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Kinesin is rapidly transported in the optic nerve as a membrane associated protein

Peter J. Morin [a], Robin J. Johnson [b] and Richard E. Fine [a,c,d]

[a] *Department of Biochemistry, Boston University School of Medicine, Boston, MA (USA),*
[b] *Department of Anatomy, Boston University School of Medicine, Boston, MA (USA),*
[c] *Department of Neurology, Boston University School of Medicine, Boston, MA (USA)*
and [d] *Edith Nourse Rogers Memorial Veterans Administration Hospital, Bedford, MA (USA)*

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We have investigated the membrane vs. cytosolic distribution of newly synthesized and total kinesin in rabbit retinal ganglion cell axons which comprise the optic nerve. We find that kinesin is rapidly transported into the axon and that this newly synthesized protein is completely membrane-associated while approximately two third of the total kinesin in the optic nerve is membrane associated. Of this membrane associated kinesin about half is resistant to removal by treatment with 100 mM Na_2CO_3 (pH 11.3) and none can be stripped by 1 M NaCl. The newly synthesized axonal kinesin is completely resistant to removal by Na_2CO_3 treatment. By these criteria, at least one third of the total and essentially all of the rapidly transported axonal kinesin appears to exist as an integral membrane protein, consistent with it functioning as the anterograde motor for rapid vesicle transport from the cell body through the axon.

Introduction

Kinesin is a microtubule-stimulated ATPase which is thought to play a role in the translocation of membranes along microtubules. It apparently consists of two identical 110–130 kDa species which contain the ATPase and microtubule binding site as well as two identical 60–65 kDa polypeptides of as yet unknown function [1]. While the interaction between kinesin and microtubules has been described in a number of systems [2–5], little is known of kinesin's interactions with membranes. One reason for this is that the routine isolation of kinesin from nervous tissue extracts has suggested that the translocator exists primarily as a cytosolic protein which engages in only transient associations with membranes during force generation.

However, several considerations suggest that kinesin may engage in a more stable interaction with transported membranes. Using motility assays, several investigators have observed substantial translocation capability of native vesicles isolated from squid axoplasm, and this activity is lost when the vesicles are washed in salt-containing buffers or exposed to proteinase activity [2,6]. Fluorescence microscopy of neuronal like cells presents a punctate and detergent sensitive distribution of kinesin, indicative of vesicular attachment in vivo [7], although a diffuse distribution has been reported in PtK1 and A6 cells [8]. Kinesin has also been reported to be a constituent of neutrophil granules [9], and, in a subcellular fractionation study of rabbit optic nerve we have observed kinesin heavy chain in association with rapidly transported vesicles isolated by immunoadsorption [10]. Recently, kinesin heavy chain has been immunocytochemically localized to detergent-sensitive particles in perinuclear arrays in sea urchin coelomocytes [11]. Finally, it has been estimated by detergent solubilization that about one third of fibroblast kinesin is associated with cellular membranes [12]. Thus there is considerable evidence for a stable attachment of kinesin to membranes, but the nature of this attachment is unknown.

Here we describe the distribution of kinesin amongst soluble and insoluble axonal fractions from the rabbit optic nerve and tract. Our data suggest that kinesin is rapidly transported within the axon and that the major-

Correspondence to: R.E. Fine, Department of Biochemistry, K-124C, Boston University School of Medicine, 80 East Concord Street, Boston, MA 02118, USA.

Abbreviations: LSB, Laemmli sample buffer; MT, microtubule; ON/OT, optic nerve/optic tract; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

ity of axonal kinesin exists in tight association with axonal membranes.

A preliminary report of this work has been previously published: Morin et al. (1988) J. Cell Biol. 107, 672a.

Materials and Methods

Materials

Radioisotopes were obtained from ICN Biomedicals (Costa Mesa, CA); Trans ^{35}S-label (containing 80% [^{35}S]methionine, 20% [^{35}S]cysteine, with a specific activity of 1000–1200 Ci/mmol), was obtained from Dupont New England Nuclear (Boston, MA); ^{125}I-goat anti-mouse IgG, ^{125}I-protein A and Sepharose CL4B-linked protein A were from Pharmacia (Newark, NJ). Adenylyl imidodiphosphate (AMP-PNP) was from Boehringer Mannheim (Chicago, IL). Taxol was generously supplied by Dr. Matthew Suffness. All other materials were reagent grade.

