that kinesin is involved in the transport of a wide range of organelles and directly implicate the tandem repeat region of the light chains in membrane binding.

MATERIALS AND METHODS

Production and Purification of Antibodies

Monoclonal antibodies were produced by a modification of a previously described procedure (Pfister *et al.*, 1989b). For the KLC-All antibody, a peptide corresponding to residues 309–318 of the rat light chain sequence (Figure 1) was coupled to keyhole limpet hemocyanin (Calbiochem, La Jolla, CA) using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce, Rockford, IL) according to the instructions of the manufacturer. For the 63–90 antibody, kinesin purified according to our previously published procedure (Wagner *et al.*, 1989) was used as the initial antigen. Fifty micrograms of coupled peptide or purified kinesin in Ribi adjuvant (monophosphoryl lipid A + trehalase dimycolate emulsion, Ribi Immunochem Research, Hamilton, MT) was injected subcutaneously into the hind limbs of five BALB/c mice (The Jackson Laboratory, Bar Harbor,

Figure 1. Epitope map of the kinesin light chain antibodies. (A) Map showing the relative positions of antibody-binding sites on the kinesin light chains. Positions of the 63-90 and L1 antibodies were determined by their ability to bind to expressed light chain fragments. These two antibodies do not recognize a kinesin light chain construct lacking the first 50 amino acids. The KLC-All-binding site is known since it is an antibody generated against a specific peptide. (B) Sequence alignment of the first 50 amino acids of the rat kinesin light chain with the corresponding sequence from squid. There is a region of high sequence conservation that is the probable binding site for the 63-90 antibody. (C) Sequence alignment of the highly conserved tandem repeats. The peptide sequence to which the KLC-All antibody was made is underlined.

ME) on six occasions at 3-d intervals. Mice were bled on d 16 and the sera were screened against purified bovine kinesin. Mice reacting with the kinesin light chains as judged by immunoblotting were killed and popliteal lymph node lymphocytes were fused with NS-1 cells. Hybridoma cultures secreting antibodies to the kinesin light chains were cloned three times by limiting dilution. Positive clones were identified by immunoblotting in multiple screens that variously used purified kinesin, specific peptide coupled to bovine serum albumen (BSA), and fusion proteins containing specific regions of the kinesin light chain as appropriate. The cloned hybridomas were then used to make ascites fluid. For some experiments, antibodies were purified from ascites fluid using the Maps II kit (Bio-Rad, Richmond, CA).

Immunoblots

Immunoblots were performed using a modification of published procedures (Gillespie and Hudspeth, 1991). Antibody fractions of interest were initially identified by screening against samples of kinesin that had been partially purified using adenylyl imidodiphosphate (AMP-PNP, Sigma, St. Louis, MO) to cosediment the kinesin with microtubules. Antibodies that reacted with authentic kinesin light chains were characterized further. Epitopes were mapped by performing immunoblots on either specific peptides conjugated to BSA (for KLC-All) or on glutathione S-transferase fusion proteins containing light chain sequences of interest.

Samples containing antigens of interest were run on SDS-polyacrylamide gels as described previously (Bloom *et al.*, 1988) and transferred 16-20 h in 10 mM CAPS buffer (pH 11) at 25 V onto

Zeta-Probe membrane (Bio-Rad). The membranes were blocked for 2 h in blocking buffer (6% casein, 1% PVP-40, 30 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, 5 mM EDTA, pH 7.4). After blocking, membranes were incubated for 2 to 4 h with primary antibody in the form of ascites fluid diluted 1:1000 in blocking buffer. Membranes were washed three times for 5 min in wash buffer [0.3% Tween 20 in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4)] and incubated for 2 h with alkaline phosphatase-conjugated goat anti-mouse IgG (Southern Biotechnologies, Birmingham, AL). The membranes were washed three times for 5 min in wash buffer followed by two 5-min washes in assay buffer (50 mM Tris, 1 mM MgCl₂, pH 9.5). The membranes were incubated for 5 min in 0.4 mM CSPD (Tropix) in assay buffer and exposed to XAR x-ray film (Kodak, Rochester, NY).

Immunofluorescence

Immunofluorescence microscopy was performed on normal rat kidney (NRK, American Type Culture Collection, Rockville, MD) cells or bovine adrenal chromaffin cells (Livett *et al.*, 1987). Cells were fixed by immersing the coverslips in a 3.7% solution of formaldehyde in PBS for 20 min followed by a 5-min incubation in 0.5% Triton X-100 in PBS. For the experiment in Figure 5D, the cells were preincubated in PEM (100 mM piperazine-*N,N*-bis(2-ethanesulfonic acid), 1 mM MgSO₄, and 1 mM EGTA, pH 7.0) containing 0.1% Triton X-100 for 5 min at 37°C prior to fixation. Cells were washed in PBS and then incubated for 30 min in 2% BSA in PBS. Cells were incubated for 1 h with primary antibody at 20 µg/ml in 10% BSA at 37°C followed by a 1-h incubation with tetramethylrhodamine iso-

thiocyanate-labeled goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) in PBS with 10% BSA. Cells were washed three times with PBS containing 0.5% Triton X-100 after each antibody incubation. Axoplasms were fixed with 3.7% formaldehyde as described previously (Brady *et al.*, 1990) and then processed as for tissue culture cells. Immunofluorescence was performed using a Bio-Rad MRC 1000 confocal setup on a Zeiss Axiovert microscope.

Immunoprecipitations of Axonal Proteins

Immunoprecipitations of rat axonal proteins were performed as described previously (Elluru *et al.*, 1995). Axonally transported proteins were labeled by injecting 1 mCi of [³⁵S]methionine (ICN Biochemicals, Cleveland, OH) into the vitreous of the right eye of a 250-g male Sprague Dawley rat. Optic nerves were removed at 12 h and homogenized in lysis buffer (50 mM NaCl, 25 mM Tris, pH 8.1, 0.5% Triton X-100, 0.5% sodium deoxycholate, 2 mM *o*-vandate, 50 mM NaF, 100 mM KPO₄, 25 mM sodium pyrophosphate, 80 mM β -glycerophosphate, 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 10 μ g/ml pepstatin, 10 μ g/ml leupeptin, 2 μ g/ml aprotinin, and 4.5 mM EDTA). Proteins were immunoprecipitated using antibodies coupled to protein A-Sepharose beads (Zymed Labs, South San Francisco, CA). The proteins were resolved using SDS-PAGE and radiolabeled proteins were detected and quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), or the gels were subjected to fluorography as described previously (Laskey and Mills, 1975). For the experiment in Figure 3, squid optic lobes or rat brains were homogenized in PEM-containing protease inhibitors, and microtubules were pelleted in the presence of Taxol and AMP-PNP. The resuspended pellets were then subjected to immunoprecipitation as described above.

Motility Studies in Isolated Axoplasm

Axoplasm was extruded from giant axons of the squid *Loligo pealeii* (Marine Biological Laboratory, Woods Hole, MA) as described previously (Brady *et al.*, 1982, 1985). Axons used in these studies were 400–600 μ m in diameter and contained $\approx$ 5 μ l of axoplasm. Purified antibodies were dialyzed into $\frac{1}{2}$ buffer (150 mM potassium aspartate, 5 mM MgCl₂, 1 mM EGTA, 10 mM HEPES, pH 7.2). The antibodies were diluted into $\frac{1}{2}$ buffer (175 mM potassium aspartate, 65 mM taurine, 35 mM betaine, 25 mM glycine, 10 mM HEPES, 6.5 mM MgCl₂, 5 mM EGTA, 1.5 mM CaCl₂, 0.5 mM glucose, pH 7.2) supplemented with 2 mM ATP, and 20 μ l of this were added to the perfusion chamber as described previously (Brady *et al.*, 1990). Preparations were observed using a Zeiss Axiomat equipped with a 100 $\times$, 1.3 aperture objective, DIC optics, and a Photonics C1966 AVEC video system with a Chalnicon video camera (Hamamatsu, Bridgewater, NJ). For some studies, a Hamamatsu Argus 20 system and model 2400 CCD video camera were used for image processing and analysis. Organelle velocities were measured using a Photonics Microscopy C2117 video manipulator (Hamamatsu) by matching the speed of cursors moving across the screen to particle movements.

