Fast Axonal Transport of Kinesin in the Rat Visual System: Functionality of Kinesin Heavy Chain Isoforms

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Submitted September 13, 1994; Accepted October 26, 1994

Monitoring Editor: Joan Ruderman

The mechanochemical ATPase kinesin is thought to move membrane-bounded organelles along microtubules in fast axonal transport. However, fast transport includes several classes of organelles moving at rates that differ by an order of magnitude. Further, the fact that cytoplasmic forms of kinesin exist suggests that kinesins might move cytoplasmic structures such as the cytoskeleton. To define cellular roles for kinesin, the axonal transport of kinesin was characterized. Retinal proteins were pulse-labeled, and movement of radiolabeled kinesin through optic nerve and tract into the terminals was monitored by immunoprecipitation. Heavy and light chains of kinesin appeared in nerve and tract at times consistent with fast transport. Little or no kinesin moved with slow axonal transport indicating that effectively all axonal kinesin is associated with membranous organelles. Both kinesin heavy chain molecular weight variants of 130,000 and 124,000 M_r (KHC-A and KHC-B) moved in fast anterograde transport, but KHC-A moved at 5–6 times the rate of KHC-B. KHC-A cotransported with the synaptic vesicle marker synaptophysin, while a portion of KHC-B cotransported with the mitochondrial marker hexokinase. These results suggest that KHC-A is enriched on small tubulovesicular structures like synaptic vesicles and that at least one form of KHC-B is predominantly on mitochondria. Biochemical specialization may target kinesins to appropriate organelles and facilitate differential regulation of transport.

INTRODUCTION

Neurons undertake a sizeable task in the creation and maintenance of axonal arbors. There is little if any protein synthesis in the axon, so polypeptides needed in axons or terminals must be transported from the cell body to their site of utilization. Because axons in humans can span a meter or more and axons in larger animals may extend even further, this task of moving material within the neuron is formidable. Classic experiments employing pulse-labeling techniques with protein precursors demonstrated that movement of material within the axon occurs in a very organized manner [reviewed by Brady (1985a)]. Efficient mechanisms exist to assure a continuing supply of essential

elements in each of the functional domains within a neuron.

Material moves within the axon as part of one of four major rate components which differ in their rate of movement and protein composition. In mammalian nerves, fast component (FC) represents the movement of membrane bounded organelles (including synaptic vesicle precursors, mitochondria, lysosomes, and their associated proteins) from the cell body toward the nerve terminal (anterograde direction) and from the nerve terminal toward the cell body (retrograde direction) at a rate of 50–400 mm/d. Slow component a (SCa) and slow component b (SCb) move only in the anterograde direction going from cell body toward the nerve terminal at a rate of 0.2–1 mm/d and 2–8 mm/d, respectively. SCa consists of microtubules and neurofilaments, whereas SCb consists of microfilaments, clathrin, and enzymes of intermediary metab-

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olism (Brady, 1985a). Although detailed descriptions exist of the composition and rates of movement for the major axonal transport rate components, less has been known about underlying molecular mechanisms. Discovery of the mechanochemical ATPase kinesin (Brady, 1985b; Vale *et al.*, 1985) provided a candidate motor molecule for movement of membrane bounded organelles in FC, but questions remained about motors for SCA and SCB. One way of obtaining insights into the role of specific motor proteins in different axonal transport rate components is to characterize the rates at which they move and to identify associated structures.

Biochemical characterization of kinesin revealed molecular weight and charge variants for both kinesin heavy and light chains (Bloom *et al.*, 1988; Pfister *et al.*, 1989; Wagner *et al.*, 1989). Based on their relative proportions, 124,000 and 130,000 M_r variants of the kinesin heavy chain, and the 64,000 and 70,000 M_r variants of the kinesin light chain were initially designated "major" and "minor" isoforms. However, *in vivo* analyses suggested that this may be an artifact of purification. Here the "minor" and "major" isoforms of the kinesin heavy chain are designated kinesin heavy chain A (KHC-A) and kinesin heavy chain B (KHC-B), respectively. Similarly, "minor" and "major" isoforms of the kinesin light chain are designated kinesin light chain A (KLC-A) and kinesin light chain B (KLC-B). These molecular weight variants of the kinesin heavy and light chains are in turn composed of multiple isoelectric species. Though the mechanisms by which these biochemical differences are generated remain to be determined, this biochemical diversity suggested that kinesin may perform multiple roles in transport of materials through the axon.

One established role for kinesin is the transport of membrane-bounded organelles along microtubules. A number of observations suggest that kinesin associates with a variety of membrane-bounded organelles *in situ*. Immunofluorescent localization of kinesin in cultured cells (Hollenbeck, 1989; Pfister *et al.*, 1989), squid giant axon (Brady *et al.*, 1990), and sea urchin blastomeres and coelomocytes (Wright *et al.*, 1991) reveals a punctate pattern that is sensitive to detergent solubilization. Further, kinesin can be localized by immunoelectron microscopy to the surface of membrane bounded organelles such as synaptic vesicles, mitochondria and coated vesicles (Leopold *et al.*, 1992). Consistent with this role, kinesin accumulates at nerve ligations at fast transport rates along with membranous components of axonal transport (Dahlström *et al.*, 1991; Hirokawa *et al.*, 1991; Morin *et al.*, 1993). However, descriptions exist of kinesin immunoreactivity with a diffuse distribution (Neighbors *et al.*, 1988) and there are suggestions that the bulk of cellular kinesin is soluble (Hollenbeck, 1989).

Regardless, several lines of evidence suggest that kinesin on the surface of membrane bounded or-

ganelles is necessary for movement of these structures. Anti-kinesin antibodies perfused into squid axoplasm inhibits both anterograde and retrograde organelle traffic (Brady *et al.*, 1990). Similarly, injection of anti-kinesin antibodies disrupts endocytic membrane compartments in macrophages (Hollenbeck and Swanson, 1990) and inhibits the movement of membrane from Golgi to the endoplasmic reticulum in NRK and Hela cells (Lippincott-Schwartz *et al.*, 1995). When kinesin synthesis is inhibited using anti-sense oligonucleotides in cultured hippocampal neurons or in the rabbit retinal ganglion, delivery of synapse associated proteins such as GAP-43 and synapsin I to nerve terminals is reduced (Ferreira *et al.*, 1992; Amaratunga *et al.*, 1993).

The consensus is that kinesin associates with membrane bounded organelles and moves them along microtubules as part of fast axonal transport. However, this view does not account for the diverse composition of the fast component, which includes multiple subclasses of membrane bounded organelles such as synaptic vesicle precursors, mitochondria and lysosomes; nor does it explain the biochemical heterogeneity of brain kinesin. Organelles move at rates that range from 50 to 200 mm/d in rat retinal ganglion cell axons and the range of rates may be even greater in other neurons. Furthermore, organelles move both from cell body to nerve terminal in anterograde transport and from nerve terminal back to cell body in retrograde transport. The roles played by various kinesin isoforms in this highly organized transport scheme and the molecular basis for regulation of kinesin activity have been largely unexamined.

There is also a possibility that kinesin associates with cellular structures that are not bounded by a membrane. If so, then kinesins may also be involved in the movement of cytoskeletal structures in slow axonal transport. Hollenbeck reported that a sizeable portion (~70%) of the cellular kinesin could be extracted using mild buffers which normally do not solubilize membrane proteins (Hollenbeck, 1989). Buffers without detergents are routinely used during purification to extract a substantial fraction of the kinesin in bovine brain (Wagner *et al.*, 1989, 1991). Therefore, kinesin could potentially have multiple roles in movement of material through the axon.

To distinguish among these putative roles for kinesin in axonal transport, the present study characterizes the axonal transport of kinesin. Rates of movement for different subclasses of membrane bounded organelles and cytoskeletal structures through the axon are known (Willard *et al.*, 1974; McQuarrie *et al.*, 1986; Oblinger *et al.*, 1987). If rate(s) of movement for kinesin in the axon can be determined, then roles for this mechanochemical enzyme in the movement of specific types of axonal structures can be assessed. To charac-

terize axonal transport of kinesin, rat retinal ganglion cells were pulse-labeled with ^{35}S -methionine and movement of kinesin into the optic nerve, tract and tectum was monitored by quantitative immunoprecipitation.

Kinesin was transported only with the fast components of axonal transport. There was no kinesin detected that moved coordinately with the cytoskeletal components of SCa and SCb. This indicates that kinesin is involved in the movement of membrane bounded organelles, but not cytoskeletal structures. Different biochemical isoforms of kinesin heavy chain moved in transport with different kinetics and these rates could be correlated with various subclasses of membrane bounded organelles. KHC-A was cotransported with a synaptic vesicle marker, whereas a subset of KHC-B variants were cotransported with mitochondrial markers. Biochemical isoforms of kinesin light chains exhibit differential transport as well, but did not correlate simply with specific organelle classes. Biochemical specialization of kinesin may be necessary to target kinesin to different subclasses of organelles or may allow the movement of various organelle subclasses to be regulated differentially. These results help define the role of kinesin in axonal transport and provide evidence for functional specialization of kinesin isoforms *in vivo*.

