Kinesin Associates with Anterogradely Transported Membranous Organelles In Vivo

Nobutaka Hirokawa,* Reiko Sato-Yoshitake,* Naoto Kobayashi,* K. Kevin Pfister, George S. Bloom, and Scott T. Brady

*Department of Anatomy and Cell Biology, University of Tokyo, School of Medicine, Hongo, Tokyo, 113, Japan; and Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical Center, Dallas, Texas 75235

Abstract. Biochemical, pharmacological and immunocytochemical studies have implicated the microtubule-activated ATPase, kinesin, in the movement of membrane bounded organelles in fast axonal transport. In vitro studies suggested that kinesin moves organelles preferentially in the anterograde direction, but data about the function and precise localization of kinesin in the living axon were lacking. The current study was undertaken to establish whether kinesin associates with anterograde or retrograde moving organelles in vivo. Peripheral nerves were ligated to produce accumulations of organelles moving in defined directions. Regions proximal (anterograde) and distal (retrograde) to the ligation were analyzed for kinesin localization by immunofluorescence, and by immunogold electron microscopy using ultracryomicrotomy. Substantial amounts of kinesin were associated with anterograde moving organelles on the proximal side, while significantly less kinesin was detected distally. Statistical analyses indicated that kinesin was mostly associated with membrane-bounded organelles. These observations indicate that axonal kinesin is primarily associated with anterograde moving organelles in vivo.

THE size and polarized morphology of the neuron requires that intracellular transport processes be highly developed. As a result, the axons of nerve cells have historically been excellent model systems for studying the mechanisms of intracellular transport.

Recent studies have identified two proteins that may serve as motor proteins for anterograde and retrograde axonal transport: kinesin and cytoplasmic dynein (Brady, 1985a; Vale et al., 1985a,b; Paschal et al., 1987; Paschal and Vallee, 1987; Lye et al., 1987; Vallee et al., 1988). Both of these proteins can be isolated from neuronal tissue and both are microtubule-activated ATPases capable of performing work in vitro. Microtubules will glide across glass coverslips coated with either kinesin or dynein, but the direction of gliding differs. With kinesin, microtubules glide in a direction consistent with anterograde transport (Vale et al., 1985b; Porter et al., 1987), while gliding with cytoplasmic dynein is consistent with retrograde transport (Paschal and Vallee, 1987). This has led to suggestions that kinesin is responsible for anterograde movement of membrane bounded organelles in the axon (Vale et al., 1985a) and cytoplasmic dynein is involved in retrograde axonal transport (Paschal and Vallee, 1987). In fact, structures of kinesin molecules (Amos, 1988; Hirokawa et al., 1989; Hisanaga et al., 1989; Scholey et al., 1989) and of kinesin bound to microspheres (Hirokawa et al., 1989) coincide well with the structures of crossbridges between membranous organelles and microtubules in axons (Smith, 1971; Hirokawa, 1982; Hirokawa et al., 1985; Miller and Lasek,

1985; Hirokawa and Yorifuji, 1986; Hirokawa et al., 1989a). While this hypothesis has many attractive attributes, little direct evidence has been available using in vivo systems until recently.

The evidence for kinesin was provided through the use of a series of monoclonal antibodies specific for kinesin heavy or light chains. These antibodies indicated by immunofluorescence microscopy that kinesin is localized to Triton X-100-soluble, punctate structures thought to represent membrane-bounded organelles in a variety of neuronal and nonneuronal cells in culture (Pfister et al., 1989a). A homologous distribution of kinesin on punctate structures was demonstrated in isolated squid axoplasm (Brady et al., 1990). Another study, based on selective detergent extraction of cultured fibroblasts, suggested that approximately one-third of the cellular kinesin pool in those cells is organelle bound (Hollenbeck, 1989). The level of resolution possible with immunofluorescence microscopy and cellular fractionation methods is limited, though, and the question of how kinesin partitions between organelles moving in the anterograde or retrograde directions remained unresolved. Accordingly, we sought new approaches to shed further light on the localization of kinesin in vivo.