ATPase and Microtubule-binding Studies

ATPase assays were performed as described previously (Wagner *et al.*, 1989). Purified kinesin at a concentration of 0.02 mg/ml was incubated with Taxol-stabilized microtubules at a concentration of 1 mg/ml and 1 mM ATP ([γ -³²P]ATP, >4000 Ci/mmol, ICN Biochemicals) diluted to 5000 cpm/nmol with cold ATP in 100 μ l of IME buffer (15 mM imidazole, 2 mM MgCl₂, and 1 mM EGTA, pH 7.0) for 20 min at 37°C. To determine the effects of KLC-All on the microtubule-stimulated ATPase activity of kinesin, kinesin was preincubated with the antibody at a concentration of 0–0.1 mg/ml (M ratios of 0–100 antibody to kinesin) for 1 h at 4°C prior to performing the assay. ATPase assays were done with kinesin that had a final purification step on a sucrose density gradient that separates kinesin

with light chains from kinesin heavy chains without light chains due to proteolysis.

Microtubule-binding assays were performed by incubating purified kinesin at a concentration of 0.02 mg/ml with Taxol-stabilized microtubules at a concentration of 1 mg/ml and 1 mM AMP-PNP in PEM buffer for 20 min at 37°C. The samples were centrifuged for 30 min at 50,000 rpm (108,920 g_{max}) over a cushion of 20% sucrose in PEM in a Beckman TLA-100.2 rotor at 4°C. Pellets and supernatants were collected, and the presence of kinesin in each fraction was determined by immunoblotting with an antikinesin antibody (H2). To determine the effects of KLC-All on the ability of kinesin to bind to microtubules, the antibody at a concentration of 0–0.1 mg/ml (M ratios of 0–100 antibody to kinesin) was preincubated with the kinesin for 1 h at 4°C prior to performing the assay.

Vesicle-binding Assays

Microsomal vesicles were purified by homogenizing fresh rat brains in five volumes of homogenization buffer (320 mM sucrose, 10 mM HEPES, 1 mM AEBSF, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin, 2 μ g/ml aprotinin, pH 7.4) using a polytron with probe PTA 20S (Brinkman Instruments, Westbury, NY) on medium setting for 40 s. The suspension was centrifuged for 15 min at 17,000 rpm (39,800 g_{max}) in a Beckman JA-17 rotor at 4°C. The supernatant (S1) was centrifuged for 40 min at 32,000 rpm (120,000 g_{max}) in a Beckman TI45 rotor at 4°C. The supernatant (S2) was layered onto 5 ml of 600 mM sucrose and 10 mM HEPES (pH 7.4), and centrifuged for 2 h at 50,000 rpm (260,000 g_{max}) in a Beckman TI70 rotor at 4°C. The pellet (P3) was resuspended in 1 ml of homogenization buffer using 10 passages through a 25-gauge hypodermic needle. The suspension was centrifuged for 10 min at 17,000 rpm in a JA-17 rotor to clear aggregates. Purified vesicle fractions were either used immediately or frozen in liquid nitrogen and stored at –80°C. The amount of endogenous kinesin in this fraction was determined by quantitative immunoblots and represented $\sim$ 0.5% of the total vesicle protein.

To assess the effects of various antibodies on the association of endogenous kinesin with membrane-bound organelles, vesicle fractions at a concentration of 1 mg/ml were preincubated with antibody at a concentration of 50 μ g/ml for 1 h at 4°C in homogenization buffer (a M ratio of 20:1 of antibody to kinesin). Samples were centrifuged for 45 min at 77,500 rpm (260,000 g_{max}) over 400 μ l of 600 mM sucrose and 10 mM HEPES (pH 7.4) in a TLA-100-2 rotor. The kinesin in the supernatants and pellets was detected by immunoblotting using a kinesin heavy chain antibody (H2). The amount of kinesin in each fraction was determined using a laser densitometer (Molecular Dynamics). For controls, the same experiment was performed using no antibody, a kinesin heavy chain antibody (H2), or two different kinesin light chain antibodies (63–90 and L1).

RESULTS

Characterization of Monoclonal Antibodies

To evaluate potential functions for kinesin light chains, we have generated and characterized a library of monoclonal antibodies against various epitopes of the light chain polypeptide. Three of the antibodies from this library (L1, KLC-All, and 63–90) were particularly useful tools for the present study (Figure 1A). L1 was obtained in our original screen for kinesin monoclonal antibodies (Pfister *et al.*, 1989b), but the location of its epitope within the light sequence was not determined at that time. Both KLC-All and 63–90 are new monoclonals that have not been previously described.

KLC-All was raised against a highly conserved epitope found in the tandem repeat domain, a 10-amino acid peptide with sequence KRGKYKEAEP located in the second tandem repeat and starting at position 309 (Figure 1, A and C). This peptide sequence is loosely conserved in all of the rat tandem repeats and is highly conserved in the second repeat for all species examined (Figure 1C). All of the amino acids in this sequence are conserved among rat, *Drosophila*, and squid, with the exception of the glutamate at position 315 that has been changed to an aspartate in *Drosophila* and squid (Cyr *et al.*, 1991; Beushausen *et al.*, 1993; Gauger and Goldstein, 1993). Sequence analysis using the program MacVector showed that this peptide as well as the corresponding peptides in the other repeats are likely to be exposed on the surface, where they have the potential to interact with membranes or with other proteins.

Immunoblots with the KLC-All antibody (Figure 2) show reactivity with both the KLC-A and KLC-B bands found in mammalian kinesin fractions (Pfister *et al.*, 1989b; Elluru *et al.*, 1995). The KLC-All antibody recognizes a similar doublet in squid kinesin fractions with apparent molecular weights of 72,000 and 66,000, respectively (Figure 2). This suggests that molecular weight variants of kinesin light chains are a widespread characteristic of the kinesins. However, kinesin light chains are difficult to visualize in immunoblots of whole tissue extracts because they bind poorly to all blotting membranes tested to date and appear to be readily displaced by other polypeptides of similar molecular weight. Although heavy chains are readily detectable, immunoblots with antibodies against kinesin light chain generally fail to detect any polypeptides even though light chains are readily detectable in immunoprecipitates from these same fractions (e.g., see Figure 3). This is clearly not a problem with antibody affinity because the same antibodies work well in a variety of immunoassays, including blots of kinesin-enriched fractions at low antigen concentration, precipitation from extracts, and immunocytochemistry.

Both L1 and 63-90 map within the first 50 amino acids from the amino terminus of the kinesin light chain polypeptide (Figure 1A). Neither antibody recognizes a light chain glutathione S-transferase fusion protein that is missing only these first 50 amino acids (Stenoien and Brady, unpublished observations). The L1 antibody probably recognizes an epitope between residues 1 and 18 of this amino-terminal fragment that is not as well conserved between rat and nonmammalian species (Figure 1B). The L1 antibody does not recognize squid kinesin light chains in immunoblots (Figure 2); therefore, this antibody was used as a negative control for some studies involving squid axoplasm. In contrast, our 63-90 antibody recognizes the kinesin light chain doublet in both rat and squid kinesin (Figure 2), indicating a conserved epitope. This

Figure 2. Immunoblots using the kinesin light chain antibodies. Kinesin was partially purified from rat brain (R) or squid optic lobe (S) using an AMP-PNP microtubule affinity sedimentation step, and 100 μ g of total protein were loaded per lane. The proteins were immunoblotted with the KLC-All, 63-90, and L1 antibodies. The KLC-All and 63-90 antibodies recognize the kinesin light chains from both rat and squid, whereas the L1 antibody only recognizes the rat kinesin light chains.

suggests that the 63-90 epitope is most likely to be localized to a highly conserved sequence between residues 18 and 50 of the light chain polypeptide (Figure 1B).