MATERIALS AND METHODS

All reagents were purchased from Sigma Chemical (St. Louis, MO) or Polysciences (Warrington, PA) unless otherwise specified. Protein concentrations were determined using Pierce (Rockford, IL) BCA reagent with bovine serum albumin as the standard. All statistical data is presented as mean $\pm$ standard deviation.

Injection of Radiolabeled Precursors

Axonally transported proteins were labeled as described earlier (Brady and Lasek, 1982; Brady, 1985a). ^{35}S -Methionine (Tran ^{35}S -label, ICN, Montreal, Quebec, Canada) was lyophilized and resuspended in water to a concentration of 0.25 mCi/ μl . Adult Sprague-Dawley rats (Sasco, The Woodlands, TX) were anesthetized with ether and 1 mCi of the radiolabel was injected into the vitreous of the right eye using a 30-gauge needle attached to a Hamilton syringe (Microliter no. 710, 22s gauge, Hamilton, Reno, Nevada) by PE-20 polyethylene tubing (Clay Adams, Parsippany, NJ).

Harvest of Radiolabeled Tissue

Animals were anesthetized with ether at specified times after injection and killed by decapitation. In fast transport studies, two radiolabeled animals per time point were killed at 4, 8, 12, 20, 24, 30, 36, 48, 60, or 96 h post-labeling, and tissue samples were pooled. The optic nerves, optic chiasm, optic tracts, lateral geniculate nuclei (LGN), and superior colliculi nuclei (SCN) were harvested (see Figure 1). Optic nerves were designated as FC-I, the optic chiasm and optic tracts were designated as FC-II, and the LGN and SCN were designated as FC-III. FC-I and FC-II were approximately 10 mm in length. For SCb studies, two radiolabeled animals per time point were killed at 2, 4, or 6 d post-labeling. Optic nerves, chiasm, and tracts were harvested, and segments from the two rats were pooled. The optic nerve and tract were sectioned into three equal

pieces. The first half of the optic nerve, the second half of the optic nerve, and the optic chiasm plus part of the optic tract were designated as SC-I, SC-II, and SC-III, respectively (see Figure 1). Each segment was about 5 mm in length.

Radiolabeled tissue was homogenized in 500–800 μl of lysis buffer (50 mM NaCl, 25 mM Tris, pH 8.1, 0.5% Triton X-100, 0.5% sodium deoxycholate, 2 mM orthovanadate, 50 mM NaF, 100 mM KPO_4 , 25 mM sodium pyrophosphate, 80 mM β -glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$ pepstatin, 2 $\mu\text{g}/\text{ml}$ aprotinin, 4.5 mM EDTA, 1 mM benzamidine, and 10 $\mu\text{g}/\text{ml}$ leupeptin) using a microhomogenizer (Micro-Metric Instruments, Tampa, FL). The insoluble particulate matter was pelleted by centrifugation at 14,000 rpm for 10 min at 4°C in an Eppendorf microcentrifuge. No kinesin was detected in this insoluble particulate fraction by immunoblot. The supernatant was collected, and the centrifugation step was repeated to ensure complete removal of insoluble material. To remove any material in the homogenate that nonspecifically bound to the protein A beads used in immunoprecipitations (described below), 100 μl of protein A-Sepharose 4B (Pharmacia Biotech, Piscataway, NJ) beads was added to the radiolabeled homogenate and incubated at 4°C for 30 min. These beads were then pelleted by centrifugation at 14,000 rpm in an Eppendorf microcentrifuge for 5 min at 4°C and discarded. An aliquot of precleared homogenate was used to determine the amount of radiolabel present by liquid scintillation counting. The rest of the homogenate was used for immunoprecipitation of kinesin, synaptophysin and hexokinase as described below.

Immunoprecipitation

The monoclonal antibodies to kinesin (H1, H2, L1, L2), chosen for this study have been extensively characterized (Hirokawa *et al.*, 1989; Pfister *et al.*, 1989; Leopold *et al.*, 1992). Except as noted, all four monoclonal antibodies were pooled for use in immunoprecipitations: H1 and H2 recognize kinesin heavy chains; L1 and L2 recognize kinesin light chains. Pooled monoclonal antibodies were bound to protein A-Sepharose 4B beads (50% slurry, Zymed Labs, S. San Francisco, CA) by mixing 15 μl of each of the four monoclonal antibodies in the form of ascites fluid with 300 μl of the protein A beads in 1 ml of binding buffer (MAPS II kit, Bio-Rad, Richmond, CA), followed by end-over-end agitation for 2 h at 4°C. Unbound antibody was removed by pelleting the beads (centrifuge at 14,000 rpm using an Eppendorf microcentrifuge for 5 min at 4°C), discarding the supernatant, and resuspending beads in 1 ml of binding buffer. This wash step was repeated twice to ensure complete removal of unbound antibody. To immunoprecipitate synaptophysin, hexokinase, or clathrin, antibody-linked beads were similarly prepared using 30 μl of G95, anti-rat brain synaptophysin rabbit polyclonal serum (from the laboratory of Paul Greengard, Rockefeller Institute), 30 μl of anti-rat brain hexokinase rabbit polyclonal serum (from the laboratory of John E. Wilson, Michigan State University), or 40 μl of OZ-71, anti-bovine brain clathrin mouse monoclonal ascites fluid (from the laboratory of Dr. Richard G. W. Anderson, University of Texas Southwestern Medical Center).

Precleared homogenate was added to the anti-kinesin antibody linked beads along with an equal volume of NET gel (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 0.25% gelatin, and 0.05% Triton X-100) plus protease inhibitors (phenylmethylsulfonyl fluoride, pepstatin, leupeptin, aprotinin, EDTA, and benzamidine). The homogenate/bead mixture was incubated at 4°C for 4–12 h with end-over-end agitation. The beads were then pelleted as above and washed four times with wash buffer I (phosphate-buffered saline, pH 7.4, 0.5% Triton X-100, 0.05% sodium deoxycholate, 0.01% sodium dodecyl sulfate, and 0.02% sodium azide) and two times with wash buffer II (125 mM Tris, pH 8.1, 500 mM sodium chloride, 0.5% Triton X-100, 10 mM EDTA, and 0.02% sodium azide). Bound kinesin was released from the beads by adding 80 μl of Laemmli sample buffer and boiling for 10 min in a boiling water bath, and the constituent polypeptides were resolved by sodium dodecyl sulfate-

Figure 1. Schematic of the experimental paradigm used in this study to characterize the axonal transport of kinesin in the rat retinal ganglion. The left side of the figure contains the time points and nerve segments used in the fast transport studies, whereas the right side of the figure contains the specifications for the SCb experiments.

polyacrylamide gel electrophoresis (SDS-PAGE). The resulting gel was Coomassie Blue-stained and fluorographed to visualize radioactive polypeptides. To determine the amount of radioactivity associated with a particular polypeptide, each radioactive band was excised from the polyacrylamide gel and quantitated in a liquid scintillation counter (as described below). Synaptophysin, hexokinase, and clathrin were sequentially immunoprecipitated from the same radiolabeled homogenate after immunoprecipitation of kinesin.

To determine whether any radiolabeled kinesin remained in the homogenate after immunoprecipitation, homogenates were immunoprecipitated three more times using the same set of anti-kinesin antibodies. The amount of radiolabeled kinesin present in each immunoprecipitate was quantitated as described above. These values were added together and defined as the total amount of radiolabeled kinesin present in the homogenate. The efficiency of the

initial immunoprecipitation was determined by dividing the amount of radiolabeled kinesin present in the initial immunoprecipitates by the total amount of radiolabeled kinesin present in the homogenate. In this manner, kinesin immunoprecipitations were determined to be 86% efficient. In contrast, hexokinase, synaptophysin, and clathrin immunoprecipitations were about 96–99% efficient.

Quantitation of Radiolabeled Kinesin at FC, SCa, and SCb Time Points

The amount of radiolabeled kinesin present in optic nerves at 4 and 30 h (FC), 4 d (SCb), or 21 d (SCa) times (Tytell *et al.*, 1981; McQuarrie *et al.*, 1986; Oblinger *et al.*, 1987) was quantitated as follows to determine how much axonal kinesin might be associated with each of these rate components. Retinas from adult male rats were labeled

with ^{35}S -methionine as described above. Two animals per time point were killed at 4 h, 30 h, 4 d, or 21 d post-labeling, and their optic nerves were harvested (FC-I, 10 mm long; see Figure 1). Labeled optic nerves were homogenized in lysis buffer, and the kinesin immunoprecipitated. Immunoprecipitates were resolved by SDS-PAGE and the resulting gel was fluorographed to visualize radioactive polypeptides. Radioactive kinesin polypeptide bands were excised and the gel pieces dissolved in 1 ml of 33% hydrogen peroxide at 60°C for 2–3 d to release radioactive polypeptides. The amount of radioactivity associated with each polypeptide was measured using a liquid scintillation counter. In some experiments, fluorographs of the immunoprecipitates were analyzed using a Molecular Dynamics Laser Densitometer. Densitometry was calibrated with an internal standard for relative measurements and all exposures were in the linear range. After immunoprecipitating kinesin from the radiolabeled homogenate, synaptophysin and clathrin were sequentially immunoprecipitated from the same radiolabeled homogenate.