In the present study, we used light and electron microscopic immunocytochemistry to evaluate the localization of kinesin in vivo under conditions that segregated organelles moving toward the axon terminal from those undergoing transport toward the neuronal cell body. Separation of the two organelle
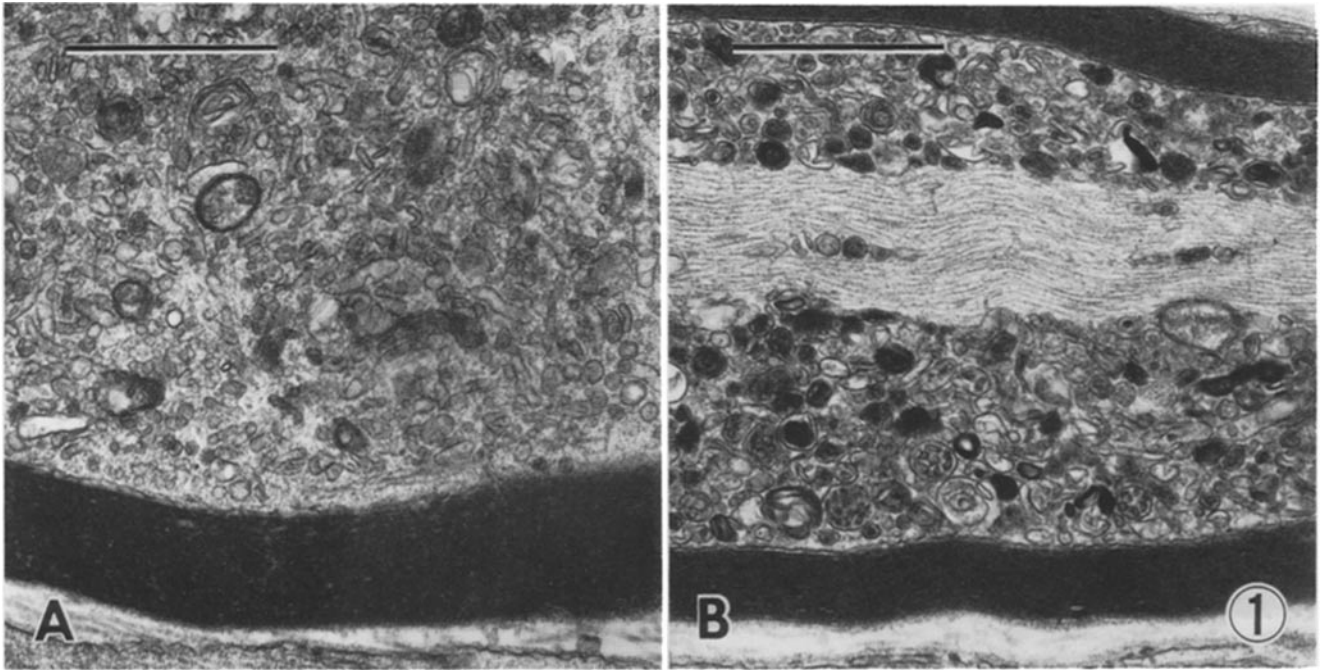
Figure 1. Electron micrographs of saphenous nerve axons after ligation. (*A*) A region proximal and very close to the ligation. Small tubulovesicular membranous organelles and mitochondria have accumulated at high density. (*B*) A region distal and very close to the ligation containing both membrane-enriched and cytoskeleton-enriched regions. Lysosomes, multivesicular bodies, and mitochondria have accumulated. Note that the mean size of organelles distal to the ligation is greater than that seen for organelles on the proximal side. In the center of this micrograph the cytoskeleton-rich domain is observed. Bar, 1 μm.

classes was accomplished by ligating a peripheral nerve. Anterograde and retrograde moving organelles are known to accumulate on the proximal and distal edges of such lesions, respectively (Smith, 1980; Tsukita and Ishikawa, 1980; Fahim et al., 1985; Hirokawa, 1990). Kinesin was found to accumulate on the proximal sides of the ligations to a much greater extent than on the distal sides. These observations suggest that kinesin is associated primarily with membrane-bounded organelles that move in the anterograde direction in the axon. Building upon previous in vitro evidence that kinesin is a motor protein for fast axonal transport, the results documented here provide the first evidence in an in vivo system that kinesin is preferentially associated with anterogradely moving membranous organelles and thus support the hypothesis that kinesin serves as a motor for fast anterograde axonal transport.

Materials and Methods

Monoclonal Antibodies

Procedures for production and purification of monoclonal antibodies against bovine brain kinesin were described previously (Pfister et al., 1989). In this study, we used two of these murine monoclonal IgG antibodies against the heavy chains of kinesin (H1, H2).

Ligation of Mouse Peripheral Nerves

Female albino mice were anesthetized and their saphenous nerves were ligated very tightly. After ligations, mice were kept within small cardboard boxes to restrict their movements. After 6–10 h, the mice were perfused transcardially with 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 as described previously (Hirokawa et al., 1990). Small pieces of nerves proximal and distal to the ligated portions were

processed for conventional thin section electron microscopy, immunofluorescence microscopy and cryoultrathin section immunocytochemistry (Hirokawa et al., 1984, 1985, 1989*b*).
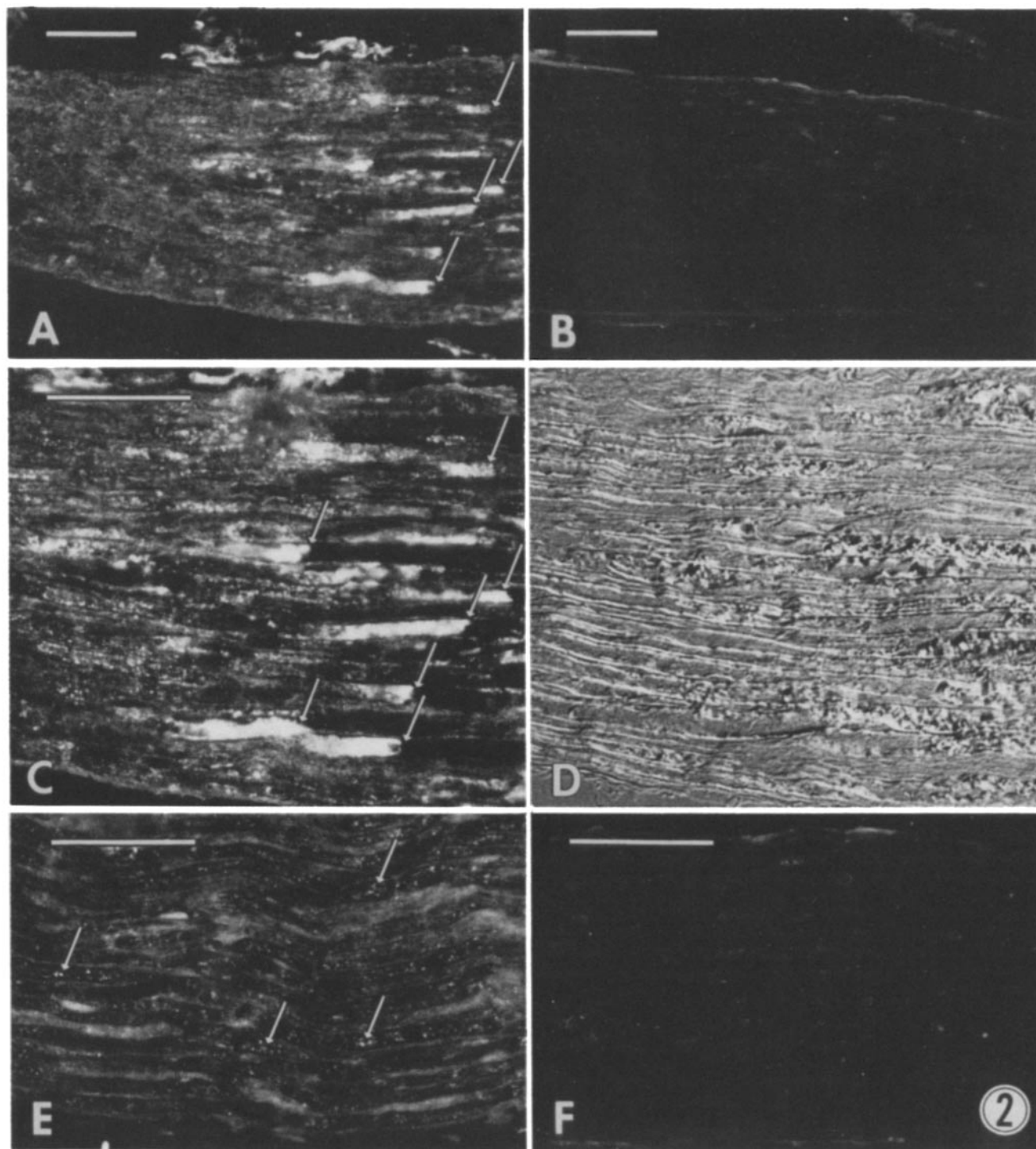
Immunofluorescence Microscopy