Immunoprecipitation of Kinesin Heavy and Light Chains by Light Chain Antibodies

Previous studies (Elluru *et al.*, 1995) demonstrated that both our L1 light chain antibody and H2 heavy chain antibody can immunoprecipitate the full complement of kinesins labeled by axonal transport in optic nerve at 12 h after labeling (Figure 3). Both of these previously described antibodies coprecipitate kinesin heavy and light chains from tissue homogenates, consistent with the identification of heavy and light chains as subunits of the native motor protein (Bloom *et al.*, 1988; Wagner *et al.*, 1989). The KLC-All and the 63-90 antibodies also immunoprecipitated radiolabeled kinesin from rat optic nerve (Figure 3). These results indicate that all four of these antibodies are

Figure 3. Immunoprecipitation of kinesin from the rat optic nerve. Shown are immunoprecipitates from rat optic nerve using three light chain antibodies (KLC-All, 63-90, and L1) as well as a kinesin heavy chain antibody (H2). All four of these antibodies are able to immunoprecipitate both the kinesin heavy chain molecular weight variants (KHC-A and KHC-B) and the light chain molecular weight variants (KLC-A and KLC-B) as previously defined (Elluru *et al.*, 1995). Proteins were labeled by injecting [35 S]methionine into the vitreous body of a rat. After 12 h, the optic nerves were dissected out, homogenized, and subjected to immunoprecipitation using the above antibodies.

Figure 4. Immunoprecipitation of kinesin from squid optic lobes and rat brain. (A) Shown is a Coomassie blue-stained gel of immunoprecipitates using the light chain antibodies KLC-All (lanes 1–3) and 63–90 (lanes 4–6) as well as a kinesin heavy chain antibody, H2 (lanes 7–9). Lanes 1, 4, and 7 are the immunoprecipitations from squid optic lobe and lanes 3, 6, and 9 are the immunoprecipitations from rat brain. Mock immunoprecipitations in the absence of extract are shown in lanes 2, 5, and 8. The positions of the kinesin heavy chain and antibody heavy and light chains are indicated. (B) Western blot of the same samples shown in A using the H2 antibody that recognizes the kinesin heavy chain. Note that the antibody heavy chains are also detected by the secondary antibody used.

able to recognize the native structure of the kinesin light chains in association with heavy chains. KLC-All antibody immunoprecipitates of these fractions do sometimes contain small amounts of a low molecular weight band of approximately 35,000. Since a similar low molecular band appears in some immunoprecipitates with the H2 antibody, this 35,000 polypeptide is thought to bind nonspecifically to either the protein A beads or to some primary antibodies.

The kinesin immunoprecipitated by KLC-All and 63–90 antibodies travels through the optic nerve and tract at rates expected for fast axonal transport and is indistinguishable from that seen with various combinations of our other monoclonal antibodies (Elluru *et al.*, 1995), suggesting that both KLC-All and 63–90 antibodies recognize the full complement of kinesin in optic nerve, including both light chain molecular weight variants. No evidence was found that either the KLC-All or the 63–90 antibody immunoprecipitated other proteins from rat brain homogenates, suggesting that kinesin light chains do not interact with the other kinesin-related proteins that have been described in brain (for review, Brady, 1995; Brady and Sperry, 1995) but are specific for conventional kinesin. Although the possibility remains that a kinesin-related protein with a molecular weight similar to the kinesin heavy chain may be interacting with the kinesin light chains, no genetic or biochemical evidence for this possibility has been obtained to date.

To determine whether the KLC-All and 63–90 antibodies recognize the native squid kinesin, immunoprecipitations were also performed on squid optic

lobe. Both of these antibodies, as well as the H2 antibody, immunoprecipitate kinesin from squid optic lobe fractions, indicating that they recognize the native structure of squid kinesin (Figure 4). The L1 antibody was not included since previous experiments demonstrated that this antibody does not recognize squid kinesin in immunoblots or by immunofluorescence microscopy. Western blots using the H2 antibody were performed on the same samples as further evidence that the KLC-All and 63–90 antibodies immunoprecipitate native squid kinesin.

Immunofluorescence with Antibodies against Kinesin Light Chains

To examine the subcellular localization of KLC-All and 63–90 immunoreactive structures, immunofluorescence was performed on chromaffin cells and NRK tissue culture cells. These antibodies stain numerous punctate structures dispersed throughout the cytoplasm (Figure 5, A–C). Staining seen with these antibodies was similar in appearance to that seen previously with the H2 antibody against kinesin heavy chains and with the L1 antibody against light chains (Pfister *et al.*, 1989b). Extraction of cells with detergent prior to fixation abolishes the immunoreactivity, indicating that these antibodies recognize kinesin bound to membranes (Figure 5D). On the basis of the number of structures stained and wide distribution of staining, both KLC-All and 63–90 appear to recognize kinesin on a wide variety of organelles.

Localization of kinesin light chains by immunofluorescence was also performed on isolated squid axoplasm with the KLC-All antibody. The staining pattern differed slightly from that seen in mammalian tissue culture cells in that the immunoreactive structures were larger and more dispersed (Figure 5E), but this staining pattern was consistent with the pattern previously reported in squid axoplasm using our H2 antibody against kinesin heavy chain (Figure 5F; Brady *et al.*, 1990). As a negative control, axoplasms were also stained with our L1 antibody. Consistent with immunoblot results (Figure 2), no specific staining of squid axoplasm was seen when the L1 antibody was used instead of KLC-All.

Effect of Antibody Perfusion on Organelle Transport in Axoplasm

The effects of KLC-All and 63–90 antibody on fast axonal transport were determined by perfusion of purified antibody fractions into squid axoplasm. KLC-All antibody concentrations between 0.1 and 2 mg/ml had a profound effect on the transport of all types of organelles, including both small organelles comparable in size to synaptic vesicles and mitochondria, within 30 min after perfusion (Table 1). There was a large decrease in both the number of vesicles moving

Figure 5. Immunofluorescence on tissue culture cells and squid axoplasm. (A) KLC-ALL staining of bovine adrenal chromaffin cells. The KLC-All stains numerous punctate structures that are dispersed throughout the cytoplasm, indicating that this antibody recognizes the kinesin on many different types of organelles. (B) 63-90 staining of bovine adrenal chromaffin cells. (C) KLC-All staining of NRK tissue culture cells. (D) KLC-All staining of NRK tissue culture cells that were extracted with Triton X-100 prior to fixation. The detergent extraction abolishes the KLC-All staining, indicating that the KLC-All-reactive structures are vesicles. (E) KLC-All staining of squid axoplasm. This antibody stains numerous punctate structures that are dispersed throughout the axoplasm. The staining differs from that seen in the tissue culture cells in that the structures are larger and more dispersed. (F) H2 staining of squid axoplasm. The H2 antibody that recognizes the squid kinesin heavy chains has a staining pattern very similar to that of KLC-All. Bar, 25 μ m.

and in the overall rate of transport. Since resolution limits preclude accurate quantitation of the number of organelles moving in these preparations, only the ves-

icle velocities were quantitated. Inhibition was observed at KLC-All antibody concentrations ≥ 0.1 mg/ml. Given that the kinesin tetramer is present at a

Table 1. Effects of KLC-All on fast axonal transport in squid axoplasm.