Kinesin preparation and antibody production

Bovine brain kinesin was prepared from 50–100 g batches of white matter as described [13] using MAP-free microtubules (MT) prepared by the method of Vallee [14]. The abundant 120 kDa protein which was specifically eluted from MTs by ATP was isolated by SDS-PAGE. The Coomassie-stained band was excised, lyophilized, and resuspended in Freud's adjuvant (complete was used for the first inoculate and incomplete thereafter). Two rabbits received multiple subcutaneous injections at 3–4 week intervals and serum was collected after the third inoculation (see Fig. 1).

Intravitreal injections and membrane preparation

Retinal isotope labelling was accomplished by introducing 0.5 mCi Trans ^{35}S-label (30–50 μl) into the posterior margin of the vitreous body of a centrally anesthetized albino rabbit (average wt. 5 lbs) which had received corneal proparocaine hydrochloride. At the appropriate time after injection, usually 2.5–3 h, rabbits were killed by lethal injection.

Retinas, optic nerve and tracts (ON/OT) were dissected free of meninges and homogenized in 2–5 ml of ice-cold 20 mM PO_4, 150 mM NaCl, 0.005% NaN_3 (PBS), containing 30 μg/ml phenylmethylsulfonylfluoride, 0.5 μg/ml leupeptin, 0.5 μg/ml aprotinin, and 10 μg/ml tosylarginine *O*-methyl ester. Colchicine, at 100 μM, and diisopropyl fluorophosphate at 1 μM, were included as indicated. Membrane and cytosolic fractions were separated (after the indicated treatment) by centrifuging, for 60 min at 150000 $\times g$ in a Beckman SW 50.1 rotor.

Triton X-114 extraction was performed according to the method of Bordier [15] except that insoluble material was removed by high-speed centrifugation prior to the phase separation. Protein was measured by the method of Bradford [16].

Immunoprecipitations

Immunoprecipitations were performed by solubilizing membrane or cytosol fractions in PBS containing 1% Triton X-100 and 0.5 mM dithiothreitol for 60 min at 4°C. Insoluble material was removed by centrifuging for 60 min at 150000 $\times g$. Anti-kinesin or preimmune serum was preincubated with sepharose CL4B-linked protein A for 16 h at 4°C. Antibody-bead complexes were washed twice with PBS containing 1% Triton X-100 before they were added to solubilized ON/OT preparations at 25 μl packed beads/ml. After overnight binding, the beads were removed by centrifugation and washed five times by resuspending in PBS containing 1% Triton X-100 and recentrifuging for 5 s in an Eppendorff microfuge. Final samples, which represent total membrane and cytosolic fractions, were suspended in Laemmli sample buffer (LSB), and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Gels were impregnated with 1 M sodium salicylate (pH 7.4), containing 20% methanol and 1% glycerol for 1 h before drying for autoradiography. Kinesin heavy chain bands were quantified by volume integration of band intensity on a LKB densitometer.

Western blotting

Gels were transferred for approximately 1000 mA h onto nitrocellulose. Filters were soaked in 3.5% carnation nonfat dry milk dissolved in PBS for 60–90 min at 37°C to saturate non specific binding sites. Antibodies were applied in PBS containing 0.7% Carnation milk for 16 h at 4° at the following dilution; anti-bovine kinesin heavy chain antiserum, 1:300; ascites SUK 4, 1:300. Filters were washed in PBS containing 1% Triton X-100 for 3 × 20 min ^{125}I-protein A or ^{125}I-goat anti-mouse was applied in PBS containing 0.7% carnation instant milk for 2 h at room temperature at a concentration of 100000 cpm/ml. Filters were washed in PBS containing 1% Triton X-100 for 3 × 20 min, dried, and exposed to film at −80°C with an intensifying screen for 1 or 2 days.