The nerves were sectioned on a cryostat (4–5-μm sections) and stained with purified monoclonal antibodies (mixture of H1 and H2, each one 20 μg/ml) or normal mouse IgG (40 μg/ml), followed by rhodamine-labeled goat anti-mouse IgG (Fisher Scientific Co., Pittsburgh, PA). Sections were preincubated 1 h with 5% skim milk in PBS and stained as described previously (Hirokawa et al., 1984, 1985).

Immunocytochemistry of Kinesin Using Ultrathin Cryosections

Procedures for immunolabeling of ultrathin cryosections were performed as described previously (Tokuyasu, 1980; Hirokawa et al., 1989*b*) with some modifications. After cryoprotection in graded series of sucrose/PBS solutions the nerves were embedded in 2% gelatin and frozen with liquid Freon 22. We used a mixture of purified H1 and H2 (20 μg/ml each) or normal mouse IgG (40 μg/ml) as the first antibodies and gold- (5 nm) labeled goat anti-mouse IgG (Janssen Life Sciences Products, Piscataway, NJ) as the second antibodies. All antibodies were diluted in PBS to desired concentrations.

Immunoblotting

Immunoreactive proteins from mouse brain were identified by immunoblotting by the method of Towbin et al. (1979), using a mixture of monoclonal antikinesin antibodies (H1 and H2) as the primary antibodies. Mouse brains were dissected and homogenized in the absence of nucleotides. Homogenates were centrifuged at 19,000 g for 30 min at 4°C. Supernatants were incubated at 37°C without nucleotide in the presence of 20 μM taxol for 15 min, then centrifuged at 19,000 g for 30 min at 30°C. The mouse brain homogenates and resulting supernatants were run on 7% SDS-polyacrylamide gels according to the method of Laemmli (1970).

Figure 2. Immunofluorescence and differential interference contrast micrographs of ligated (*A-D*) and nonligated (*E* and *F*) mouse saphenous nerves stained with anti-kinesin antibodies (*A-E*) and non immune mouse IgG (*F*). (*A*) Axons proximal to the ligation (ligation is on the right edge). (*B*) Axons distal to the ligation (ligation is on the left edge). (*C*) Higher magnification of *A*. Staining is very bright at regions close to the ligation (*arrows*). (*D*) Differential interference contrast micrograph of the same field in *C*. (*E*) Nonligated axons stained with anti-kinesin monoclonal antibodies. Arrows indicate punctate staining. (*F*) Nonligated axons stained with normal mouse IgG. Schwann cell cytoplasm is faintly stained, but axons are unlabeled. Bar, 50 μm.

Morphometric Measurements

Electron micrographs were printed at 200,000×. The density of gold particles were obtained in various definable regions of the micrographs taking into account the magnification factor of the prints as described previously (Hirokawa et al., 1990).