Antibody (mg/ml)	No. of experiments	Anterograde velocity ($\mu\text{m/s}$)	Retrograde velocity ($\mu\text{m/s}$)
KLC-ALL (0.01)	2	1.48 ± 0.07 (n = 11)	1.30 ± 0.06 (n = 9)
KLC-ALL (0.05)	2	1.33 ± 0.14 (n = 7)	1.09 ± 0.13 (n = 7)
KLC-ALL (0.1-0.5)	8	1.11 ± 0.21 (n = 29)	0.91 ± 0.18 (n = 21)
KLC-All (1.0-2.0)	9	0.80 ± 0.29 (n = 32)	0.74 ± 0.27 (n = 33)
L1 (0.1-2.0)	4	1.48 ± 0.14 (n = 17)	1.23 ± 0.15 (n = 17)
63-90 (1.0-2.0)	4	1.85 ± 0.10 (n = 9)	1.28 ± 0.07 (n = 10)

KLC-ALL or control antibodies were perfused into squid axoplasm at the above concentrations and the effects on organelle velocity were observed. Velocities were measured by matching the rate of a calibrated cursor to the rates of multiple particles moving in randomly selected fields of view in AVEC-DIC. All measurements were performed 30–60 min after perfusion with antibody. Each measurement (n) represents the average velocity of particles moving in each field of view at one time point. Velocities are expressed as means $\pm$ SD.

concentration of $\approx 0.5 \mu\text{M}$ in squid axoplasm (Brady *et al.*, 1990), these concentrations of antibody represent antibody:kinesin M ratios ranging from approximately 1 to 20. Lower concentrations of KLC-All antibody ranging from 0.01 to 0.05 mg/ml had little or no effect on organelle transport during incubations of up to 60 min. Interestingly, this antibody affected anterograde and retrograde traffic equally, as seen previously with the H2 antibody (Brady *et al.*, 1990). A control antibody that does not recognize squid kinesin light chain (L1) had no effect on motility when perfused at comparable concentrations (Table 1). The extent of inhibition with KLC-All antibody increased with increasing antibody concentration up to 2 mg/ml, the highest concentration used. At this antibody concentration, virtually all motility had stopped by 60–70 min after perfusion (Figure 6).

There were qualitative differences in the inhibition caused by KLC-All and that previously reported with H2 (Brady *et al.*, 1990). Inhibition with KLC-All antibody resulted in an increase of Brownian motion for particles in axoplasm, suggesting that KLC-All had caused vesicles to disassociate from the microtubules. Consistent with this explanation, very few vesicles could be detected moving along or associating with individual microtubules at the axoplasm periphery. In contrast, inhibition with the H2 antibody led to vesicles that continued to move along these microtubules but at slower rates. At late stages of experiments with KLC-All perfusion, essentially all vesicles throughout

the axoplasm appeared to be undergoing Brownian movements, indicating that they were no longer attached to microtubules.

Another important difference between KLC-All and H2 is the concentration dependence of inhibition. H2 was most effective at concentrations that were approximately equimolar to kinesin. Increasing H2 concentration to M excess gradually decreased the extent of inhibition and motility was never completely inhibited at any antibody concentration tried. The concentration dependence of the H2 antibody suggested that this antibody inhibits kinesin by cross-linking adjacent kinesin molecules and reducing efficiency of movement. As the concentration of antibody increases, each binding site on kinesin is occupied by a single antibody so that cross-linking does not take place. Since inhibition by KLC-All continued to increase as the antibody concentration increased, some mechanism of inhibition other than cross-linking must be invoked to explain KLC-ALL effects.

Although the 63–90 antibody was comparable to KLC-All and H2 in its ability to recognize squid kinesin (see Figure 2; Brady *et al.*, 1990), perfusion of isolated axoplasm with 63–90 antibody did not have a comparable effect on organelle transport (Figure 6 and Table 1). At concentrations of 1–2 mg/ml, purified 63–90 antibody had no detectable effect on transport in either anterograde or retrograde directions. At these concentrations, KLC-All effectively eliminated all movement. Even though maximal inhibition with H2 antibody occurred in the concentration range of 0.1–0.6 mg/ml, there was still a significant reduction in transport rates at concentrations >1.0 mg/ml (Brady *et al.*, 1990). The lack of inhibition by 63–90 antibody suggests that antibody binding at this site does not impair kinesin functions important for microtubule-based motility of organelles.

Effect of Light Chain Antibodies on Kinesin Function In Vitro

The inhibition of transport in isolated axoplasm by the KLC-All antibody indicated that kinesin light chains were important for kinesin function in vivo. The physiological functions of kinesin are the result of three distinct activities of this motor protein: binding to microtubules, microtubule-activated hydrolysis of ATP, and association with the membrane surface of transported organelles. To determine whether kinesin light chains play a role in any of these kinesin activities, three kinds of in vitro assays were done. These assays assessed the effects of light chain antibodies on nucleotide-sensitive microtubule binding of kinesin, microtubule-activated kinesin ATPase, and association of kinesin with purified vesicle fractions.

The effect of KLC-All on the binding of kinesin to microtubules in the presence of AMP-PNP was exam-

Figure 6. Time course for antibody inhibition. The velocities of organelles were measured by matching the speed of a calibrated cursor to the organelle velocities seen on the video screen. (A) In these experiments ($n = 6$), KLC-All at a concentration of 2 mg/ml was added to squid axoplasm. The KLC-All antibody had a profound effect on both anterograde and retrograde organelle traffic, and, at this concentration of antibody, almost all organelles ceased moving by 1 h after perfusion. (B) To control for nonspecific effects of antibodies, the effects of the 63-90 antibody, which also recognizes squid kinesin light chains, were evaluated. When the 63-90 antibody was perfused into squid axoplasm at similar concentrations, there was no effect on organelle motility ($n = 3$). Motility continued in both anterograde and retrograde directions at levels comparable to those seen with no-antibody controls or with an antibody (L1) that does not recognize any squid proteins in immunoblots.

ined. Comparable amounts of kinesin were pelleted with microtubules in the presence and absence of antibody (Figure 7A). Kinesin light chains do not appear to affect the ability of kinesin to interact with microtubules. The effect of light chain antibodies on kinesin ATPase activity in the presence of microtubules was also evaluated. The KLC-All antibody did not have a detectable effect on the microtubule-stimulated ATPase activity of kinesin (Figure 7B and Table 2).

The effect of KLC-All antibody on the basal ATPase activity of kinesin in the absence of microtu-

bules was also examined. A slight decrease in the basal ATPase activity was observed (Table 2) but the significance of this change is uncertain since the basal rate is so low to begin. Regardless, this form of kinesin ATPase activity is not thought to play a significant role in motility. Since no effect of KLC-All antibody incubation could be seen in these two types of *in vitro* assays for kinesin function, the light chains do not appear to inhibit vesicle motility by affecting either the microtubule-binding or ATPase activities of kinesin directly.

Figure 7. KLC-All has no effect on the microtubule-binding and ATPase activities of kinesin. Kinesin (2 μg) was preincubated with 0–10 μg of KLC-All antibody for 1 h prior to performing each assay. (A) The amount of kinesin bound to microtubules in the presence of increasing antibody concentration was determined by centrifuging kinesin with Taxol-stabilized microtubules and 1 mM AMP-PNP. The amount of kinesin bound in the presence of antibody ($n = 4$ for each concentration) was expressed as a ratio of that bound in the absence of antibody. (B) The microtubule-stimulated ATPase activity of kinesin was determined in the presence and absence of antibody. The ATPase activity of kinesin in the presence of antibody ($n = 4$) was expressed as a ratio of the ATPase activity in the absence of antibody.

Table 2. Effect of KLC-All antibody on the microtubule-stimulated and basal ATPase activities of kinesin.