The absolute mass of kinesin in the optic nerve of several animals was determined to insure that differences in the amount of radiolabeled kinesin at different time points post-labeling were due to differences in specific activity, not to differences in the amount of kinesin present in the axons of different animals. Optic nerves from two non-radiolabeled rats were harvested and homogenized in lysis buffer. Insoluble particulate matter was removed as above for immunoprecipitations. Specific volumes of supernatant were dot-blotted onto nitrocellulose membrane along with known amounts of purified bovine brain kinesin to generate a standard curve. Membranes were incubated with all four monoclonal antibodies to kinesin (H1, H2, L1, and L2) and developed according to the method of Papasozomenos and Binder (1987). Briefly, membranes were blocked for 1 h with 5% (w/v) Carnation Instant Milk in borate-buffered saline (BBS: 100 mM boric acid, 25 mM sodium borate, 75 mM sodium chloride, pH 8.2). Primary antibody was applied in 5% milk/BBS overnight followed by three 10-min washes with BBS. Secondary antibody (rabbit anti-mouse immunoglobulin G, Jackson Immunoresearch Laboratories, 315-005-003, West Grove, PA) was applied for 2 h in 5% milk/BBS followed by three 10-min washes in BBS. ^{125}I -Protein A (IM 144, Amersham, Arlington Heights, IL) was applied in 5% milk/BBS for 2 h at a concentration of 0.05 $\mu\text{Ci}/\text{ml}$. Membranes were finally washed three times in BBS + 0.1% Triton X-100. The amount of radioactivity bound to each protein dot was quantitated using a Molecular Dynamics (Sunnyvale, CA) PhosphorImager. Absolute mass of kinesin present in the optic nerve was determined by comparison of kinesin in homogenate blots to the standard curve of purified bovine brain kinesin.

SDS-PAGE and Two-Dimensional Gel Electrophoresis

SDS-PAGE was performed according to a modification of the method of Laemmli (1970) using 4–10% gradient polyacrylamide gel with 0–6 M urea (Bloom *et al.*, 1988). Polypeptide patterns were visualized by staining gels with Coomassie Blue (Serva, Garden City Park, NY). Radioactive polypeptides were detected by fluorography after impregnation of the gel with the scintillant, 2,5-diphenyloxazole (Laskey and Mills, 1975). Two-dimensional gel electrophoresis was performed according to a modification of the method of O'Farrell (1975) as described previously (Brady *et al.*, 1984) using a mix of pH 3–10 and 5–8 ampholines (Pharmacia Biotech) to produce a pH range of 4–7. Proteins separated by isoelectric focusing were then separated by SDS-PAGE as described above.

Normalization Procedures

To correct for variations in the amount of radioactivity incorporated into the retina and transported into the optic nerve and to facilitate comparisons between marker proteins of different abundance, immunoprecipitated disintegrations/min were normalized to the

amount of material being transported in a given experiment before plotting. Normalization of data for FC time points was accomplished by dividing the amount of radioactivity in a protein band or aliquot of homogenate for a given experiment by the total amount of radioactivity present in that protein band or aliquot of homogenate for all 10 time points and then representing this as a percentage (% dpm seg A = $[\text{dpm}_{\text{seg A}} \div \Sigma(\text{dpm}_{\text{all times}})] \times 100$). With the use of this procedure, the relative amount of a given polypeptide in a segment at each time point is readily defined.

The normalization procedure for SCb experiments differed slightly from that used for FC experiments since there were fewer time points in these experiments. For a given SCb experiment, data were normalized by dividing the amount of radioactivity (dpm) in a protein band or aliquot of homogenate from one segment by the total amount of radioactivity present in that protein band or homogenate summed over all three segments and representing that value as a percentage (% dpm seg A = $[\text{dpm}_{\text{seg A}} \div \Sigma(\text{dpm}_{\text{all seg}})] \times 100$). Since the amount of kinesin declined substantially between 2, 4, and 6 d, only the shape of the SCb curves representing the distribution of kinesin was considered.

RESULTS

Biochemical Diversity of Kinesin in the Rat Retina

Biochemical variants of kinesin heavy and light chains present in rat retinas had not previously been defined, although they were presumed to be similar to those purified from bovine brain. To characterize the repertoire of molecular weight and charge variants of kinesin present in rat retina before analyzing axonal transport kinetics in the optic nerve, rat retinas were radiolabeled by intraocular injection of ^{35}S -methionine. Retinas were harvested 2 h post-injection, the kinesin present was immunoprecipitated, and immunoprecipitates were resolved by two-dimensional gel electrophoresis. Rat retinas contained two molecular weight variants of kinesin heavy chain and two molecular weight variants of kinesin light chain with charge variants at each molecular weight (Figure 2). Relative molecular weights of heavy and light chain variants were similar to the analogous polypeptides from purified bovine brain kinesin, although the relative amount of each isoform varied. KHC-A and KHC-B were used to refer to kinesin heavy chains of 130,000 and 124,000 M_r , respectively. Similarly, KLC-A and KLC-B refer to molecular weight variants of kinesin light chain of 70,000 and 64,000 M_r . As in purified bovine brain kinesin fractions (Wagner *et al.*, 1989), the amount of labeled KHC-B was greater than KHC-A and the amount of labeled KLC-B was greater than KLC-A. In two-dimensional PAGE, each molecular weight variant of kinesin heavy and light chains were seen to be composed of up to five isoelectric species.

To determine that these labeled polypeptides were subunits of true kinesins and not kinesin related proteins and to characterize the specificity of our antibodies for neuronal kinesins, each of the four monoclonal antibodies was used individually to immunoprecipitate axonally transported proteins from optic nerve at

Figure 2. Fluorograph of a representative two-dimensional gel electrophoretic separation of radiolabeled rat retina kinesin. The rat retina contains two molecular weight variants of the kinesin heavy chain, KHC-A (large open arrow at $\approx 130,000 M_r$) and KHC-B (large solid arrow at $\approx 124,000 M_r$); and two molecular weight variants of the kinesin light chain, KLC-A (large open arrow at $\approx 70,000 M_r$) and KLC-B (large solid arrow at $\approx 64,000 M_r$). Furthermore, each of these heavy and light chain molecular weight variants comprise several isoelectric species (small vertical arrows for both KHC and KLC). A polypeptide of approximately 80–85,000 M_r is occasionally seen in immunoprecipitates of retina (large triangle), but is not detected in immunoblots of either SDS-PAGE or two-dimensional PAGE and is presumed to be precipitated nonspecifically.

4 h and 2 d (Figure 3). The H2 antibody appears to precipitate the full range of labeled kinesin subunits

Figure 3. Fluorographs demonstrating the isoform specificity of monoclonal antibodies to the kinesin heavy and light chains. Rat retinal ganglion cells were pulse-labeled with ^{35}S -methionine, and the kinesin present in the optic nerve at (A) 4 h or (B) 2 d post-labeling was immunoprecipitated using only one of our monoclonal antibodies to the kinesin heavy (H1 or H2) or light (L1 or L2) chain. Immunoprecipitates were resolved by SDS-PAGE and the resulting gels fluorographed to visualize radioactive polypeptides. In all cases, antibodies to kinesin heavy chain also coprecipitate light chain subunits and antibodies to the light chain coprecipitate the heavy chains indicating these are true kinesins and not kinesin related proteins. Apparent in immunoprecipitates by H2, L2, and L1 are two molecular weight variants of both kinesin heavy and light chains. The relative amounts of the heavy and light chain molecular weight variants differ both with the specific mAb utilized and the time point post-labeling at which the immunoprecipitation was performed. The H2 antibody appears to immunoprecipitate the full range of both heavy and light chains detected in the nerve, while the H1 antibody precipitates labeled kinesin only at 2 d. This suggests that H1 immunoreactive kinesin appears in the optic nerve at a later time than H2 immunoreactivity.

(both heavy and light chains) detectable at both 4 h and 2 d, while the other three antibodies each precipitate a subset of the radiolabeled proteins seen with the H2 antibody. Most, and possibly all, of the different heavy chain isoforms detectable in SDS-PAGE were precipitated by either L2 or L1. The fact that both heavy and light chains are immunoprecipitated by antibodies to either heavy chain or light chain alone indicates that all of the anti-kinesin immunoreactivity labeled by axonal transport in these studies corresponds to true kinesins rather than kinesin related proteins.

Although the H2 antibody appeared to precipitate all labeled kinesin at all time points, our H1 antibody does not recognize those heavy chain isoforms that were radiolabeled in optic nerve at 4 h (Figure 3A). In addition, comparisons between the H2 and H1 lanes in Figure 3B indicate that H1 precipitates only a fraction of the KHC-B present in optic nerve at 2 d. This suggests that the KHC-B band includes at least two distinct immunologic forms, both of which are recognized by H2, but only a subset of which are recognized by H1. In addition, immunoprecipitation with antibodies that distinguish among kinesin isoforms suggest that at least some neuronal kinesin holoenzymes comprise two immunologically identical heavy chains rather than a mixture of different heavy chain isoforms.