Five of these regions were proximal and close to ligations, and eight others were located near the lesions on the distal sides. In all thirteen of these regions, four different domains were analyzed: (*a*) extracellular space, especially area filled with collagen fibers; (*b*) myelin sheaths; (*c*) cytoskeleton-enriched domains, which were filled with microtubules and neurofilaments; and (*d*) regions where membranous organelles (tubulovesicular
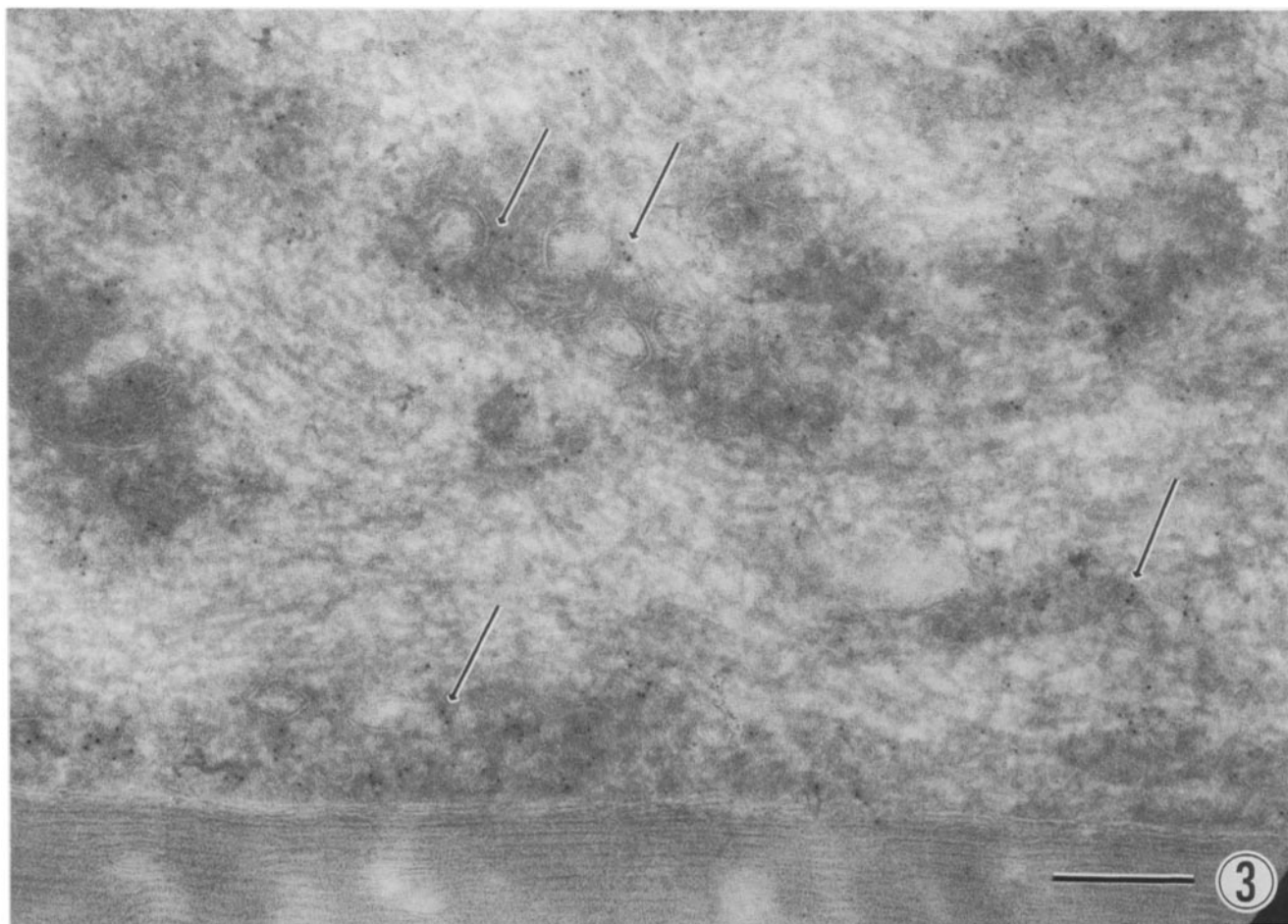
Figure 3. Cryoultrathin section of axon proximal to the ligation stained with anti-kinesin antibodies and 5-nm gold-labeled second antibodies. In this area membranous organelles are moderately accumulated in between cytoskeleton enriched regions. Gold particles are preferentially found in areas where membranous organelles have accumulated and tend to be associated with the organelles (*arrows*). Bar, 200 nm.

membranous organelles and mitochondria in the proximal region, and multivesicular bodies, presumptive lysosomes, and mitochondria in the distal region) had accumulated. The total area examined for each of these regions ranged from 30 to 74 μm^2, and the average number of gold particles per μm^2 was determined for each region.

Results

Electron Microscopy of Axons after Ligation

Peripheral (saphenous) nerves of female albino mice were ligated under anesthesia. 6–10 h after ligation, the mice were fixed by perfusion and the nerves were dissected. The proximal and distal portions of the ligated nerves were processed for electron microscopy. As noted in previous studies, different classes of organelles accumulated in the regions proximal and distal to the ligated portion (Smith, 1980; Tsukita and Ishikawa, 1980; Fahim et al., 1985; Hirokawa et al., 1990). Tubulovesicular membranous structures and mitochondria were primary constituents in regions of the axon proximal to the ligation (Fig. 1 *A*), while larger, more varied membrane bounded organelles such as presumptive lysosomes, multivesicular bodies and mitochondria (often in a distorted state) were most common in regions distal to the ligation (Fig. 1 *B*).