Experiment	Antibody concentration	No. of experiments	Specific activity
+MTs	0 mg/ml	4	325 ± 27
+MTs	0.01 mg/ml	4	323 ± 37
+MTs	0.025 mg/ml	4	337 ± 15
+MTs	0.05 mg/ml	4	318 ± 31
+MTs	0.1 mg/ml	4	316 ± 43
−MTs	0 mg/ml	4	6.3 ± 1.6
−MTs	0.1 mg/ml	4	1.9 ± 0.6

Purified kinesin at a concentration of 0.02 mg/ml was incubated with 1 mM ATP in 100 μ l of IME buffer for 20 min at 37°C to determine the basal ATPase activity (−MTs). The microtubule-stimulated ATPase activity (+MTs) was determined by including Taxol-stabilized microtubules (1.0 mg/ml) in the assay. To determine the effect of KLC-All on ATPase activity of kinesin, the KLC-All antibody at a concentration of 0.01–0.1 mg/ml was preincubated with the kinesin at 4°C for 1 h prior to performing the assay. The specific activity is given as nmol of ATP hydrolyzed per min per mg of kinesin $\pm$ SD.

Observations of increased Brownian motion of vesicles in isolated axoplasm suggested that KLC-All antibody might cause disassociation of vesicles from microtubules. This could result either from release of kinesin from microtubules or by release of kinesin from vesicles. Since KLC-All did not alter kinesin interactions with microtubules (Figure 7), we evaluated the ability of this antibody to displace kinesin from purified vesicle fractions. KLC-All, 63–90, L1 (light chain antibodies), or H2 (heavy chain antibody) were incubated with a purified microsomal vesicle fraction for 1 h and then the fractions were centrifuged to pellet the vesicles and associated proteins. No kinesin heavy chain could be detected in the supernatant of fractions exposed to 63–90, L1, or H2 antibodies (Figure 8). None of these three antibodies could be distinguished from the control with no antibody. In contrast, a substantial amount of kinesin heavy chain was released into the supernatant after incubation with KLC-All (Figure 8). Under the conditions of this experiment, at least 30% of the vesicle kinesin was released from the vesicle surface by incubation with KLC-All (Figure 8B). Addition of a fusion protein containing the kinesin light chain tandem repeats in M excess blocked release of vesicle kinesin by the KLC-All antibody and the tandem repeat fusion protein bound to vesicles without displacing kinesin (Stenoien and Brady, unpublished data). No other antibody released more kinesin than the no-antibody control incubations. This result suggested that binding of KLC-All antibody to kinesin light chains destabilizes kinesin interactions with a membrane surface.

Finally, parallel immunoblots were done on comparable fractions using an antibody that recognized cy-

toplasmic dynein (a generous gift from J.R. McIntosh) to determine whether KLC-All could also release cytoplasmic dynein from the vesicles. Preliminary studies suggest that the distribution of cytoplasmic dynein after KLC-All treatment did not differ from that seen with no antibody or with the H2 antibody. No cytoplasmic dynein was detected in the supernatant and the amount in the pellet fractions did not seem to be affected by antibody treatment. More detailed studies will be needed to determine whether there is any physical interaction between kinesin and cytoplasmic dynein on vesicle surfaces.

DISCUSSION

To explore the function of kinesin light chains in fast axonal transport, we generated a library of monoclonal antibodies that recognize different epitopes in the light chain sequence. Antibodies against conserved sequences in kinesin light chains in different organisms are particularly valuable as tools because these epitopes are likely to represent functionally important domains. One of these new antibodies, KLC-All, recognizes a peptide sequence found in the highly conserved tandem repeat region of the light chains. These repeat domains are present in all kinesin light chains reported to date with remarkable sequence conservation (Bloom and Endow, 1995). A second antibody, 63–90, recognizes a conserved epitope in the light chain amino terminus. KLC-All and 63–90 each immunoprecipitate kinesin from both rat and squid extracts, indicating that they recognize the native structure of kinesin subunits in evolutionarily divergent organisms. This cross-reactivity has facilitated use of these antibodies for evaluation of kinesin light chain function in vivo. Both antibodies stain punctate, detergent-extractable structures in tissue culture cells and in squid axoplasm. They display a staining pattern similar to that seen with our H2 heavy chain antibody that is also widely cross-reactive and which stains a wide variety of organelles (Pfister *et al.*, 1989b Brady *et al.*, 1990).

Once reactivity of KLC-All and 63–90 antibodies with native squid kinesin was established, effects on fast axonal transport could be assessed by perfusion of each antibody into isolated squid axoplasm. The 63–90 antibody had no effect on motility at any concentration tested, suggesting that this domain does not play a critical role in motile activity. However, the preservation of amino acid sequence in this domain from squid to rat suggests that selective pressure to preserve some essential function has served to maintain that identity. The conserved sequence recognized by 63–90 antibody is located near the amino terminus of the light chain, which is thought to be important for light chain–heavy chain interactions. Perhaps the 63–90 epitope is important for assembly of kinesin light chain with

the heavy chain. Precedent for a conserved region adjacent to a domain containing heptad repeats playing a role in assembly of coiled coil α helical structures can be found in intermediate filaments, where a phosphorylation in the amino terminus head regulates assembly (Chou *et al.*, 1989). Molecular genetic and immunochemical analyses should be able to evaluate this possibility in the near future.

In contrast, the KLC-All antibody inhibited both anterograde and retrograde traffic with a similar time course and similar kinetics. At concentrations of antibody that were approximately equimolar to the concentration of kinesin (0.5 μ M) present in squid axoplasm, KLC-All antibody was as effective as the H2 antibody at inhibiting movement. At higher antibody concentrations, the effects of KLC-All and H2 were very different. With H2, inhibition decreases slightly at higher antibody concentrations (antibody:kinesin ratios >5 ; Brady *et al.*, 1990). This decrement in antibody effect suggested that inhibition by H2 might result from cross-linking of adjacent kinesins by the bivalent antibodies, thereby reducing mobility. As the M ratio of antibody:epitope increases, antibodies saturate binding sites without cross-linking adjacent molecules. Inhibition by steric interference would thus be reduced and motor protein movements would not be compromised to the same extent. In contrast, inhibition with KLC-All continued to increase with increasing antibody concentration even at the highest levels tested. This suggested that binding at the KLC-All epitope directly disrupts some aspect of kinesin function.

Kinesin motor function requires three distinct activities: nucleotide-sensitive microtubule binding, microtubule-activated ATPase, and specific membrane interactions. Disrupting any one of these would inhibit organelle transport. Both microtubule- and ATP-binding sites are localized to the kinesin head domain, which is formed by the amino terminus of the heavy chain (Hirokawa *et al.*, 1989; Yang *et al.*, 1990). Membrane binding is thought to be mediated by the carboxyl terminal of the heavy chain and associated light chains at the tail end of the motor protein (Hirokawa *et al.*, 1989). Although the epitope for KLC-All is in the tail domain on kinesin light chains, kinesin in solution folds to place the tail and associated light chains adjacent to the head domain (Hirokawa *et al.*, 1989; Hackney *et al.*, 1992). Such folding might allow light chains to influence either microtubule-binding or ATPase activities.

However, KLC-All binding had no obvious effect on *in vitro* assays of either ATPase or microtubule-binding activities of kinesin. The absence of any effect reduces the likelihood that light chain tandem repeats regulate head domain activities directly. In contrast, coincubation with vesicle fractions containing kinesin

Figure 8. The effects of KLC-All on the binding of endogenous kinesin to vesicles. (A) KLC-All at a concentration of 50 μ g/ml was incubated with purified microsomal vesicles at a concentration of 1 mg/ml for 1 h at 4°C. After centrifugation at $260,000 \times g$, the supernatants and pellets were probed for the presence of kinesin using the H2 antibody on Western blots. A substantial amount of kinesin was released into the supernatant in the presence of KLC-All. For controls, three other kinesin antibodies were used: 63-90, L1, and H2. These antibodies were indistinguishable from the no-antibody control. (B) Quantitation of kinesin released. A total of six experiments were performed and quantitated by scanning the blots with a laser densitometer (Molecular Dynamics). KLC-All was able to release 30% of the endogenous kinesin. These experiments indicate that KLC-All may inhibit organelle motility by disrupting the association of kinesin with organelles.

demonstrated that KLC-All binding released kinesin from the membrane surfaces, whereas 63-90, L1 and H2 antibodies had no effect on kinesin binding. This result suggests that the mechanism for KLC-All inhibition of fast axonal transport is release of kinesin from vesicles induced by antibody binding. Consistent with this explanation, vesicles begin to undergo Brownian motion and do not remain attached to microtubules in the presence of KLC-All antibody. In contrast, vesicles continued to move after H2 antibody perfusion on both peripheral and interior microtubules, albeit at slower rates. The effect of KLC-All also differs from that of AMP-PNP, which acts by blocking ATP hydrolysis and stabilizing microtubule binding. AMP-PNP causes vesicles to become firmly attached to the microtubules both in the interior and on isolated microtubules at the periphery.