Quantitation of Radiolabeled Kinesin at FC, SCA, and SCb Time Points in the Optic Nerve

Characterization of kinesin axonal transport required determination of whether radiolabeled kinesin was present in the 10-mm long optic nerve of retinal ganglion cell axons at times post-labeling when FC, SCA, and SCb proteins were labeled. These time points were calculated from published rates for movement of

Figure 4. Fluorograph (A) and quantitation of fluorograph (B) demonstrating the amount of radiolabeled kinesin present in the optic nerve at FC (4 and 30 h), SCb (4 d), and SCa (21 d) time points, as determined by quantitative immunoprecipitation and analyzed with a Molecular Dynamics Densitometer. Synaptophysin, an integral membrane protein, and clathrin serve as markers for FC and SCb, respectively. The predominance of radiolabeled kinesin was found at the FC time points. A small amount of radiolabeled kinesin was found at SCb time points, and none was detected at SCa time points. Error bars represent the standard deviation with $n = 3$.

FC, SCa, and SCb in the rat visual system (Tytell *et al.*, 1981; McQuarrie *et al.*, 1986; Oblinger *et al.*, 1987). The times used in initial experiments were 4 and 30 h post-labeling for FC, 4 d for SCb, and 21 d for SCa. At each of these time points, there was a significant amount of radiolabeled protein present in the optic nerve.

Optic nerves were assayed for kinesin and two marker proteins: synaptophysin and clathrin heavy chain. As seen in Figure 4, A and B, the synaptophysin is radiolabeled at substantial levels in segment FC-I at 4 and 30 h post-labeling, but significantly less synaptophysin was detectable at 4-d and none at 21-d time points. Synaptophysin was thus present in the optic nerve primarily at FC time points, consistent with the fact that synaptophysin is an integral membrane protein (Johnston *et al.*, 1989; Sudhof and Jahn, 1991) which must be moved in FC. The faint bands that appear at various molecular weights at the 4-d time point in the synaptophysin immunoprecipitation (Figure 4A) are radiolabeled SCb proteins which nonspecifically bind to the protein A-Sepharose beads used in immunoprecipitation and appear after relatively long exposures.

In contrast to synaptophysin, radiolabeled 180-kDa clathrin heavy chain was significantly labeled in optic nerve only at the 4-d SCb time point (Figure 4, A and B), consistent with previous reports (Garner and Lasek, 1981). A small portion of axonal clathrin ($\leq 10\%$ of that present in SCb) may be present in FC (Black *et al.*, 1991), but levels of FC clathrin were below the level of detectability. As with synaptophysin, clathrin immunoprecipitates from the 4-d time point contain several faint radioactive bands corresponding to SCb proteins that are immunoprecipitated nonspecifically. However, some additional radiolabeled bands appear that may represent polypeptides which interact with clathrin heavy chains including clathrin light chains (Black *et al.*, 1991) and the HSC70/clathrin uncoating ATPase (de Waegh and Brady, 1989). Previous studies have shown that radiolabeled tubulin and neurofilaments are present in rat optic nerve at 21 d post-labeling (Brady and Black, 1986; Oblinger *et al.*, 1987) as appropriate for characterization of SCa polypeptides. These results demonstrate that 4-h, 30-h, 4-d, and 21-d time points are appropriate for characterization of FC, SCb, and SCa proteins.

Table 1. Quantitation of total kinesin in optic nerve

A. Total optic nerve protein (μg)	B. Total nerve kinesin (μg)	C. Kinesin as % of total nerve protein ($\text{B/A} \times 100$)
2829.3 $\pm$ 24.3	12.1 $\pm$ 1.2	0.43%

Total nerve kinesin (the sum of axonal and nonneuronal kinesin) was measured to permit estimates of kinesin specific activity in these experiments. Kinesin levels in optic nerve were similar to those previously reported for mammalian brain (Wagner *et al.*, 1989). Each optic nerve was dissected and homogenized as described in Methods. The amount of total protein in a nerve homogenate was determined using the Pierce BCA reagent. The mass of kinesin present in each optic nerve was determined by quantitative dot blotting. Both total nerve protein and total nerve kinesin are presented here as the mean $\pm$ standard deviation with $n = 3$.

Radiolabeled kinesin was present in optic nerves primarily at the two FC time points (Figure 4, A and B). Lower levels were detected at the SCb time point (4 d), but no radiolabeled kinesin was detectable at the SCa time point (21 d). Differences in the amount of radiolabeled kinesin present at FC and SCb time points were not due to variations in the total amount of kinesin present in optic nerves of different rats.

Measurement of total kinesin present in the optic nerve of several rats revealed little variability. Each optic nerve contains $12.1 \pm 1.2 \mu\text{g}$ of kinesin representing 0.43% of the total protein present (Table 1), consistent with previous estimates of kinesin abundance in brain (Wagner *et al.*, 1989). The 4- and 30-h radiolabeled kinesin comprise 86% of the total kinesin found at the four time points (Table 2). These data suggest that the bulk of axonal kinesin is transported with FC, but do not exclude the possibility that a small amount of kinesin moves with SCb.

The radioactivity associated with heavy and light chain molecular weight variants of kinesin was quantitated to detect differences in their relative amounts at FC and SCb time points. While both molecular weight variants of kinesin heavy and light chains were present at FC and SCb time points, relative amounts differed over time (Figure 4A and Table 2). Radiolabeled KHC-A was highest at the 4-h time point, decreasing at later times (Table 2). In contrast, KHC-B was barely detectable at 4 h, reached a maximum at 30 h, and then decreased at 4 d. Differences were less pronounced between molecular weight variants of the light chain. Relative amounts of both KLC-A and KLC-B in optic nerve rose between 4 and 30 h, then

Table 2. Quantitation of radiolabeled kinesin in fast and slow axonal transport

	4 h (dpm) mean $\pm$ SD ^a	30 h (dpm) mean $\pm$ SD ^a	4 d (dpm) mean $\pm$ SD ^a	21 days (dpm) mean $\pm$ SD ^a
KHC-A	463 $\pm$ 54	367 $\pm$ 46	21 $\pm$ 2	0
KHC-B	20 $\pm$ 10	755 $\pm$ 88	223 $\pm$ 28	2 $\pm$ 3
KLC-A	62 $\pm$ 13	253 $\pm$ 14	78 $\pm$ 7	7 $\pm$ 7
KLC-B	227 $\pm$ 28	440 $\pm$ 41	61 $\pm$ 19	11 $\pm$ 10
KHC/KLC ^b	1.67	1.62	1.76	—
	FC (4 + 30 h)	SCb (4 d)	SCa (21 d)	
Kinesin dpm mean $\pm$ SD (% of total)	2587 $\pm$ 294 (86%)	383 $\pm$ 46 (13%)	20 $\pm$ 20 (1%)	

Optic nerve kinesin radiolabeled by axonal transport was measured as a first estimate of kinesin in fast and slow axonal transport. Most radiolabeled kinesin was in the optic nerve at short injection sacrifice intervals, when only rapidly transported materials are labeled. Only a small fraction was labeled at 4 d, when proteins moving with SCb become significantly labeled and essentially all radiolabeled kinesin was gone by the time that SCa proteins were labeled in the nerve. This suggests that the bulk of axonal kinesin is associated with membranous structures. No kinesin was detectable in SCa, but the possibility that a small fraction of kinesin moves with SCb cannot be eliminated by this experiment. Note that molecular weight variants for KHC and KLC were differentially labeled at these time points, but the ratio of total KHC to total KLC remained constant. When the number of methionines in KHC and KLC are taken into account, this ratio is consistent with a 1:1 stoichiometry for KHC and KLC in vivo (Bloom *et al.*, 1988; Kuznetsov *et al.*, 1988).

^a For these experiments, all animals were injected at the same time with the same lot of radiolabeled methionine. After animals for all four time points had been sacrificed, nerve homogenates immunoprecipitated from all four time points were analyzed in the same SDS-PAGE gel. Radioactivity associated with both molecular weight variants for each kinesin subunit were quantitated. This procedure minimizes variability between the four time points and eliminates differences due to radioisotope decay. Data in this table is presented as the mean $\pm$ standard deviation with $n = 3$.

^b The ratio of kinesin heavy chains to light chains at each of the four time-points was calculated by dividing the radioactivity associated with KHC-A plus KHC-B by the radioactivity associated with KLC-A plus KLC-B.

decreased between 30-h and 4-d time points. Although the ratio of the two KLC forms varied, there was no simple relationship between time point and abundance of a radiolabeled KLC molecular weight variant.

Although relative proportions of KHC-A and KHC-B change more dramatically than KLC-A and KLC-B, the ratio of kinesin heavy to light chains remained essentially constant over the 4-h to 4-d time interval (Table 2). This is consistent with a heterotetrameric structure for kinesin, although the specific isoforms of constituent polypeptides change. A simple mechanism to explain differences in KHC-A and KHC-B levels over time is that these variants move at different rates.

Kinetics for Transport of Kinesin Heavy and Light Chains in the Visual System

A more detailed kinetic analysis was undertaken to establish whether kinesins moved as part of FC and/or SCb. First, the time course of appearance for different radiolabeled kinesin variants in the optic nerve was examined with a higher time resolution. The presence of radiolabeled kinesin in the optic nerve was evaluated at 4, 8, 12, 20, 24, 30, 36, 48, 60, and 96 h. Previous studies indicate that significant amounts of radiolabeled SCb proteins do not appear in the optic nerve before 2 d post-labeling (Oblinger *et al.*, 1987). Thus, radiolabeled proteins present in optic nerve before 48 h post-labeling represent FC while radiolabeled proteins in the optic nerve after 48 h will include increasing amounts of proteins moving at SCb rates.