Cell culture and immunocytochemistry

Chick embryo neurons were grown on Titertek slides according to the described method [17]. After four days in culture, the cells were fixed in 4% paraformaldehyde in PBS containing 0.05% Triton X-100. All subsequent incubation media contained this detergent at the same concentration. Following fixation for 30 min at 4°C, cells were washed three times for 5 min each in PBS/Triton and then incubated in 1% BSA/Triton for 1 h at 4°C. This solution was then replaced by PBS/Triton containing 0.1% BSA and

either nonimmune rabbit serum or rabbit anti-bovine kinesin antiserum at a dilution of 1:200. Cells were incubated overnight at 4°C in primary antibody. They were subsequently washed in three changes of PBS/Triton of 10 min each. Primary antibody was detected using a protein A-gold (5 nm) conjugate (1:100 for 60 min) followed by washing, fixation with 2% glutaraldehyde, further washing and silver intensification using an IntenSE kit from Janssen Life Sciences Products.

Results

Kinesin purification and antibody production

Kinesin was purified from bovine brain white matter depleted of endogenous microtubules and microtubule binding proteins as described [2]. After ATP elution, a major 120 kDa band is visualized in the supernatant (Fig. 1, lane 2). Excision of the 120 kDa band from a preparative gel and subsequent immunization resulted in the production of an antibody which recognizes a 120 kDa band in cytosolic extracts of whole axons (Fig. 1, lane 4) and reacted with the 120 kDa component of kinesin purified by Bio-Gel A-5 and hydroxyapatite column chromatography and assayed for microtubule-stimulated ATPase activity. The band recognized by this antibody is identical to that recognized by the SUK 4 monoclonal antibody against the N-terminal portion of the kinesin heavy chain, kindly provided by Dr. John Scholey [18]. Our antibody also recognized a 120 kDa band in chick and rabbit brain extracts (data not shown).

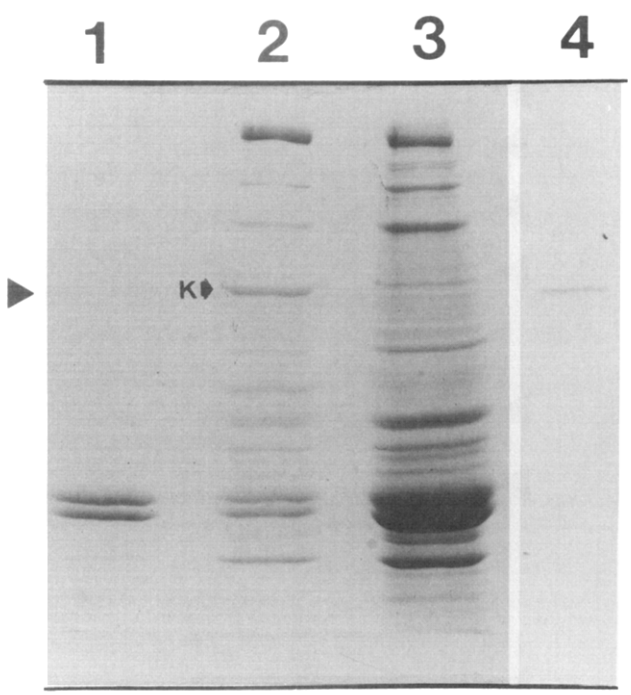

Fig. 1. Preparation of bovine kinesin and rabbit polyclonal kinesin antiserum. Kinesin was prepared by AMP-PNP-dependent microtubule binding as described [13]. Aliquots of residual microtubules, ATP eluate, and pre-elution wash are shown in lanes 1–3, respectively. Kinesin isolated by SDS-PAGE (lane 2) was excised and used to raise rabbit anti bovine kinesin heavy chain antiserum, PF-2. The antiserum Western blots a single band from rabbit ON/OT cytosol (lane 4) and from bovine brain cytosol (not shown) at a dilution of 1:300 (as shown) and 1:5000 (not shown).

Rapid transport of kinesin in the rabbit optic nerve

The rabbit optic nerve preparation provides an excellent system to determine the transport kinetics of a given protein. After injection of a bolus of radiolabeled amino acid into the vitreous humor overlying the retina, rapidly transported proteins made in the retinal ganglion cells traverse the 3 cm optic nerve within a few hours since the maximal rate of anterograde rapid transport is on the order of 1 cm/h [19].