These observations are consistent with proposals that kinesin light chains are involved in the binding of

kinesin to membrane components (Hirokawa *et al.*, 1989; Cyr *et al.*, 1991). Furthermore, they indicate that the tandem repeat domain of the light chain plays an important role in this binding. The nature of that binding and the mechanism by which KLC-All antibody releases kinesin remain to be determined. The presence of kinesin in the supernatant fraction of tissue homogenates has been used to argue that kinesin is present in an equilibrium between soluble and bound fractions (Hollenbeck, 1989). However, the kinesin that remains bound to vesicles after the first fractionation step is very tightly associated (Leopold *et al.*, 1992; Schnapp *et al.*, 1992), inconsistent with the existence of a kinesin equilibrium between soluble and bound states. The simplest mechanism to explain antibody-induced release of kinesin from vesicles is that KLC-All binds to kinesin light chain on the membrane surface, which destabilizes the interaction between a membrane component and vesicle-associated kinesin. Further study will be required to determine whether the vesicle component interacting with kinesin light chains is a protein receptor, membrane lipid, or some combination of the two.

Over the last decade, kinesin has been widely accepted as a motor for fast anterograde axonal transport, based on its action as a plus-end-directed motor for moving microtubules in motility assays in vitro (Vale *et al.*, 1985b; Porter *et al.*, 1987; Howard *et al.*, 1989). By contrast, cytoplasmic dynein acts as a minus-end-directed motor for microtubules in the same type of assay, making it a prime candidate for a fast retrograde motor (Paschal and Vallee, 1987; Paschal *et al.*, 1987). Although the simplicity of this model is attractive, a variety of observations suggest that the situation in intact axons may be more complicated.

Pharmacological studies of fast axonal transport in squid axoplasm using agents expected to differentiate between kinesin and dynein-mediated transport have not done so. AMP-PNP perfused into squid axoplasm at concentrations expected to inhibit kinesin but not cytoplasmic dynein inhibits both anterograde and retrograde transport (Lasek and Brady, 1985). Another agent that has been suggested to distinguish between kinesin and dyneins is *N*-ethylmaleimide (NEM). NEM inhibits both kinesin (Pfister *et al.*, 1989a) and cytoplasmic dynein (Paschal *et al.*, 1987), but the concentrations of NEM needed to inhibit in vitro motility of microtubules is higher for kinesin than for dynein (Paschal *et al.*, 1987; Porter *et al.*, 1987). To inhibit either anterograde and retrograde axonal transport in squid axoplasm, NEM must be added at the higher concentration needed to inhibit kinesin and then both directions of transport are inhibited (Pfister *et al.*, 1989a).

Immunological approaches have also failed to inhibit either anterograde or retrograde transport selectively. First, kinesin is present on both anterograde and retrograde-moving organelles, although levels are

significantly lower on retrograde organelles (Dahlström *et al.*, 1991; Hirokawa *et al.*, 1991). Dynein is also detectable in both anterograde and retrograde transport (Hirokawa *et al.*, 1990). Second, a unique antibody against tubulin inhibited both directions of organelle transport (Johnston *et al.*, 1987), presumably by interfering with motors binding to a microtubule. Third, perfusion of our H2 antibody directed against kinesin heavy chain affected both anterograde and retrograde transport at a similar concentration and with a similar time course (Brady *et al.*, 1990). The characteristics of inhibition by H2 antibody suggested an indirect mechanism of inhibition, probably due to cross-linking of adjacent kinesin molecules, raising the possibility that H2 inhibits retrograde transport by cross-linking kinesin on the retrograde organelles and sterically interfering with an adjacent retrograde motor. A similar model might be invoked to explain inhibition of retrograde traffic with AMP-PNP. The small amount of kinesin present on the retrograde organelles could form a rigor attachment to microtubules in the presence of AMP-PNP, preventing continued movement by a retrograde motor (i.e., "chaining" the retrograde vesicle in place). Alternatively, inhibition of retrograde traffic might be caused by nonspecifically clogging the passageways for retrograde organelle transport when the anterograde traffic is halted (i.e., "forming a traffic jam").

The effect of the KLC-All antibody on both anterograde and retrograde transport makes the cross-linking explanation unlikely. KLC-All appears to inhibit by releasing kinesin from vesicles, which is not compatible with cross-linking or chaining any motors. Similarly, the traffic jam explanation is inconsistent with the characteristics of KLC-All inhibition. The most compelling evidence stems from observations of transport along the peripheral microtubules. There was an almost complete lack of organelles moving or attached to isolated microtubules at the periphery of the axoplasm in the presence of KLC-All antibody despite the absence of any apparent physical obstacles. Moreover, organelles that had stopped moving in the interior of the axoplasm with KLC-All were observed to undergo Brownian motion consistent with disassociation. These would not seem to constitute a significant physical barrier to movements mediated by motors that are not released by KLC-All.

Another possibility is that a physical interaction may exist between kinesin and cytoplasmic dynein on vesicles, such that inhibition of kinesin or release of kinesin might have a parallel effect on cytoplasmic dynein. If this is the case, then KLC-All might release cytoplasmic dynein from vesicles as well. However, preliminary studies using parallel immunoblots of vesicle-binding assay fractions with an antibody against the heavy chain of cytoplasmic dynein do not indicate a decrease in the amount of vesicle-associated

cytoplasmic dynein following treatment with either KLC-All or H2 antibodies. At present, the available data do not provide a clear explanation for why pharmacological and immunological treatments directed against one class of microtubule motors frequently block both directions of transport.

A final issue raised by these experiments is the role that other motor proteins may play in fast axonal transport. A number of kinesin-related proteins have been identified in mammalian brain (for review, Brady and Sperry, 1995), and these have been proposed to play a role in translocating various types of organelles (Aizawa *et al.*, 1992; Kondo *et al.*, 1994; Sekine *et al.*, 1994; Nangaku *et al.*, 1994; Noda *et al.*, 1995; Yamazaki *et al.*, 1995). Others have described vesicle transport mediated by unconventional myosins and proposed that these may play a role in some forms of vesicle transport (Kuznetsov *et al.*, 1992; Bearer *et al.*, 1993; Langford *et al.*, 1994).

Despite these reports, results presented here and a number of other lines of evidence suggest that kinesin may be important for movement of a wide range of organelle types. The possibility exists that the KLC-All antibody is able to recognize kinesin light chain-related proteins that could be associated with kinesin heavy chain-related proteins and could thus affect the motility generated by kinesin-related proteins. This does not appear to be the case since the KLC-All antibody only recognizes light chains found on conventional kinesin in immunoblots and does not immunoprecipitate any kinesin-related proteins. Finally, conventional kinesin has been localized to a variety of organelles using antibodies against both the heavy and light chains of kinesin (Pfister *et al.*, 1989b; Brady and Pfister, 1991; Leopold *et al.*, 1992), providing further evidence for its role in translocating many types of organelles.