Immunofluorescence Microscopic Localization of Kinesin

Proximal and distal portions of ligated nerves were stained with a mixture of two previously characterized monoclonal antibodies (H1 and H2) raised against bovine brain kinesin (Pfister et al., 1989a; Hirokawa et al., 1989a). The specificity of these antibodies for kinesin heavy chains in homogenates of mouse nerve tissues was determined by immunoblots (data not shown). No other polypeptides were noted in immunoblots, similar to the results seen in other mammalian sources of kinesin (Pfister et al., 1989a) and, in the case of H2, also in squid (Brady et al., 1990). Immunofluorescence microscopy revealed that kinesin antibodies strongly stained regions of the nerve proximal to the ligation (Fig. 2 *A*). By contrast, staining in the regions distal to the ligated portions (Fig. 2 *B*) was significantly lower than that seen on the proximal side. Staining on the proximal sides followed a gradational pattern, increasing in brightness as the regions closer to the ligated portions were examined. This characteristic was apparent even though the ligated regions themselves had been damaged to some extent (Fig. 2, *A*, *C*, and *D*). The diameter of nerves is widened at regions close to the ligated parts (Fig. 2, *A* and *C*), with the accumulations of organelles noted in the electron microscopic studies. At higher magnifi-

298
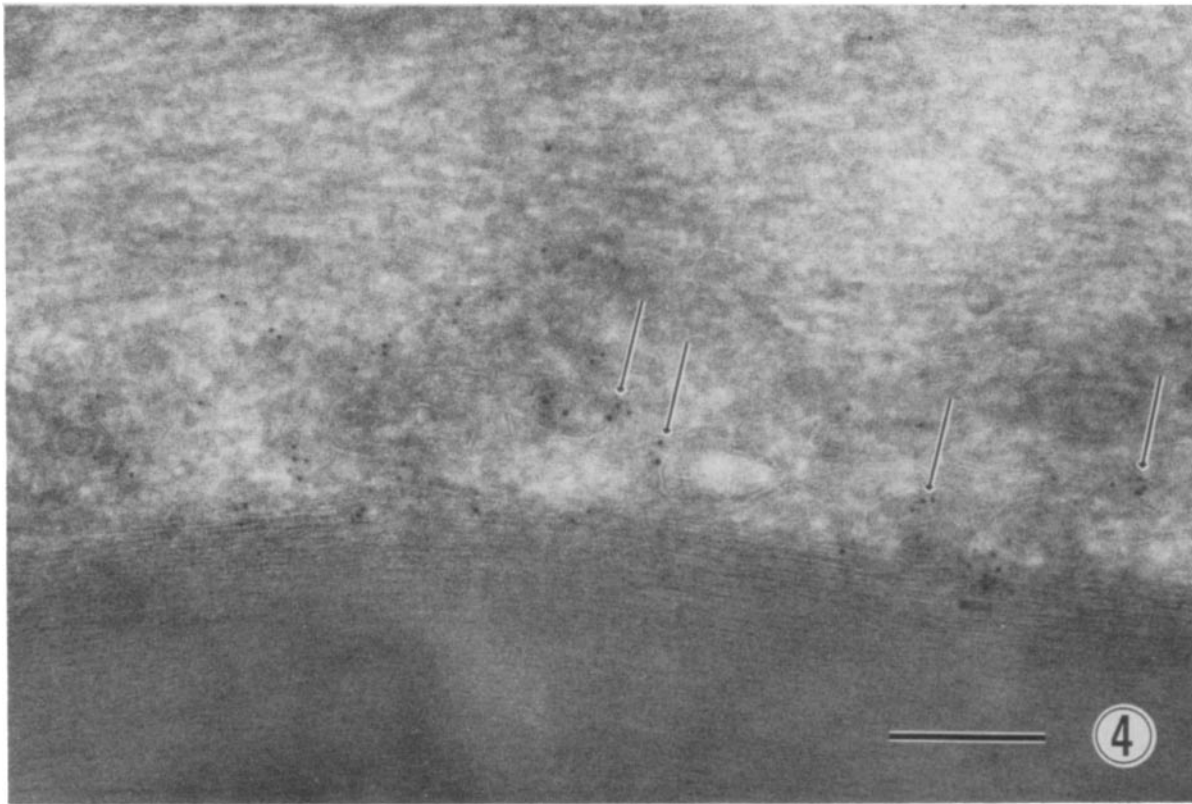
Figure 4. Cryoultrathin section of an axon far proximal (~1.5 mm) to the ligation stained with anti-kinesin antibodies and 5-nm gold-labeled second antibodies. In this area membranous organelles are relatively sparsely distributed, but gold particles tend to be associated with the organelles (*arrows*). Bar, 200 nm.

cation (Fig. 2 *C*), the fluorescent staining pattern could be resolved into a more punctate pattern in axonal regions more distant from the ligation (>200 μm). Comparisons between fluorescent (Fig. 2 *C*) and differential interference contrast microscopy (Fig. 2 *D*) images of the same field demonstrated that the bulk of the kinesin-positive materials was within axonal profiles rather than in the myelin or extracellular space.

When nonligated control nerves were labeled with these antibodies, punctate structures in the axoplasms were stained, and the overall staining intensity was lower than in the ligated nerves (Fig. 2 *E*). This pattern is similar to the patterns obtained in tissue culture cells (Pfister et al., 1989*a*) and in isolated axoplasm from the squid (Brady et al., 1990). In control sections treated with normal mouse IgG as the primary antibody, we observed only faint background staining (Fig. 2 *F*). The immunofluorescence results indicate that kinesin accumulates anterogradely at the ligation and suggest, therefore, that kinesin is preferentially associated with anterogradely moving structures. Kinesin accumulates at substantially lower levels with retrograde moving material on the distal side of the ligation.

Electron Microscopic Immunolocalization of Kinesin

The immunofluorescence analysis was consistent with the bulk of the kinesin moving toward the axon terminal with membrane bounded organelles. However, soluble proteins move with slow axonal transport (Hammerschlag and Brady, 1989; Brady, 1985*b*; Grafstein and Forman, 1980) and would also accumulate on the proximal side of a ligation,

though at a slower rate. Therefore, the localization of kinesin was also determined at the electron microscopic level using cryoultramicrotomy. As indicated above, different classes of membranous organelles preferentially accumulated in regions proximal and distal to the ligation (Fig. 1). Regions enriched in membrane bounded organelles were readily distinguished from cytoskeletal domains in the area at short distances away from the ligation, where membranous organelles were moderately accumulated (Fig. 3).