Figure 5 illustrates the relative amounts of total radiolabeled protein and kinesin in optic nerve over this time course. Data in Figure 5 and the remaining graphs (see Figures 7, 9, 10B, 11B, and 12B) were normalized to facilitate comparison of results from multiple experiments as described in MATERIALS AND METHODS. The relative amount of radiolabel in the optic nerve rose rapidly by 4 h post-labeling and remained near constant up to 30 h post-labeling. Between the 30- and 96-h time points, the relative amount of radiolabel present gradually increased again. The first peak of radiolabel to appear in the optic nerve was composed of mostly FC proteins, while the second increase included an increasing amount of SCb proteins.

The initial appearance of radiolabeled kinesin in optic nerve was similar to the initial appearance of total radiolabeled protein (Figure 5). Significant amounts of radiolabeled kinesin appeared in the optic nerve at 4 h and remained at that level up to 24 h post-labeling. The relative amount of radiolabeled kinesin then drops slightly at 30 h and remains fairly constant up to 48 h post-labeling after which levels decline. Kinesin approximates the behavior of total

Figure 5. Quantitation of total radiolabeled protein and radiolabeled kinesin in optic nerve over a 96-h time interval. The data represented have been normalized to facilitate the comparison of results from multiple experiments. Normalization was accomplished by dividing the amount of radioactivity in a polypeptide band or aliquot of homogenate, by the amount of radioactivity present in that band or aliquot of homogenate from all 10 time points and then expressing this number as a percentage (% dpm time A = $[\text{dpm}_{\text{time A}} \div \Sigma(\text{dpm}_{\text{all times}})] \times 100$). Significant amounts of radiolabel appear as early as 4 h post-labeling, signifying the entry of FC proteins into the optic nerve. Between 30 and 96 h, the amount of radiolabel again increases, indicating the entry of SCb proteins into the optic nerve. Radiolabeled kinesin seems to approximate the entry of FC proteins, but not SCb proteins into the optic nerve. Error bars represent the standard deviation with $n = 4$.

radiolabel only at early time points which correlated with the appearance of FC proteins in optic nerve. At time points greater than 30 h, kinesin levels declined while total radiolabel continued to increase. Since significant amounts of SCb proteins have not entered the optic nerve before 30 h, these results suggested that kinesin was transported with FC but not SCb.

Kinesin immunoprecipitates from the optic nerve at each time point were resolved by SDS-PAGE. A representative Coomassie Blue-stained polyacrylamide gel and corresponding fluorograph are shown in the top and bottom panels of Figure 6, respectively. Heavily stained bands at ~55 and ~29 kDa in the top panel are immunoglobulin heavy and light chains. Kinesin heavy chain appears as a band at ~124 kDa and is present at relatively even levels in all 10 samples, indicating that the total amount of kinesin per nerve is constant. The 130,000 M_r molecular variant is not readily apparent in Coomassie Blue-stained gels. Kinesin light chains were also not easily visualized by Coomassie Blue, due both to their lower relative mass and to the presence of other polypeptides. The polypeptide band at ~150 kDa was precipitated nonspecifically and appeared in immunoprecipitations with antibodies against a wide variety of proteins.

A fluorograph of this gel is presented in the bottom panel of Figure 6. As in Figure 4A, two molecular weight variants of both the kinesin heavy chain

Figure 6. Coomassie Blue-stained gel (top panel) and corresponding fluorograph (bottom panel) demonstrating differences in the timing of appearance of the two molecular weight variants of the kinesin heavy chain, KHC-A and KHC-B, in the optic nerve. Kinesin was immunoprecipitated from segment FC-I at increasing time points post-labeling. The immunoprecipitates were then resolved by SDS-PAGE, and the gels were fluorographed to visualize radioactive polypeptides. Only the 124,000 M_r variant of the kinesin heavy chain, KHC-B, can be visualized on the Coomassie Blue-stained gel. However, both molecular weight variants of the kinesin heavy and light chains are visible in the fluorograph. KHC-A appears as early as 4 h post-labeling, whereas KHC-B appears between 12 and 20 h post-labeling. KLC-A and KLC-B, on the other hand, seem to behave similarly. The 150,000 M_r polypeptide (arrow) is immunoprecipitated non-specifically and is seen in immunoprecipitations using a wide variety of antibodies (our unpublished results).

(KHC-A and KHC-B) and light chain (KLC-A and KLC-B) were present in immunoprecipitates. Radiolabeled KHC-A and KHC-B isoforms differed in the time of their first appearance in the optic nerve. Quantitation of these gels (Figure 7, A and B) indicated that labeled KHC-A appeared first, peaked between 4 and 12 h, then quickly declined. In contrast, labeled KHC-B did not begin to appear at significant levels in optic nerve until 8–12 h, peaked between 24 and 30 h, then remained relatively constant up to 60 h before declining. The two molecular weight variants of the light chain were more similar in their kinetics, appearing in the optic nerve as early as 4 h, reaching a maximum at 8–12 h post-labeling and remaining more or less constant until 48 h when they began to decline. Both KLC-A and KLC-B exhibited multiple peaks and the ratio between the two differed at various time points. As expected for subunit polypeptides, kinetics for the combination of KLC-A and KLC-B approximated the

Figure 7. Quantitation of the appearance of the kinesin heavy (A) and light (B) chain molecular weight variants in the optic nerve. The radioactive polypeptides from gels in Figure 5 were cut out and quantitated as described (see MATERIALS AND METHODS). The data presented have been normalized as described in Figure 4. The difference in the timing of appearance of KHC-A and KHC-B in the optic nerve is readily apparent. Furthermore, the differences in the shapes of the curves for KHC-A and KHC-B suggest differences in the rate of movement of these two polypeptides into the optic nerve. The light chain molecular weight variants, KLC-A and KLC-B, seem to behave similarly. Error bars represent the standard deviation with $n = 7$.

combined behavior of heavy chain molecular weight variants which resulted in a constant ratio of heavy chain to light chain across the 96 h time course.

Relative rates and direction of movement for the kinesin polypeptides can be estimated from transport of radiolabeled kinesin between three consecutive segments of the rat visual system (Figures 8 and 9). FC-I and FC-II segments were each approximately 10 mm long and FC-III included the synaptic terminals of these axons. Since KHC-A and KHC-B exhibited different behaviors in optic nerve, their appearance in each segment was quantitated separately while light chain molecular weight variants were combined. Although these methods are not well suited for accurate measurement of velocity—particularly for the fastest rates—estimates can be based on the time of first appearance for a band into the FC-II segment. Using

Figure 8. Fluorographs demonstrating the movement of radiolabeled kinesin from segment FC-I, to segment FC-II, and finally into segment FC-III. Radiolabeled kinesin was immunoprecipitated from three contiguous segments of the optic nerve, tract, and terminals, at increasing time points, over a 96 h time interval. The immunoprecipitates were then resolved by SDS-PAGE, and the resulting gels fluorographed to visualize the radioactive polypeptides. Radiolabeled kinesin appears first in FC-I, then FC-II, and then finally in FC-III, suggesting movement in the anterograde direction. Both heavy chains and light chains exhibit complexity in their transport kinetics as reflected in different ratios of molecular weight variants and possibly multiple peaks.

these criteria, the front of KHC-A must be moving at a rate ≥ 80 mm/d and the front of KHC-B was moving at a rate of ≥ 40 mm/d. Both rates are within the range reported for FC in mammalian optic nerve. However, inasmuch as KHC-A was already detectable in the FC-I segment at the earliest time point used, these values are likely to underestimate actual rates.

The times at which the peak of a kinesin heavy chain appeared in each segment also indicated that kinesin was moving with FC in the anterograde direction. KHC-A peaked in the FC-I segment at 8 h, the FC-II segment at 20 h, and the FC-III segment at 24 h (Figures 8 and 9A). The sequential appearance of KHC-A in these three segments at increasing distances from the retinal ganglion indicated that KHC-A was moving in the anterograde direction. Very little radiolabeled KHC-A was left in any of these segments after the peak had passed indicating that radiolabeled KHC-A peak moved through the axon in axonal transport without significant deposition of KHC-A in the axon.

KHC-B also moved in the anterograde direction as part of FC, but at a fraction of the KHC-A rate (Figures

Figure 9. Quantitation of the movement of KHC-A (A), KHC-B (B), and KLC-A plus KLC-B (C) between segments FC-I, FC-II, and FC-III. The radioactive polypeptides from gels in Figure 8 were cut out and quantitated. The data presented has been normalized as described in Figure 4. KLC-A and KLC-B were grouped together because these two polypeptides exhibited similar rates of movement. Apparent in these graphs is the appearance of all four polypeptides, KHC-A, KHC-B, KLC-A, and KLC-B, in segment FC-I, then segment FC-II, and finally in segment FC-III within FC time points. Because these segments occur at increasing distances from the retinal ganglion, this observation suggests that all four polypeptides move in the anterograde direction as part of the FC. However, the differences in the shapes of the curves for KHC-A and KHC-B suggest differences in the rate of movement of these two polypeptides. Error bars represent the standard deviation with $n = 7$.