The light chains of kinesin have been relatively neglected components of the kinesin motor. Cloning them from a variety of organisms (Cyr *et al.*, 1991; Beushausen *et al.*, 1993; Cabeza-Arvelaiz, *et al.*, 1993; Wedaman *et al.*, 1993; Fan and Amos, 1994) indicates regions with an exceptional degree of sequence conservation, but their specific functions have been largely speculative. The immunological dissection of kinesin light chain function described in these studies has provided direct evidence that specific domains of the light chain are involved in kinesin binding to membrane surfaces. Specifically, the highly conserved tandem repeat domains were found to play a critical role in membrane binding, whereas the amino termini of kinesin light chains do not seem to be involved. Although other regions of the light chain (Cyr *et al.*, 1991) and heavy chain (Skoufias *et al.*, 1994) of kinesin may also be important for membrane interactions, the high degree of conservation in these domains suggests a highly specific association that is necessary for kine-

sin binding to membrane-bound organelles. Inhibition of transport by an antibody to this domain confirms that kinesin plays a role in the transport of a wide range of membrane organelles. Future studies on the kinesin light chains should provide more details on kinesin interactions with membrane surfaces and establish the molecular identity of membrane-based ligands for the tandem repeat domain.

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REFERENCES

- Aizawa, H., Sekine, Y., Takemura, R., Zhang, Z., Nangaku, M., and Hirokawa, N. (1992). Kinesin superfamily in murine central nervous system. *J. Cell Biol.* 119, 1287-1296.
- Amaratunga, A., Morin, P., Kosik, K., and Fine, R. (1993). Inhibition of kinesin synthesis and rapid anterograde axonal transport in vivo by antisense oligonucleotide. *J. Biol. Chem.* 268, 17427-17430.
- Bearer, E.L., DeGiorgis, J.A., Bodner, R.A., Kao, A.W., and Reese, T.S. (1993). Evidence for myosin motors on organelles in squid axoplasm. *Proc. Natl. Acad. Sci. USA* 90, 11252-11256.
- Beushausen, S., Kladakis, A., and Jaffe, H. (1993). Kinesin light chains—identification and characterization of a family of proteins from the optic lobe of the squid *Loligo pealeii*. *DNA Cell Biol.* 12, 901-909.
- Bloom, G.S., and Endow, S.A. (1995). Motor proteins 1: kinesins. *Protein Profile* 2, 1109-1171.
- Bloom, G.S., Wagner, M.C., Pfister, K.K., and Brady, S.T. (1988). Native structure and physical properties of bovine brain kinesin and identification of the ATP-binding subunit polypeptide. *Biochemistry* 27, 3409-3416.
- Brady, S.T. (1985). A novel brain ATPase with properties expected for the fast axonal transport motor. *Nature* 317, 73-75.
- Brady, S.T. (1995). A kinesin medley: biochemical and functional heterogeneity. *Trends Cell Biol.* 5, 159-164.
- Brady, S.T., Lasek, R.J., and Allen, R.D. (1982). Fast axonal transport in extruded axoplasm from squid giant axon. *Science* 218, 1129-1131.
- Brady, S.T., Lasek, R.J., and Allen, R.D. (1985). Video microscopy of fast axonal transport in isolated axoplasm: a new model for study of molecular mechanisms. *Cell Motil.* 5, 81-101.
- Brady, S.T., and Pfister, K.K. (1991). Kinesin interactions with membrane bounded organelles in vivo and in vitro. In: *Motor Proteins*, ed. J. Kendrick-Jones and R.A. Cross, Cambridge, United Kingdom: The Company of Biologists, 103-108.
- Brady, S.T., Pfister, K.K., and Bloom, G.S. (1990). A monoclonal antibody against kinesin inhibits both anterograde and retrograde fast axonal transport in squid axoplasm. *Proc. Natl. Acad. Sci. USA* 87, 1061-1065.