The rapid transport of kinesin was investigated by immunoprecipitation from the ON/OT of rabbits, injected intravitreally (0.5 mCi/eye) with [^{35}S]Met/Cys 2.5 h prior to being killed using rabbit anti-bovine kinesin heavy chain antiserum (Fig. 2A). Total membrane and cytosolic fractions were separated by ultracentrifugation of homogenates and solubilized for immunoprecipitation as described in Materials and Methods. As expected of a rapidly transported protein, the newly synthesized kinesin heavy chain can be immunoprecipitated from the membrane fraction (lane 1), but not the cytosol fraction (lane 2). A species of slightly reduced mobility precipitates from both fractions but constitutes only a small percentage of the total. We have observed a similar splitting of the kinesin heavy chain when Western blotting with this antiserum, but the effect is present inconsistently and its significance is not clear.

Since the newly synthesized and rapidly transported kinesin heavy chain is membrane associated, we tested the ability of Na_2CO_3 treatment at high pH to remove it from the membrane. This treatment has been shown to solubilize all peripherally associated membrane proteins as well as to lyse any intact membrane vesicles [21]. Total membranes from [^{35}S]Met/Cys-labeled ON/OT were prepared as described above. After extraction with 1 M Na_2CO_3 (pH 11.3), the samples were adjusted to pH 7.4 with HCl and kinesin was immunoprecipitated as described in Fig. 2. As shown in Fig. 3, the radiolabelled kinesin heavy chain is not released to an extent demonstrable by densitometry. We do see a significant amount of radioactivity at the top of the separating gel, predominantly in the Na_2CO_3-treated membrane fraction while we see substantially less with control membranes. This distribution is suggestive of an aggregative or crosslinking effect of the harsh Na_2CO_3 treatment on kinesin heavy chains.

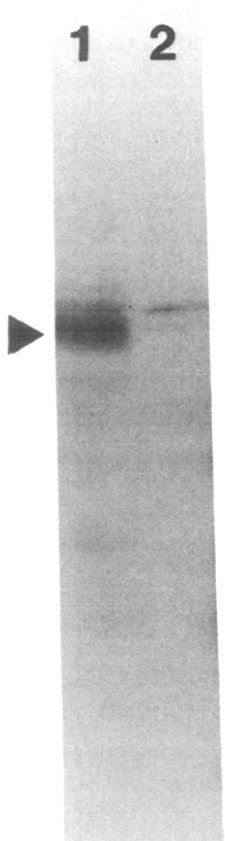

Fig. 2. Rapid transport of kinesin heavy chain. At 2.5 h post intravitreal injection of 0.5 mCi [^{35}S]Met/Cys, radiolabelled proteins have been rapidly transported from retinal ganglion cells into their axons which comprise the ON/OT. Using the antiserum described above, kinesin heavy chain was immunoprecipitated from total solubilized membranes (lane 1) and cytosol (lane 2) of ON/OT preparations and the immunoprecipitates subjected to SDS-PAGE followed by autoradiography as described in Materials and Methods. Molecular mass marker: 116 kDa.

Cytosolic vs. membrane kinesin in the optic nerve / optic tract

The distribution of total kinesin in the ON/OT was determined by blotting membrane and cytosolic fractions with anti-bovine kinesin antiserum. To assess the contribution of microtubule bound kinesin to the membrane pool, samples were incubated on ice with or without colchicine (100 μM) for 15 min prior to centrifugation for 1 h at 4°C. As shown in Fig. 4, the majority of kinesin heavy chain (75% as determined by densitometry), remains in the membrane fraction under these conditions. We have found that with longer incubations and in reduced ionic strength buffers, substantially more of the kinesin partitions into the cytosolic fraction. In a typical preparation, such as that shown in Fig. 4, at least two thirds of the kinesin heavy chain immunoreactivity is membrane associated.