8 and 9B). As with KHC-A, KHC-B appeared sequentially in segments FC-I, FC-II, and FC-III, consistent with anterograde transport. Comparing the slopes of entry for KHC-B into each segment with the slopes of entry of KHC-A into corresponding segments suggested that KHC-A moved at five to six times the rate of KHC-B. Therefore, if the front of the KHC-A wave moved at rate of 240 mm/d, a typical rate reported for the front of fast transport in rat retinal ganglion cell axons, then the corresponding fraction of KHC-B moved at a rate of 40–50 mm/d, toward the lower end of the range for transport of FC proteins in the rat visual system. Therefore, differences in time of appearance for KHC-A and KHC-B in the optic nerve (Figures 4A, 6, and 7A) could be readily explained by slower net transport rates for KHC-B.

Because KLC-A and KLC-B did not differ significantly in their time of first appearance in the FC-I segment (see Figures 4A, 6, and 7B), they were combined for analysis of movement from one segment to another (Figure 9C). As with KHC-A and KHC-B, the kinesin light chains entered segment FC-I, FC-II, and FC-III sequentially, indicating anterograde transport. The rate of entry and exit of the kinesin light chains in each segment were similar to KHC-A and KHC-B combined (data not shown), suggesting that the light chains also moved at FC rates. This was consistent with the fact that heavy to light chain ratios did not change over the 4–96-h interval (Table 2).

Comparison of Kinesin Transport with the Transport of SCb Proteins

While the kinetics of kinesin transport between 4 and 96 h demonstrated that axonal kinesin moves with the FC, the possibility remained that a small amount of kinesin was also transported with SCb. To determine whether any kinesin moved at SCb rates, the distribution of kinesin at longer injection sacrifice intervals was considered. The time points for SCb experiments were 2, 4, and 6 d after labeling. The slower rates permitted use of segments that were only 5 mm in length for improved spatial resolution (SC-I, SC-II, and SC-III). Distribution of total radioactivity and of kinesin polypeptides in segments SC-I, SC-II, and SC-III at these time points are shown in Figure 10, A and B. Data in Figure 10B was normalized to facilitate comparison between kinesin and total SCb protein data. The amount of radiolabeled kinesin in the nerve drops severalfold from the 2-d to the 6-d time point, while the total amount of radiolabel associated with SCb proteins increased. As a result, only the shapes of these curves should be compared, not their magnitude.

At 2 d, the peak of total radiolabeled proteins was in SC-I (Figure 10B). At 4 d, the peak in SC-I declined and the relative amount of label in SC-II increased, consis-

tent with movement in the anterograde direction. By 6 d, an even larger fraction of total radiolabeled proteins had moved into SC-II and a significant amount had begun to enter SC-III. Movement of radiolabeled proteins from SC-I into SC-II and SC-III over the course of 6 d was consistent with published rates of 1–2 mm/d for movement of SCb proteins in the rat visual system.

The distribution of radiolabeled kinesin heavy and light chains differed significantly from that of total radiolabeled proteins in SCb (Figure 10, A and B). Kinesin heavy chains peaked in segment SC-II at 2 d and in segment SC-III at the 4- and 6-d time points. For kinesin light chains, there was no discernible peak at 2 and 6 d and only a slight peak in SC-III at 4 d. No peak of kinesin heavy or light chains was seen to move along the nerve at SCb rates, suggesting that no axonal kinesin moved with SCb. This is consistent with the observation that the amount of radiolabeled kinesin present in the rat visual system declines substantially over the 2–6 d interval in contrast to both total radiolabel and specific SCb proteins such as synapsin or clathrin which increased. The position of the kinesin peaks and the declining amounts suggest that kinesin present in the optic nerve and tract at SCb time points represented the trailing end of FC.

Comparison of the Rate of Movement of KHC-A and KHC-B to Synaptic Vesicle and Mitochondrial Markers

At least two populations of kinesin were identified in anterograde FC moving at different rates: one enriched in KHC-A moving at a faster rate and a second enriched in KHC-B moving slower. One plausible explanation for these results is that KHC-A and KHC-B were associated with different subclasses of membrane bounded organelles having different average rates. Both radiolabel studies (Lorenz and Willard, 1978) and video microscopy (Brady *et al.*, 1982, 1985) demonstrated that small tubulovesicular structures moved in axonal transport at a rate severalfold over that of mitochondria. If KHC-A and KHC-B represented kinesin associated with small tubulovesicular organelles and mitochondria respectively, protein markers for these structures should display transport kinetics similar to the corresponding kinesin variant. Synaptophysin was used as a marker for one class of small tubulovesicular structures, synaptic vesicles and their precursors (Jahn *et al.*, 1985; Sudhof and Jahn, 1991), and hexokinase was used as a marker for brain mitochondria (Wilson, 1985). The kinetics of axonal transport for synaptophysin and hexokinase in the optic nerve (Figure 2) was compared with those of KHC-A and KHC-B (Figures 6 and 7A).

After immunoprecipitation of kinesin, the same radiolabeled samples were used for immunoprecipitation of hexokinase and synaptophysin (see MATERIALS AND

Figure 10. Fluorograph (A) and quantitation of fluorograph (B) demonstrating that kinesin does not move at SCb rates. Kinesin was immunoprecipitated from three contiguous 5-mm segments of the optic nerve and tract (SC-I, SC-II, and SC-III), the immunoprecipitates resolved by SDS-PAGE, and the resulting gels fluorographed to visualize the radioactive polypeptides. These radioactive polypeptide bands were then cut out and quantitated. The data presented has been normalized using a method different than that used in other figures in this report. Normalization was accomplished by dividing the amount of radioactivity in a polypeptide band or aliquot of homogenate in a particular segment, by the amount of radioactivity present in that protein band or aliquot of homogenate from all three segments at that time point and then representing this number as a percentage (% dpm seg A = $[dpm_{seg A} \div \Sigma(dpm_{all seg})] \times 100$). The fluorograph in (A) shows that the amount of kinesin continues to decline at 4 and 6 d, while total radioactivity in the nerve continues to increase between 2 and 6 d. Since the radioactivity in the nerve at times between 2 and 6 d is primarily associated with SCb, the distribution of radiolabeled kinesin was compared with the distribution of total radioactivity at the same time in (B). The total radiolabeled proteins appear to be primarily in segment SC-I at 2 d, then increases in segment SC-II at 4 d, and finally begins to be present in significant amounts in segment SC-III at 6 d, consistent with SCb rates. In contrast, radiolabeled kinesin heavy and light chains have a very different distribution at all three time points and do not approximate the behavior of the SCb proteins, suggesting that kinesin is not associated with this component. Error bars represent the standard deviation with $n = 3$.

METHODS). As above, immunoprecipitates were resolved by SDS-PAGE and fluorographed to visualize radioactive polypeptides. Normalized results are shown in Figure 11B (synaptophysin) and Figure 12B (hexokinase). Significant amounts of synaptophysin were present in the optic nerve as early as 4 h, remained relatively constant through the 12 h time point, and then declined (Figure 11, A and B). The rate of entry and exit from the optic nerve for synaptophysin were similar to KHC-A, although synaptophysin does not seem to peak as high as KHC-A. The normalized values suggest that a

fraction of the synaptophysin trails behind the peak, although this represented a very small amount of radiolabeled synaptophysin. These data suggested that most synaptophysin moved through the axon with kinetics similar to KHC-A.

The fact that curves for synaptophysin and KHC-A were not identical was in fact consistent with previous analyses of coordinate transport for polypeptides (Garner and Lasek, 1982). The only time that absolute identity of transport distribution can be obtained is when there is a stoichiometric complex between two

Figure 11. Fluorograph (A) and quantitation of fluorograph (B) demonstrating the cotransport of KHC-A and the synaptic vesicle marker, synaptophysin. The homogenates used in Figure 5 for immunoprecipitating kinesin were then used to immunoprecipitate synaptophysin and hexokinase (see Figure 12). Immunoprecipitates were resolved by SDS-PAGE and the resulting gels fluorographed to visualize the radioactive polypeptides. Radioactive polypeptide bands were then cut out and quantitated. The data presented has been normalized as described in Figure 4. Both synaptophysin and KHC-A enter the optic nerve at similar time points and the width of the two peaks are comparable, suggesting that these two polypeptides are cotransported. The differences may reflect the fact that synaptic vesicle precursors are not the only small tubulovesicular structures moving at the fastest rate and others may be more homogeneous in their transport. Error bars represent the standard deviation with $n = 7$.

polypeptides. In this case, synaptic vesicle precursors are only one of a number of different small tubulovesicular structures that move at or near the maximum rate. The presence of other populations of organelles with slightly different sizes and kinetics is a level of complexity that cannot be avoided in this kind of analysis. Unfortunately, this level of analysis does not have the resolution to separate these different populations. The fact that KHC-A and synaptophysin enter and leave the optic nerve with similar kinetics was consistent with cotransport. Regardless, these data imply a primary association of KHC-A with small tubulovesicular structures moving at or near the maximum rate for fast axonal transport.