- Brady, S.T., and Sperry, A.O. (1995). Biochemical and functional diversity of microtubule motors in the nervous system. *Curr. Opin. Neurobiol.* 5, 551–558.
- Cabeza-Arvelaiz, Y., Shih, L.C.N., Hardman, N., Asselbergs, F.A., Bilbe, G., Schmitz, A., White, B., Siciliano, M., and Lachman, L.B. (1993). Cloning and molecular characterization of the human kinesin light chains gene. *European Molecular Biology Laboratory nucleotide library*.
- Chou, Y.H., Rosevear, E., and Goldman, R.D. (1989). Phosphorylation and disassembly of intermediate filaments in mitotic cells. *Proc. Natl. Acad. Sci. USA* 86, 1885–1889.
- Cyr, J.L., Pfister, K.K., Bloom, G.S., Slaughter, C.A., and Brady, S.T. (1991). Molecular genetics of kinesin light chains: Generation of isoforms by alternative splicing. *Proc. Natl. Acad. Sci. USA* 88, 10114–10118.
- Dahlström, A., Pfister, K.K., and Brady, S.T. (1991). The axonal transport motor kinesin is bound to anterogradely transported organelles: quantitative cytofluorimetric studies of fast axonal transport in the rat. *Acta Physiol. Scand.* 141, 469–476.
- Elluru, R., Bloom, G.S., and Brady, S.T. (1995). Fast axonal transport of kinesin in the rat visual system: functionality of the kinesin heavy chain isoforms. *Mol. Biol. Cell* 6, 21–40.
- Fan, J., and Amos, L.A. (1994). Kinesin light chain isoforms in *Caenorhabditis elegans*. Sequence note. *J. Mol. Biol.* 240, 507–512.
- Feiguin, F., Ferreira, A., Kosik, K.S., and Caceres, A. (1994). Kinesin-mediated organelle translocation revealed by specific cellular manipulations. *J. Cell Biol.* 127, 1021–1039.
- Ferreira, A., Niclas, J., Vale, R.D., Banker, G., and Kosik, K.S. (1992). Suppression of kinesin expression in cultured hippocampal neurons using antisense oligonucleotides. *J. Cell Biol.* 117, 595–606.
- Gauger, A.K., and Goldstein, L.S. (1993). The *Drosophila* kinesin light chain. Primary structure and interaction with kinesin heavy chain. *J. Biol. Chem.* 268, 13657–13666.
- Gho, M., McDonald, K., Ganetzky, B., and Saxton, W.M. (1992). Effects of kinesin mutations on neuronal functions. *Science* 258, 313–316.
- Gillespie, P.G., and Hudspeth, A.J. (1991). Chemiluminescence detection of proteins from single cells. *Proc. Natl. Acad. Sci. USA* 88, 2563–2567.
- Hackney, D.D., Levitt, J.D., and Suhan, J. (1992). Kinesin undergoes a 9S to 6S conformational transition. *J. Biol. Chem.* 267, 8696–8701.
- Hackney, D.D., Levitt, J.D., and Wagner, D.D. (1991). Characterization of $\alpha 2\beta 2$ and $\alpha 2$ forms of kinesin. *Biochem. Biophys. Res. Commun.* 174, 810–815.
- Hirokawa, N., Pfister, K.K., Yorifuji, H., Wagner, M.C., Brady, S.T., and Bloom, G.S. (1989). Submolecular domains of bovine brain kinesin identified by electron microscopy and monoclonal antibody decoration. *Cell* 56, 867–878.
- Hirokawa, N., Sato-Yoshitake, R., Kobayashi, N., Pfister, K.K., Bloom, G.S., and Brady, S.T. (1991). Kinesin associates with anterogradely transported membranous organelles in vivo. *J. Cell Biol.* 114, 295–302.
- Hirokawa, N., Yoshida, Y., Sato-Yoshitake, R., and Kawashima, T. (1990). Brain dynein (MAP1C) localizes on both anterogradely and retrogradely transported membranous organelles in vivo. *J. Cell Biol.* 111, 1027–1037.
- Hollenbeck, P.J. (1989). The distribution, abundance, and subcellular localization of kinesin. *J. Cell Biol.* 108, 2335–2342.
- Hollenbeck, P.J., and Swanson, J.A. (1990). Radial extension of macrophage tubular lysosomes supported by kinesin. *Nature* 346, 864–866.
- Howard, J., Hudspeth, A.J., and Vale, R.D. (1989). Movement of microtubules by single kinesin molecules. *Nature* 342, 154–158.
- Johnston, K.M., Brady, S.T., Kooy, D.v.d., and Connolly, J.A. (1987). A unique tubulin antibody which disrupts particle movement in squid axoplasm. *Cell Motil. Cytoskeleton.* 7, 110–115.
- Kondo, S., Sato-Yoshitake, R., Noda, Y., Aizawa, H., Nakata, T., Matsuura, Y., and Hirokawa, N. (1994). KIF3A is a new microtubule-based anterograde motor in the nerve axon. *J. Cell Biol.* 125, 1095–1107.
- Kuznetsov, S.A., and Gelfand, V.I. (1986). Bovine brain kinesin is a microtubule-activated ATPase. *Proc. Natl. Acad. Sci. USA* 83, 8530–8534.
- Kuznetsov, S.A., Langford, G.M., and Weiss, D.G. (1992). Actin-dependent organelle movement in squid axoplasm. *Nature* 356, 722–725.
- Kuznetsov, S.A., Vaisberg, E.A., Rothwell, S.W., Murphy, D.B., and Gelfand, V.I. (1989). Isolation of a 45 kDa fragment from the kinesin heavy chain with enhanced ATPase and microtubule-binding activities. *J. Biol. Chem.* 264, 589–595.
- Kuznetsov, S.A., Vaisberg, E.A., Shanina, N.A., Magretova, N.A., Chernyak, N.M., and Gelfand, V.I. (1988). The quaternary structure of bovine brain kinesin. *EMBO J.* 7, 353–356.
- Langford, G.M., Kuznetsov, S.A., Johnson, D., Cohen, D.L., and Weiss, D.G. (1994). Movement of axoplasmic organelles on actin filaments assembled on acrosomal processes: evidence for a barbed-end-directed organelle motor. *J. Cell Sci.* 107, 2291–2298.
- Lasek, R.J., and Brady, S.T. (1984). Adenylyl imidodiphosphate (AMPPNP), a nonhydrolyzable analogue of ATP, produces a stable intermediate in the motility cycle of fast axonal transport. *Biol. Bull.* 167, 503.
- Lasek, R.J., and Brady, S.T. (1985). Attachment of transported vesicles to microtubules in axoplasm is facilitated by AMP-PNP. *Nature* 316, 645–647.
- Laskey, R.A., and Mills, A.D. (1975). Quantitative film detection of 3H and 14C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* 56, 335–341.
- Leopold, P.L., McDowall, A.W., Pfister, K.K., Bloom, G.S., and Brady, S.T. (1992). Association of kinesin with characterized membrane-bounded organelles. *Cell Motil. Cytoskeleton* 23, 19–33.
- Livett, B.G., Mitchelhill, K.I., and Dean, D.M. (1987). Adrenal chromaffin cells—their isolation and culture. In: *In Vitro Methods for Studying Secretion*, ed. A.M. Poisner and J.M. Tifará, New York: Elsevier, 171–175.
- Nangaku, M., Sato-Yoshitake, R., Okada, Y., Noda, Y., Takemura, R., Yamazaki, H., and Hirokawa, N. (1994). Kif1b, a novel microtubule plus end-directed monomeric motor protein for mitochondria transport. *Cell* 79, 1209–1220.
- Noda, Y., Sato-Yoshitake, R., Kondo, S., Nangaku, M., and Hirokawa, N. (1995). KIF2 is a new microtubule-based anterograde motor that transports membranous organelles distinct from those carried by kinesin heavy chain or KIF3A/B. *J. Biol. Chem.* 270, 157–167.
- Paschal, B.M., Shpetner, H.S., and Vallee, R.B. (1987). MAP1C is a microtubule-activated ATPase which translocates microtubules in vitro and has dynein-like properties. *J. Cell Biol.* 105, 1273–1282.
- Paschal, B.M., and Vallee, R.B. (1987). Retrograde transport by the microtubule-associated protein MAP1C. *Nature* 330, 181–183.
- Pfister, K.K., Wagner, M.C., Bloom, G.S., and Brady, S.T. (1989a). Modification of the microtubule-binding and ATPase activities of

- kinesin by *N*-ethylmaleimide (NEM) suggests a role for sulfhydryls in fast axonal transport. *Biochemistry* 28, 9006–9012.
- Pfister, K.K., Wagner, M.C., Stenoi, D., Bloom, G.S., and Brady, S.T. (1989b). Monoclonal antibodies to kinesin heavy and light chains stain vesicle-like structures, but not microtubules, in cultured cells. *J. Cell Biol.* 108, 1453–1463.
- Porter, M.E., Scholey, J.M., Stemple, D.L., Vigers, G.P.A., Vale, R.D., Sheetz, M.P., and McIntosh, J.R. (1987). Characterization of the microtubule movement produced by sea urchin egg kinesin. *J. Biol. Chem.* 262, 2794–2802.
- Saxton, W.M., Hicks, J., Goldstein, L.S.B., and Raff, E.C. (1991). Kinesin heavy chain is essential for viability and neuromuscular functions in *Drosophila*, but mutants show no defects in mitosis. *Cell* 64, 1093–1102.
- Schnapp, B.J., Reese, T.S., and Bechtold, R. (1992). Kinesin is bound with high affinity to squid axon organelles that move to the plus-end of microtubules. *J. Cell Biol.* 119, 389–399.
- Sekine, Y., Okada, Y., Noda, Y., Kondo, S., Aizawa, H., Takemura, R., and Hirokawa, N. (1994). A novel microtubule-based motor protein (Kif4) for organelle transports whose expression is regulated developmentally. *J. Cell Biol.* 127, 187–201.
- Skoufias, D.A., Cole, D.G., Wedaman, K.P., and Scholey, J.M. (1994). The carboxy-terminal domain of kinesin heavy chain is important for membrane binding. *J. Biol. Chem.* 269, 1477–1485.
- Vale, R.D., Reese, T.S., and Sheetz, M.P. (1985a). Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell* 42, 39–50.
- Vale, R.D., Schnapp, B.J., Mitchison, T., Steuer, E., Reese, T.S., and Sheetz, M.P. (1985b). Different axoplasmic proteins generate movement in opposite directions along microtubules in vitro. *Cell* 43, 623–632.
- Wagner, M.C., Pfister, K.K., Bloom, G.S., and Brady, S.T. (1989). Copurification of kinesin polypeptides with microtubule-stimulated Mg-ATPase activity and kinetic analysis of enzymatic processes. *Cell Motil. Cytoskeleton* 12, 195–215.
- Wedaman, K.P., Knight, A.E., Kendrick Jones, J., and Scholey, J.M. (1993). Sequences of sea urchin kinesin light chain isoforms. *J. Mol. Biol.* 231, 155–158.
- Wright, B.D., Terasaki, M., and Scholey, J.M. (1993). Roles of kinesin and kinesin-like proteins in sea urchin embryonic cell division: evaluation using antibody microinjection. *J. Cell Biol.* 123, 681–689.
- Yamazaki, H., Nakata, T., Okada, Y., and Hirokawa, N. (1995). KIF3A/3B: a heterotrimeric kinesin superfamily protein that works as a microtubule plus end-directed motor for membrane organelle transport. *J. Cell Biol.* 130, 1387–1399.
- Yang, J.T., Saxton, W.M., Stewart, R.J., Raff, E.C., and Goldstein, L.S.B. (1990). Evidence that the head of kinesin is sufficient for force generation and motility in vitro. *Science* 249, 42–47.