It is also apparent from Fig. 4 that our antiserum (as well as SUK 4, data not shown) recognizes two additional peptides in the ON/OT. The smaller band runs at about 45 kDa. This peptide is present in significant quantities regardless of the buffer or reducing conditions. Its consistent presence in the membranes despite generous compliments of proteinase inhibitors (as indicated), suggests that it may be a normal constituent of axonal membranes. The 45 kDa component corresponds to the molecular weight of a proteolytic fragment consisting of the N-terminal region of kinesin heavy chain which is generated in vitro by incubation of kinesin with chymotrypsin and which contains the MT and ATP binding sites of kinesin [3,18]. Despite the addition of diisopropylfluorophosphate, a very potent serine proteinase inhibitor, to the excized ON/OT before homogenization we always observe this band in our axonal membranes. It is never seen, however, in cytosolic fractions and also behaves as an integral membrane protein by the criteria mentioned above. The larger protein runs at about 180 kDa and is

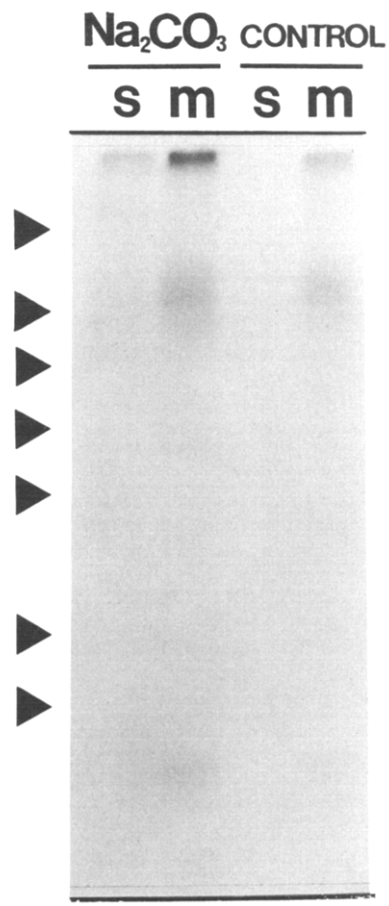

Fig. 3. Immunoprecipitation of radiolabeled ON/OT kinesin from Na_2CO_3 extracted membranes. ON/OT membranes were incubated for 1 h at 0°C in 100 mM Na_2CO_3 (pH 11.3), or in control PBS (pH 7.4). The membranes were separated by centrifugation and resuspended to their original volume. All samples were re-adjusted to pH 7.4 before solubilization in 1% Triton X-100 and immunoprecipitation using PF-2 antiserum. The immunoprecipitates were subjected to SDS-PAGE followed by autoradiography as described in Materials and Methods. Membrane pellets (m) supernatants (s). Molecular mass markers: 200, 116, 84, 58, 48, 36 and 26 kDa.

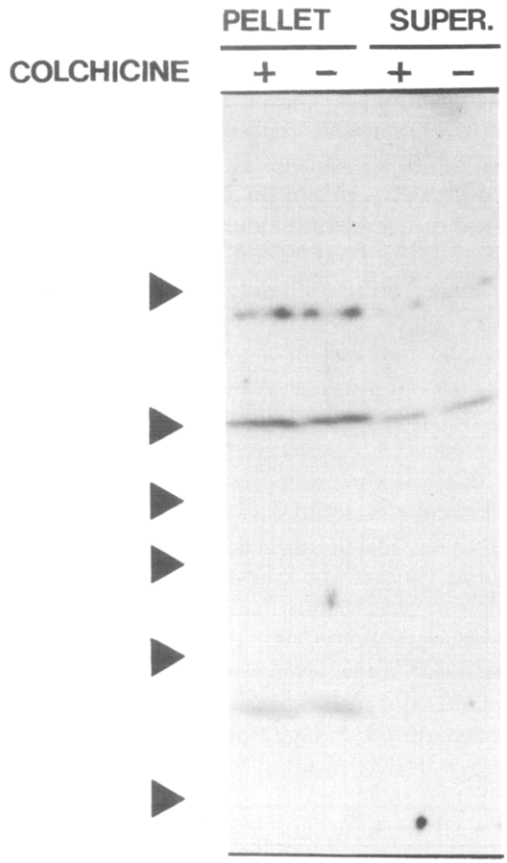

Fig. 4. Distribution of kinesin in the ON/OT. Homogenates were incubated for 15 min at 0°C in the presence of 100 μM colchicine before separation of membranes by centrifugation for 1 h at 4°C. Equal volume aliquots were compared by Western blotting with PF-2 antiserum as described in Materials and Methods. Molecular mass markers: 200, 116, 84, 58, 48 and 36 kDa.