First, we determined the fraction of gold particles in different regions of the axon. In areas proximal to the ligations gold particles were preferentially localized in the membrane-enriched regions and were very closely associated with the membranous organelles (Fig. 3). To determine the degree of enrichment, we calculated the relative density of gold particles for the myelin sheath, the extracellular space, membrane-enriched areas, and cytoskeleton-enriched areas in the regions such as shown in Fig. 3. As shown in Table I, gold particles were primarily localized to the membrane-enriched areas. Fig. 4 shows axoplasm far proximal (~1.5 mm) to the ligation, where membranous organelles were sparsely distributed and the cytoskeletal arrangement was not distorted. In this area of the nerve most gold particles also appeared to be preferentially associated with membranous organelles.

In contrast to the situation seen on the proximal edges of lesions, fewer gold particles were observed close to the distal borders, where retrograde moving organelles accumulated. Morphometric analysis suggested that kinesin is also preferentially localized in membranous organelles enriched region

Table I. Gold Particles Per Unit Area in Sections
of the Nerves Proximal and Distal to the Ligation
(Less Than ~200 µm)

Region	Number of gold particles/µm²	
	Proximal (n = 5)	Distal (n = 8)
Extracellular space	1.50 ± 0.25	1.53 ± 0.32
Myelin sheath	0.75 ± 0.20	0.72 ± 0.20
Cytoskeleton	15.01 ± 3.14	1.73 ± 0.51
Membranous organelles enriched region	140.19 ± 15.03	2.54 ± 1.01

n refers to numbers of separate regions of nerves examined; total area examined for each parameter ranged from 30 to 74 µm².

Table II. Gold Particles Per Unit Area in Sections
of the Nerves Far Proximal and Distal to the Ligation
(~1.5-2 mm away)

Region	Number of gold particles/µm²	
	Proximal (n = 4)	Distal (n = 4)
Extracellular space	0.87 ± 0.20	0.80 ± 0.18
Myelin sheath	0.62 ± 0.19	0.42 ± 0.08
Axoplasm	37.78 ± 7.25	3.97 ± 0.63

n refers to numbers of separate regions of nerves examined; total area examined for each parameter ranged from 30 to 74 µm².

in these distal regions (Table I), but the extent of enrichment was very low by comparison to the proximal sides. These data corroborated the results obtained by immunofluorescence (Fig. 2), indicating again that kinesin is far more abundantly associated with organelles moving in the anterograde direction than with those undergoing retrograde transport.

Regions of axoplasm far proximal (Fig. 4) and distal to the lesions (~1.5-2 mm away), where the membranous organelles are relatively sparsely distributed, were also analyzed quantitatively. Table II summarizes gold particles per unit area in these sections. Comparing these data with those in the proximal and distal region it is clear that kinesin is accumulating at the proximal region.

Immunoelectron microscopy of sections incubated with control antibodies resulted in the detection of very few, randomly dispersed gold particles (Fig. 5). Similarly, background levels of gold particles associated with myelin or extracellular space (Fig. 3, 4, and 5, and Table I) were extremely low, indicating that nonspecific labeling with immunogold reagents was negligible in these studies. Taken together, the immuno-EM data presented here strongly indicate that kinesin preferentially associated with anterogradely transported organelles.

Discussion

Inhibition of fast axonal transport by ligation of a nerve results in the segregation of anterograde from retrograde

Figure 5. Cryoultrathin section of an axonal region proximal to the ligation stained with normal mouse IgG and 5-nm gold-labeled second antibodies. Very few gold particles are detectable. Bar, 200 nm.

moving membrane-bounded organelles. On both the proximal and distal sides of a ligation, two types of intracellular regions are generated. One type, termed a membrane-enriched region, contains large numbers of membrane-bounded organelles with known directions for movement. The second type is devoid of membrane bounded organelles, but rich in cytoskeletal structures (neurofilaments and microtubules), and is termed a cytoskeleton-enriched region. In effect, the cell has established three morphologically distinct subcellular domains via physiological processes: an anterograde membrane-enriched region, a retrograde membrane-enriched region, and a cytoskeleton-enriched region.

We have used this preparation to evaluate the extent to which kinesin partitions with anterograde moving organelles, retrograde moving organelles, and cytoskeletal or freely diffusible fractions of the axonal cytoplasm. Using immunofluorescence microscopy and quantitative electron microscopic immunocytochemistry, kinesin was found to be preferentially associated with the regions where membrane-bounded organelles had accumulated on the proximal sides of ligations. Significantly less kinesin was associated with retrograde moving organelles that became enriched on the distal sides of the ligations, and very low levels of kinesin were detectable in cytoskeleton-enriched regions. These observations provide in vivo evidence that kinesin is preferentially associated with anterogradely transported membrane-bounded organelles, and in light of its mechanochemical properties (Vale et al., 1985b) suggest strongly that kinesin is bound to the organelles and responsible for their movement toward the axon terminal. It should be noted though, that there might be a pool of soluble kinesin which is appreciable in total size, but present at low concentration and may not have been detected by immuno-EM.