In contrast to synaptophysin, the amount of radiolabeled hexokinase in the optic nerve was negligible at

Figure 12. Fluorograph (A) and quantitation of fluorograph (B) demonstrating the cotransport of KHC-B and the mitochondrial marker, hexokinase. Hexokinase enters the optic nerve at a time somewhat after the first appearance of KHC-B, but with a similar rate. Two-dimensional gel electrophoresis indicates that the broad KHC-B peak represents the overlap of at least two peaks of KHC-B with different isoelectric points (manuscript in preparation). These patterns suggest the hexokinase moving at FC rates is cotransported with the second half of the KHC-B peak. However, unlike KHC-B, the amount of hexokinase in the optic nerve also increase at SCb time points. This is due to hexokinase being transported in SCb, in addition to FC. Inasmuch as the amounts of hexokinase transported in SCb are severalfold higher than the amounts transported in FC, the data presented in this figure have been normalized across a 48-h interval only, so that KHC-B can be compared only with FC hexokinase. Error bars represent the standard deviation with $n = 7$.

4 h, gradually increased between 12 and 30 h post-labeling, and then increased again between 30 and 48 h post-labeling (Figure 12, A and B). The rate of entry of hexokinase into segment FC-I is similar, but not identical to the rate of entry for KHC-B at early time points. Two differences can be seen between the transport of hexokinase and KHC-B. First, KHC-B could be detected in segment FC-I earlier than hexokinase. Two-dimensional gel electrophoretic analysis of KHC-B at different time points in the optic nerve indicate that this peak encompassed several charge variants with similar molecular weight appearing in FC-I at different times (see Figure 2 and Elluru (1994)) and the kinetics for the slower moving of these isoforms may match the transport kinetics of hexokinase. Second, the amount of radiolabeled hexokinase con-

tinues to increase between 30 and 96 h, whereas the amount of radiolabeled KHC-B remains relatively constant and then begins to decrease. The continued increase in radiolabel associated with hexokinase stems from the fact that, unlike kinesin, hexokinase is also transported in SCb. As a result, the levels of this protein increase in the optic nerve at SCb time points. Transport of hexokinase in both FC and SCb required normalization of data over a shorter time, 4–48 h, to compare the entry of FC hexokinase with the entry of KHC-B into the optic nerve. A manuscript is currently in preparation detailing the kinetics of both FC and SCb hexokinase.

Taken together, these results demonstrate that axonally transported kinesin is associated with FC, corresponding to the movement of membrane bounded organelles, and effectively rules out a role for kinesin in the transport of cytoskeletal structures. Different biochemical variants of kinesin have different kinetics in axonal transport, which appear to reflect associations of kinesin with different classes of membrane bounded organelles. Specifically, KHC-A appears to be associated with small tubulovesicular organelles and a portion of KHC-B seems to be associated with mitochondria.

DISCUSSION

Much of our knowledge about the molecular motor kinesin was obtained from in vitro characterizations, leaving many questions unanswered about the functions and properties of kinesin in neurons. Among these open questions are identification of cargoes moved by kinesin; characterization of interactions between kinesin and cellular structures; determination of functional significance for kinesin isoforms; and definition of mechanisms for regulation of kinesin based movements. Many of these issues can be addressed by examining the axonal transport of kinesin itself using a variant of classical pulse-chase methods (Brady, 1985a).

Kinesin was first identified as an ATPase having properties consistent with a molecular motor for fast axonal transport (Brady, 1985b; Vale *et al.*, 1985; Kuznetsov and Gelfand, 1986). While evidence that kinesin was involved in movement of membrane bounded organelles has accumulated, kinesin is routinely purified from soluble extracts of brain (Wagner *et al.*, 1989) and other tissues (Scholey *et al.*, 1985; Urrutia *et al.*, 1991; Balczon *et al.*, 1992). Moreover, kinesin purified from brain includes multiple isoforms of both heavy and light chains (Wagner *et al.*, 1989) which can be distinguished biochemically (see Figure 2) and immunochemically [see Figure 3 and Pfister *et al.* (1989)]. The functional significance of different isoforms and the associations of kinesin in situ were largely unexamined.

Several lines of evidence indicate that a tight association can exist between kinesin and membrane bounded organelles in cells, including immunolocalization of kinesin on membrane bounded organelles (Pfister *et al.*, 1989; Leopold *et al.*, 1992) and inhibition of organelle movement by specific antibodies to kinesin (Brady *et al.*, 1990; Hollenbeck and Swanson, 1990; Lippincott-Schwartz *et al.*, 1995) or by antisense oligonucleotides to block synthesis of kinesin (Ferreira *et al.*, 1992; Amaratunga *et al.*, 1993). Despite indications of a role in membrane bounded organelle transport, little was known about interactions between kinesin and membrane surfaces. Since a substantial fraction of total kinesin is soluble following homogenization (Bloom *et al.*, 1988; Wagner *et al.*, 1989) or gentle extraction (Hollenbeck, 1989), the extent to which kinesin can interact in vivo with non-membranous structures such as the cytoskeleton was unknown.

The axonal transport paradigm is particularly well suited for identifying specific long-term associations of polypeptides with intracellular structures in situ (Brady and Lasek, 1982). A substantial literature has shown that proteins associated with membranous structures move with FC, whereas proteins interacting with non-membranous structures such as cytoskeletal elements move with SCa and/or SCb. Proteins with multiple associations move at multiple rates. There are two unique advantages to using axonal transport to study neuronal proteins (Brady and Lasek, 1982; Brady, 1985a). First, cells need not be lysed and fractionated to characterize intracellular protein interactions. Lysing of cells leads to an obligatory dilution of intracellular contents, which in turn disrupts equilibrium interactions in the cell. Therefore, if a protein is readily solubilized as a side effect of dilution, then this protein would appear as a "soluble" protein and weak or labile interactions would not be detected. The axonal transport paradigm is not affected by artifacts due to cell lysis and dilution of intracellular contents. For example, axonal transport has been used to demonstrate that proteins such as enolase, calmodulin and phosphofructokinase—which are "soluble" by the cell lysis and fractionation methods—move as part of SCb (Brady and Lasek, 1981; Brady *et al.*, 1981; Oblinger *et al.*, 1988). Second and equally important, axonal transport provides information about structural and metabolic dynamics of neuronal proteins. To characterize the axonal transport kinetics of kinesin, rat retina was pulse-labeled with ³⁵S-methionine and movement of radiolabeled kinesin through the retinal ganglion cell axons was monitored by quantitative immunoprecipitation.

Biochemical Variants of Kinesin in Rat Retina

Before characterizing axonal transport of kinesin in the visual system, the repertoire of biochemical vari-

ations for kinesin had to be defined in the visual system. As seen in Figure 2, rat retina contained molecular weight and charge variants for both heavy and light chains of kinesin similar to those found for purified bovine brain kinesin (Wagner *et al.*, 1989). Relative proportions of the molecular weight variants were similar to that of bovine brain kinesin, providing a basis for analyzing the transport of kinesin isoforms.

Previously, the nomenclature for molecular weight variants of kinesin heavy and light chains was based on the apparent abundance of different forms in kinesin purified from bovine brain. Molecular weight variants of kinesin heavy and light chains were designated as "major" and "minor" based on their relative amount. Immunoprecipitation of axonally transported kinesin suggested that the relative proportions of different molecular weight variants depended on the time post-labeling (see Figure 6). At early times, the "minor" heavy chain molecular weight variant (KHC-A; 130,000 Da) was more prominent than the "major" form (KHC-B; 124,000 Da), but at later times KHC-B predominated over KHC-A.

The molecular origin of these kinesin isoforms has not been determined. A single kinesin light chain gene has been identified which generates three transcripts by alternative splicing (Cyr *et al.*, 1992). Most species have a single gene for the kinesin heavy chain, along with a variety of genes encoding kinesin related proteins (Goldstein, 1993). However, a second gene for kinesin heavy chain expressed preferentially in neuronal tissues of human and rodents was described recently (Niclas *et al.*, 1994; B.W. Richards and S.T. Brady, unpublished observations). In addition, kinesin heavy and light chains may be phosphorylated *in vivo* (Hollenbeck, 1993; Matthies *et al.*, 1993). Certainly, some kinesin heavy and light chains in axonal transport are phosphorylated (Elluru *et al.*, 1990) (our manuscript in preparation). Both translational and post-translational mechanisms are likely to contribute to biochemical heterogeneity of kinesin purified from mammalian brain. However, correlating different transcripts and post-translational modifications to biochemically defined isoforms will require generation of specific probes that distinguish among the different isoforms.

Characterization of Kinesin Axonal Transport

The major rate components of axonal transport were labeled and examined at appropriate times in optic nerve. The presence of labeled marker proteins was used to confirm the labeling of a given rate component: synaptophysin (FC), clathrin (SCb), or tubulin (SCa). Radiolabeled kinesin was detectable in optic nerve when radiolabeled FC (4 and 30 h) and SCb (4 d) proteins are present, but not when SCa proteins were present (21 d). Approximately 86% of total radiola-

beled kinesin was present in the optic nerve at FC time points before cytoplasmic proteins associated with SCb were significantly labeled in the optic nerve. The presence of kinesin in the FC was consistent with the association of kinesin with membrane-bounded organelles and suggested that interactions between kinesin and FC organelles were relatively stable.