significantly more abundant when samples are electrophoresed under nonreducing conditions. In addition, this immunoreactivity copurifies with kinesin

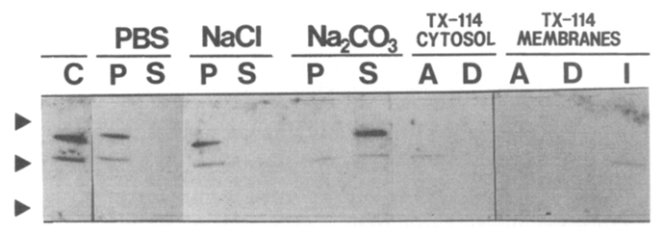

Fig. 5. Characterization of kinesin's membrane attachment. ON/OT homogenates were prepared as described above in PBS containing proteinase inhibitors and 100 μM colchicine. After separation of membrane and cytosol by centrifugation at 150000 × g for 1 h, membrane aliquots were subjected to extraction in PBS (Control), 1 M NaCl, 100 mM Na_2CO_3 (pH 11.3), or Triton X-114 as described [15], except that an insoluble fraction (I) was removed by centrifugation prior to phase separation (see Materials and Methods). Equal volume aliquots of residual pellets (P), and extracted supernatants (S), fractions from the salt washes, and aqueous (A), detergent (D), and insoluble (I), fractions from the Triton X-114 extractions were compared by Western blotting with PF-2 antiserum. Molecular mass markers: 200, 116 and 84 kDa.

through microtubule affinity and Bio-Gel A-5 column chromatography, when these procedures are performed in the absence of reducing agents (data not shown).

Characterization of the membrane attachment of kinesin

The observation that the majority of the total and essentially all of the rapidly transported kinesin is membrane bound, has led us to begin to characterize the association. We have begun by subjecting crude preparations of axonal membranes to conditions which typically release peripheral membrane proteins. Homogenates of ON/OT were pelleted, and resuspended in 1 M NaCl, 100 mM Na_2CO_3 (pH 11.3), or PBS, and left on ice for 1 h, before pelleting again. Membranes and supernatants were analyzed by Western blotting and the efficacy of these treatment was confirmed by