Although kinesin and dynein exhibit a variety of pharmacological differences in ATPase and microtubule gliding assays, comparable distinctions between anterograde and retrograde transport in vivo have not been readily demonstrated. A variety of pharmacological and biochemical treatments that differentially affect kinesin and dynein have failed to distinguish between anterograde and retrograde movements of membrane-bounded organelles in squid axoplasm (for further discussion see Pfister et al., 1989b; Brady et al., 1990; Leopold et al., 1990). Vanadate and EDTA appear to affect turnaround of membrane-bounded organelles at a crush in frog myelinated nerves (Smith, 1988), but it is unclear whether these agents exert a direct effect on the motor proteins or on regulatory processes. An antibody specific for the heavy chain of kinesin interferes with bidirectional transport in isolated axoplasm (Brady et al., 1990), but no specific immunological probes for dynein function have been reported to date. UV-vanadate cleavage studies have been reported to affect retrograde transport preferentially in some in vitro assays for organelle movement (Schroer et al., 1989; Schnapp and Reese, 1989), but UV-vanadate treatments inhibited bidirectional movements in a nonneuronal preparation in which kinesin has not yet been identified (Euteneuer et al., 1989).

The immunoelectron microscopic methods used here effectively address a major issue: the extent to which kinesin is associated with anterograde and retrograde moving membrane-bounded organelles. Kinesin is present at significantly higher levels in the anterograde membrane-enriched regions than in any other region, indicating a substantial enrichment of kinesin in association with these organelles. By contrast, the retrograde membrane-enriched regions contain kinesin at levels only slightly above the background levels seen in the myelin sheath or extracellular matrix (see Figs. 3–5 and Table I). The observation that the highest concentrations of kinesin in the axon is on anterograde moving organelles is consistent with a role for kinesin as the motor for fast anterograde axonal transport.

Although the level of gold particles detectable in the retrograde membrane-enriched regions were low as compared to that seen in the corresponding anterograde regions, the present study suggests a possibility that a small amount of kinesin is associated with some retrograde moving organelles. A different situation exists for cytoplasmic dynein, which was recently found to accumulate in both anterograde and retrograde membrane-enriched regions of similar preparations (Hirokawa et al., 1990) using antibodies raised against brain dynein. This raises the intriguing possibility that included among the cargo transported by kinesin to the axon terminal is an inactive form of the motor protein responsible for the movement of similar cargo in the opposite direction.

The concentration of kinesin associated with membrane-bounded organelles in situ does make sense in a physiological context. In vitro studies of kinesin ATPase activity have shown that the hydrolysis of ATP by a soluble fraction of kinesin is substantially increased by addition of microtubules (Wagner et al., 1989; Kutznetsov and Gelfand, 1986; Cohn et al., 1987). As a result, the presence of a significant pool of freely diffusible kinesin inside a cell with a microtubule based cytoskeleton would be expected to result in the hydrolysis of a substantial amount of cellular ATP without accomplishing useful work. If, however, most kinesin in the cell were associated with membrane-bounded organelles that are to be moved, then hydrolysis of ATP by kinesin would be closely coupled to movement of organelles.

The demonstration that kinesin is most highly concentrated in the area enriched with anterograde organelles strengthens that kinesin is a motor protein for organelle motility toward the axon terminal. By extension, it seems likely that a general in vivo function of kinesin is to transport membranous organelles toward the "plus" ends of microtubules in other parts of the neuron and in nonneuronal cells. Though this suggestion was made earlier based on in vitro motility assays (Vale et al., 1985b), at least one member of the kinesin superfamily protein was recently shown to be a minus-end directed motor (Walker et al., 1990). This unexpected finding stresses the importance of thoroughly investigating the directionality of force production by mechanochemical enzymes such as kinesin, both in vitro and in vivo approaches.

The paucity of kinesin associated with retrograde organelles reduces the likelihood of kinesin playing a direct role in retrograde organelle motility. However, one of the anti-kinesin antibodies used in the present study (H2) was also found to inhibit bidirectional organelle transport in extruded squid axoplasm (Brady et al., 1990), suggesting that the situation could be more complex in other, unrelated organisms. We suspect that more substantial amounts of kinesin are present on retrograde moving organelles in the squid giant axon than in mammalian peripheral nerve. This might

permit organelle-bound anti-kinesin to interfere sterically with both kinesin and dynein in the squid axon and thereby inhibit anterograde, as well as retrograde motility in that system. This apparent distinction between the mouse and squid underscores the importance of studying the microtubule-based motor proteins in a variety of in vivo settings for gaining a full understanding of their functions and mechanisms of action in the cell.

References

Amos, L. A. 1987. Kinesin from pig brain studied by electron microscopy. *J. Cell Sci.* 87:105–111.

Bloom, G. S., M. C. Wagner, K. K. Pfister, and S. T. Brady. 1988. Native structure and physical properties of bovine brain kinesin, and identification of the ATP-binding subunit polypeptide. *Biochemistry.* 27:3409–3416.

Brady, S. T. 1985a. A novel brain ATPase with properties expected for the fast axonal transport motor. *Nature (Lond.).* 317:73–75.

Brady, S. T. 1985b. Axonal Transport: methods and applications. *In* Neuromethods 1. General Methods. A. Boulton and G. Baker, editors. 419–476.

Brady, S. T., K. K. Pfister, and G. S. Bloom. 1990. A monoclonal antibody against the heavy chain of kinesin inhibits both anterograde and retrograde axonal transport in isolated squid axoplasm. *Proc. Natl. Acad. Sci. USA.* 87:1061–1065.

Cohn, S. A., A. L. Ingold, and J. M. Scholey. 1987. Correlation between the ATPase and microtubule translocating activities of sea urchin egg kinesin. *Nature (Lond.).* 328:160–163.