This result contrasted with previous reports based on extraction protocols in which 70% of fibroblast kinesin was soluble (Hollenbeck, 1989). Less than 14% of axonal kinesin was labeled in the optic nerve at times when soluble proteins like glycolytic enzymes are the major labeled species. Even this fraction of kinesin is unlikely to represent a cytoplasmic pool of axonal kinesin, because it did not move coordinately with SCb proteins. Using smaller nerve segments and larger intervals between time points, movement of SCb proteins from one nerve segment to another can be easily demonstrated (Figure 10B). In such studies, no peak of radiolabeled kinesin moved coordinately with SCb proteins, and the amount of radiolabeled kinesin in the nerve continued to decline as it was cleared from the nerve. Because radiolabeled kinesin in the optic nerve 4–6 d after labeling did not move at SCb rates, it is likely to be the trailing edge of FC kinesin.

Thus, virtually all axonal kinesin appeared to be associated with membrane bounded organelles *in vivo*. The observed differences between these results and those obtained by extraction of cultured cells (Hollenbeck, 1989) presumably result from the different methodologies employed. Although previous estimates were based on careful cell lysis and gentle detergent extraction (Hollenbeck, 1989), even the gentlest cell lysis leads to an obligatory dilution of intracellular contents and has the potential to disrupt physiologically important protein-protein interactions. Changes in intracellular compartmentation may also play a role. Although a substantial fraction of cellular kinesin is initially solubilized under gentle conditions, subsequent extraction of organelle fractions with high ionic strength and high pH fails to solubilize the kinesin still associated with organelles (Leopold, 1992; Leopold *et al.*, 1992; Schnapp *et al.*, 1992).

The axonal transport data imply that most extractable brain kinesin was originally associated with membrane-bounded organelles. The ease of initial solubilization may provide important clues about the nature of kinesin interactions with the membrane surface. Either a large fraction of neuronal kinesin is weakly bound to membranes or disruption of cellular compartmentation during homogenization or extraction may modify kinesins and release them from the membrane surface. Modifications of kinesin during extraction have been previously demonstrated, including both proteolysis (Hackney *et al.*, 1991) and phosphorylation (Hollenbeck, 1993). At least one re-

port (Sato Yoshitake *et al.*, 1992) suggested that phosphorylation of kinesin by protein kinase A (PKA) inhibits kinesin binding to membrane bounded organelles. However, the physiological significance of PKA phosphorylation is open to question since kinesin is normally phosphorylated in cells lacking PKA activity (Hollenbeck, 1993). Whether one of these modifications or an as yet undefined post-translational modification of kinesin regulates its binding to membrane surfaces *in vivo* remains to be determined.

The biochemical properties of kinesin moving in axonal transport over a 96-h time course varied systematically (Figure 6, A and B). For heavy chains, the higher molecular weight KHC-A first appeared in the optic nerve at 4 h and peaked by 12 h, whereas significant labeling of the lower molecular weight KHC-B form did not appear until 12 h. The labeling of KHC-A and KHC-B in optic nerve with different kinetics is best explained by a slower rate of entry into the optic nerve for KHC-B than for KHC-A. Differences in rates of synthesis for KHC-A and KHC-B or in commitment of these forms to axonal transport appear unlikely, because their rate of movement from optic nerve to optic tract differed as well (see Figures 8 and 9).

When the appearance of radiolabeled kinesin in three consecutive 10-mm segments was monitored at 10 closely spaced time points post-labeling, movement of FC proteins from one segment to another could be followed. These studies demonstrated that KHC-A and KHC-B both move in the anterograde direction as part of FC, but that they have different mean rates. No peaks of kinesin were seen to move in the retrograde direction, consistent with previous studies showing accumulation of kinesin primarily on the proximal side of a block (Dahlström *et al.*, 1991; Hirokawa *et al.*, 1991). A small amount of kinesin immunoreactivity (9–10% of that on the proximal side) did accumulate distally with retrograde transport (Dahlström *et al.*, 1991), so our data cannot exclude the possibility of a small fraction of kinesin moving in the retrograde direction, but no evidence of a retrograde moving fraction of kinesin was seen in these experiments.

Functional Implications of Kinesin Heavy Chain Molecular Weight Variants in the Axon

Different rates of transport for molecular weight variants of kinesin heavy chain imply that kinesin isoforms have distinct physiological functions. One attractive explanation stems from the fact that different classes of membrane bounded organelles move at different rates. Small tubulovesicular structures, such as synaptic vesicle precursors, may move five times as fast as larger membrane-bounded organelles such as mitochondria and lysosomes [for example, see Lorenz and Willard (1978) and Allen *et al.* (1982)]. Biochemically distinct kinesins could be associated with differ-

ent organelle types. Consistent with this, KHC-A was cotransported with a synaptic vesicle marker, while a fraction of KHC-B was cotransported with a mitochondrial marker (see Figures 11 and 12). This suggested that kinesins containing the KHC-A polypeptide are found predominantly on small tubulovesicular structures like synaptic vesicles or their precursors, while at least a portion of kinesin containing the KHC-B isoform are enriched on mitochondria. Previous immunochemical studies of purified membrane fractions demonstrate that both of these organelle fractions contain kinesin on their surfaces (Leopold *et al.*, 1992).

Different rates for KHC-A and KHC-B are unlikely to result from differences in their ability to move. Video microscopy (Allen *et al.*, 1982; Brady *et al.*, 1982; Brady *et al.*, 1983) indicates that small tubulovesicular structures move smoothly with few or no pauses at near maximal rates. A slower rate for mitochondria results because they make frequent pauses, presumably due to interactions with the axoplasmic matrix. Net rates of movement for organelles in axoplasm appeared to be a function of organelle size, and both large and small organelles move at similar rates along isolated microtubules (Brady *et al.*, 1983; Allen *et al.*, 1985; Johnston *et al.*, 1987). Different kinetics for KHC-A and KHC-B are more likely to reflect a difference in their organelle associations than a difference in their efficiency as motors.

Very little has been known about the functional consequences of biochemical variants for kinesin heavy and light chains. The apparent correlation between different kinesin isoforms and distinct classes of membrane bounded organelles suggest at least two explanations. Different kinesins may be important either for differential regulation of kinesin activity or for targeting of kinesin to organelles which must be moved in the FC. These two possibilities are not mutually exclusive and both are consistent with characteristics of axonal transport. Not all membrane-bounded organelles of the neuron move in axonal transport (Ellisman and Lindsey, 1982), and different organelles must be targeted to axons, dendrites, presynaptic terminals, and the various other domains of a neuron. For example, mitochondria must be delivered to neuronal domains with high utilization of ATP, whereas synaptic vesicles go only to presynaptic terminals. Some mechanism must exist to assure that different organelles reach their appropriate destinations.

The polypeptide compositions of different organelles vary widely, and there may be no proteins in common between a synaptic vesicle and a mitochondrion, yet both have kinesin associated with their surfaces (Leopold *et al.*, 1992) and move with FC. Other organelles like the Golgi complex and the nucleus have no detectable kinesin (Pfister *et al.*, 1989; Leopold

et al., 1992; Fath *et al.*, 1994) and do not move in axonal transport. Some mechanism must exist to target kinesin only to those organelles which are moved in axonal transport. The existence of different kinesin isoforms on different classes of organelle could represent such a mechanism. Previous sequence analysis demonstrated the existence of three kinesin light chain transcripts produced by alternative splicing and these differences were proposed to be involved in targeting kinesins to different organelles (Cyr *et al.*, 1991). Demonstration that different light chains are present on different organelles would confirm this hypothesis. Unfortunately, these three light chain variants are not sufficiently different to be resolved by molecular weight and available light chain antibodies cannot distinguish among them. Appropriate antibodies are currently being generated to address this question. Experiments described in this report do indicate that different heavy chains may be associated with different organelle classes. Mechanisms by which different kinesin heavy chains are targeted to specific organelles could involve biochemical properties of heavy chains themselves or may require specific light chains as well.

Much remains to be learned about biochemical differences between different isoforms of kinesin heavy and light chains. Differences may result from both variations in primary sequence and post-translational modifications. There are two kinesin heavy chain genes expressed in mammalian brain (Niclas *et al.*, 1994), and additional transcripts could be generated by alternative splicing. Previous work established that the single gene for the kinesin light chain in rat generates three different transcripts through alternative splicing (Cyr *et al.*, 1991). Further studies will be required to correlate specific gene products with the kinesin heavy and light chain isoforms seen in SDS-PAGE. In addition, at least a portion of kinesin heterogeneity is due to post-translational modification, since neuronal kinesin may be phosphorylated (Elluru *et al.*, 1990; Sato Yoshitake *et al.*, 1992; Hollenbeck, 1993; Matthies *et al.*, 1993). These biochemical differences require further characterization to understand how they lead to differences in transport or targeting of membrane bounded organelles in the neuron.

ACKNOWLEDGMENTS

We thank Dr. Philip Leopold, Columbia University, Dr. Mark Wagner, University of Michigan, and Dr. Kevin Pfister, University of Virginia, for helpful discussions and technical advice during the preparation of this paper. We also thank Dr. Richard Anderson, University of Texas Southwestern, Dr. John Wilson, Michigan State University, and Dr. Paul Greengard, Rockefeller University, for their generous contribution of antibodies used in this study. The research described in this report was supported in part by National Institutes of Health grants NS-23868 and NS-23320 (to S.T.B.) and NS-30485 (to G.S.B.), Council for Tobacco Research grant 3258, Welch Foundation grant 1237, National Institutes of General Med-

ical Sciences grant GM-08014, and the Muscular Dystrophy Association.

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