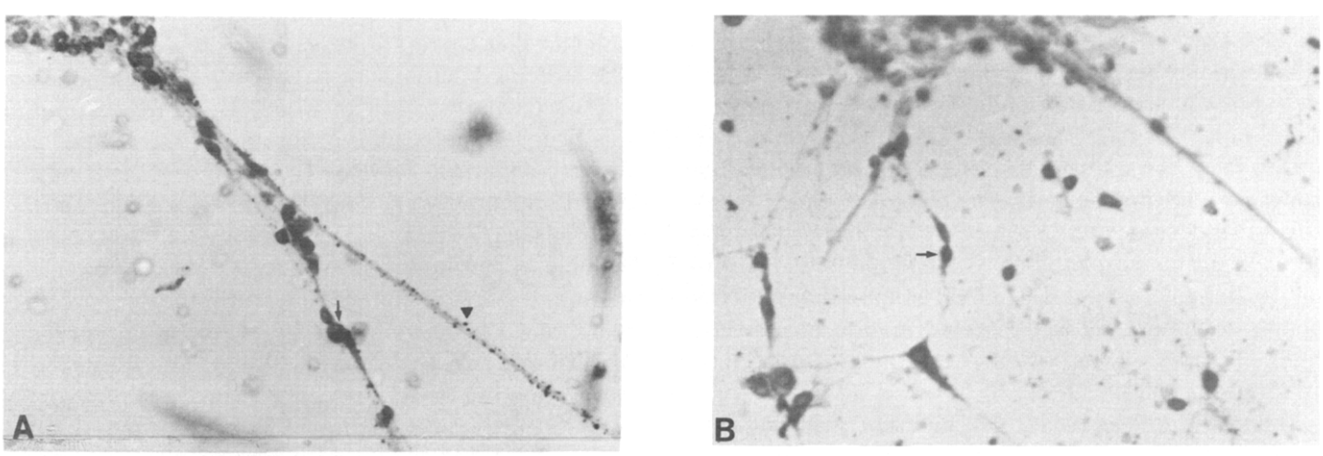

Fig. 6. Localization of kinesin in primary cultured chick embryo neurons. Cells were cultured and stained with a protein A-gold (5 nm) conjugate as described in Materials and Methods. (A) The primary antibody was PF-2, a rabbit anti-bovine kinesin heavy chain antiserum described in Fig. 1, at a dilution of 1:200. Axonal process staining is predominantly punctate (arrowhead) while cell body staining is continuous and diffuse (arrow) Magnification: 264×. (B) The primary antibody was a preimmune rabbit serum at a dilution of 1:200. Cell body staining is also continuous (arrow) while there is no staining of the processes. Magnification: 232×.

documenting the fate of the α-subunit of the G0 protein by Western blotting (not shown).

As shown in Fig. 5, 1 M NaCl treatment results in a membrane/supernatant distribution indistinguishable from the control (PBS) treatment. Na_2CO_3 treatment, a more stringent criterion for identifying integral and peripheral membrane proteins, released about half of the 120 kDa heavy chain into the supernatant. The 180 kDa immunoreactive protein behaved identically to kinesin in PBS and salt, but appears to be quantitatively released by Na_2CO_3. An aliquot of the original cytosol fraction was also blotted for comparison (lane C).

The failure of Na_2CO_3 treatment to release more than 50% of the kinesin from axonal membranes suggests that some of the kinesin may be attached via an integral membrane linkage. We therefore performed Triton X-114 extractions on the cytosolic and membrane fractions and again analyzed the subfractions by Western blotting. As shown in Fig. 5 (lanes 7–12), 100% of the cytosolic species partitions into the aqueous phase and the 180 kDa band is lost altogether. The membrane associated kinesin, however, appears to be insoluble in Triton X-114, even in the presence of colchicine (as shown), and even with sonication (not shown).

Distribution of kinesin in cultured neurons

We have used our polyclonal antiserum to observe the morphological distribution of kinesin in primary cultured chick neurons. As shown in Fig. 6 A, B, the punctate staining of the neuronal processes is specific for the immuno serum. The dark staining of the neuronal cell bodies seen with both anti-kinesin and preimmune sera indicates that this staining is nonspecific.

Discussion

Kinesin has been strongly implicated, but not definitively proven, to be the molecular motor for anterograde rapid axonal transport. The observation that kinesin itself is rapidly transported can be added to a growing body of circumstantial evidence. Since cytoskeletal and associated proteins are transported into the axon en masse in the slow components, it is difficult to imagine why a large, MT-stimulated ATPase capable of generating MT-directed motion of vesicles, might be rapidly transported if it were not providing the force for translocation.

The membrane association of kinesin correlates with its rapid transport, as the rapid component has long been known to be membrane associated [22]. This finding also supports the morphological data presented by Pfister [7], which indicated that kinesin present in the processes of certain cell lines is particle associated. The immunocytochemistry presented here extends these observations to primary cultured neurons and the contrast in staining between axon (discrete) and cell body (diffuse), suggests that distinct pools of kinesin can exist within a neuron. This observation parallels published reports on kinesin in fibroblasts [8,12] and cultured neuronal and nonneuronal cell types [7]. One discrepancy should be noted here; Hollenbeck [12] observes heavy, diffuse staining with a kinesin antibody in cultured chick embryo dorsal root neurons. It is possible that the use of detergents in our staining protocol resulted in the selective extraction of axoplasmic kinesin. In any event, the punctate staining which we have observed is consistent with a predominantly visicular distribution.

Biochemical characterization of the kinesin distribution in the ON/OT defines three pools of heavy chain. Roughly one third of the kinesin is soluble (Fig. 4), and partitions completely into the aqueous phase of a Triton X-114 extraction (Fig. 5). Since these fractions were prepared under MT-depolymerizing conditions (i.e., ice cold, and in the presence of colchicine and in the absence of Ca^{2+} chelators), some or all of this kinesin may derive from a MT-associated pool. The remaining two thirds are membrane associated, stable to 1 M NaCl washing, and insoluble in Triton X-114. About 50% of the membrane associated kinesin can be removed by Na_2CO_3 extraction. The remaining kinesin (about one third of the total), behaves as an integral membrane protein by this criterion.

The possibility that the 'integral membrane' kinesin is an artifact caused by trapping of kinesin in sealed vesicles is rendered highly unlikely for two reasons. Firstly, Na_2CO_3 treatment at high pH has been shown to totally disrupt intact vesicles causing the formation of sheets and releasing any intravesicular proteins [21]. Secondly, negative stained preparations of our axonal membranes examined by EM after Na_2CO_3 treatment indicate that there are no vesicles present, only sheet-like structures. (Johnson, R.J., unpublished results).

The only other cell in which kinesin's distribution has been evaluated is the fibroblast [12]. Insofar as a direct comparison between these cell types is appropriate, it appears that the membrane vs. soluble distribution is exactly reversed, assuming that none of the 'soluble' kinesin which we detect derives from the disrupted cytoskeleton. In any event, the proportion of membrane associated kinesin in the optic nerve is about twice that reported in cultured fibroblasts. However, when the loosely associated kinesin is removed by Na_2CO_3 extraction, the final proportions are identical, with one third of the total kinesin on the membrane. It is possible that the saponin treatment employed to liberate soluble kinesin in fibroblasts also released loosely associated kinesin from organelles. Alternatively this pool may not exist in fibroblasts, or its

detection in the optic nerve may represents an artifact arising from myelin contamination or some other feature of the preparation.

Our current feeling is that this pool of kinesin does exist in the optic nerve because of its stability to NaCl washing. The larger proportion of membrane-associated kinesin *in the axon* may be explained by the absence of the large soluble fraction observed in the cell body morphologically.

Functional correlates of these pools of kinesin are difficult to assign. This is especially true since the contribution made by the slow components as well as a possible glial contribution are unknown. However, the radiolabelled kinesin in the ON/OT at 2.5 h post intravitreal injection derives solely from the retinal ganglion neurons. This rapidly transported kinesin has been shown by immunoprecipitation to be membrane bound and totally resistant to Na_2CO_3 extraction (Figs. 2 and 3); it is therefore represented in only one of the three pools described. If the kinesin present in the other pools is neuronal, then it either has been slowly transported into the axon or represents rapidly transported kinesin which, for some reason, is no longer membrane bound or as tightly adherent. One possibility is that kinesin's membrane association is regulated in such a way that it is rendered tenuous, or removed, once the rapid transport vesicle with which it is associated has arrived at its destination. This possibility is consistent with kinesin's discrete distribution amongst axonal membranes [10], and with its absence from synaptic vesicles (Morin, P.J., unpublished observation). It is interesting to speculate that this regulation might be interrelated with the mechanisms involved in regulating and specifying vesicle fusion. In this regard, the finding of a 45 kDa fragment of kinesin in immunoblots of axonal membranes, which is not seen in immunoprecipitates of newly synthesized axonal kinesin, suggests that this fragment may result from the degradation of intact axonal kinesin heavy chains, at some stage after kinesin enters the axon. In light of our previous work which has indicated that a population of rapidly transported vesicles contain plasma membrane proteins and presumably fuse with axolemma [10], a proteolytic cleavage is a conceivable mechanism by which kinesin could be inactivated after the transported vesicle reaches its destination.

In conclusion, the data presented here suggests that kinesin is synthesized as a cytosolic protein in the cell body of a neuron and that it is rapidly transported into the axon as a membrane protein. By the criterion of Na_2CO_3 stability, this rapidly transported kinesin behaves as an integral membrane protein. Only the newly synthesized (Na_2CO_3 stable), kinesin can be assigned a neuronal origin with certainty and this pool accounts for roughly one third of the total. This pool is likely to function as the anterograde motor for microtubule mediated vesicle transport. Two other pools of kinesin have been biochemically defined in the optic nerve: one is soluble and the other is membrane associated but can be removed by Na_2CO_3, but not by NaCl treatment. The functions of these latter pools are as yet not understood.

Acknowledgements

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