Euteneuer, U., K. B. Johnson, and M. Schliwa. 1989. Photolytic cleavage of cytoplasmic dynein inhibits organelle transport in Reticulomyxa. *Eur. J. Cell Biol.* 50:34–40.

Fahim, M., R. L. Lasek, S. T. Brady, and A. Hodge. 1985. Identification of the membranous organelles moving in fast axonal transport in squid giant axon: a correlative electron microscopy and video microscopy study. *J. Neurocytol.* 14:689–704.

Grafstein, B., and D. S. Forman. 1981. Intracellular transport in neurons. *Physiol. Rev.* 60:1167–1283.

Hammerschlag, R., and S. T. Brady. 1988. The cytoskeleton and axonal transport. *In* Basic Neurochemistry, G. Siegel, R. W. Alberts, B. W. Agranoff, and P. Molinoff, editors. Raven Press, Ltd., New York. 457–478.

Hirokawa, N. 1982. The cross-linker system between neurofilaments, microtubules and membranous organelles in frog axons revealed by quick-freeze, freeze-fracture, deep-etching method. *J. Cell Biol.* 94:129–142.

Hirokawa, N., M. A. Glicksman, and M. B. Willard. 1984. Organization of mammalian neurofilament polypeptides within the neuronal cytoskeleton. *J. Cell Biol.* 98:1523–1536.

Hirokawa, N., G. S. Bloom, and R. B. Vallee. 1985. Cytoskeletal architecture and immunocytochemical localization of microtubule-associated proteins in regions of axons associated with rapid axonal transport. The beta, beta′-iminodipropionitrile-intoxicated axon as a model system. *J. Cell Biol.* 101:227–239.

Hirokawa, N., and H. Yorifuji. 1986. Cytoskeletal architecture in the reactivated crayfish axons with special reference to the crossbridges between microtubules and membrane organelles and among microtubules. *Cell Motil. Cytoskeleton.* 6:458–468.

Hirokawa, N., K. K. Pfister, H. Yorifuji, M. C. Wagner, D. T. Brady, and G. S. Bloom. 1989a. Submolecular domains of bovine brain kinesin iden-

tified by electron microscopy and monoclonal antibody decoration. *Cell.* 56:867–878.

Hirokawa, N., K. Sobue, K. Kanda, A. Harada, and H. Yorifuji. 1989b. The cytoskeletal architecture of the presynaptic terminal and molecular structure of synapsin 1. *J. Cell Biol.* 108:111–126.

Hirokawa, N., Y. Yoshida, R. Sato-Yoshitake, and T. Kawashima. 1990. Brain dynein localizes on both anterogradely and retrogradely transported membranous organelles. *J. Cell Biol.* 111:1027–1037.

Hisanaga, S., H. Murofushi, K. Okuhara, R. Sato, Y. Masuda, H. Sakai, and N. Hirokawa. 1989. The molecular structure of adrenal medulla kinesin. *Cell Motil. Cytoskeleton.* 12:264–272.

Hollenbeck, P. J. 1989. The distribution, abundance and subcellular localization of kinesin. *J. Cell Biol.* 108:2335–2342.

Kuznetsov, S. A., and V. I. Gelfand. 1986. Bovine brain kinesin is a microtubule-activated ATPase. *Proc. Natl. Acad. Sci. USA.* 83:8530–8534.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Leopold, P. L., R. Snyder, G. S. Bloom, and S. T. Brady. 1990. Nucleotide specificity for the bidirectional transport of membrane bounded organelles in isolated axoplasm. *Cell Motil. Cytoskeleton.* 15:210–219.

Lye, R. J., M. E. Porter, J. M. Scholey, and J. R. McIntosh. 1987. Identification of a microtubule-based cytoplasmic motor in the nematode C. elegans. *Cell.* 51:309–318.

Miller, R. H., and R. J. Lasek. 1985. Cross-bridges mediate anterograde and retrograde vesicle transport along microtubules in squid axoplasm. *J. Cell Biol.* 101:2181–2193.

Paschal, B. M., H. S. Shpetner, and R. B. Vallee. 1987. MAP1C is a microtubule-activated ATPase which translocates microtubules in vitro and has dynein-like properties. *J. Cell Biol.* 105:1273–1282.

Paschal, B. M., and R. B. Vallee. 1987. Retrograde transport by the microtubule-associated protein MAP1C. *Nature (Lond.).* 330:181–183.

Pfister, K. K., M. C. Wagner, D. L. Stenoien, S. T. Brady, and G. S. Bloom. 1989a. Monoclonal antibodies to kinesin heavy and light chains stain vesicle-like structures, but not microtubules, in cultured cells. *J. Cell Biol.* 108:1453–1463.

Pfister, K. K., M. C. Wagner, G. S. Bloom, and S. T. Brady. 1989b. Modification of the microtubule-binding and ATPase activities of kinesin by N-ethylmaleimide (NEM) suggests a role for sulfhydryls in fast axonal transport. *Biochemistry.* 28:9006–9012.

Porter, M. E., J. M. Scholey, D. L. Stemple, G. P. A. Vigers, R. D. Vale, M. P. Sheetz, and J. R. McIntosh. 1987. Characterization of the microtubule movement produced by sea urchin egg kinesin. *J. Biol. Chem.* 262:2794–2802.

Schnapp, B. J., and T. S. Reese. 1989. Dynein is the motor for retrograde axonal transport of organelles. *Proc. Natl. Acad. Sci. USA.* 86:1548–1552.

Scholey, J. M., J. E. Heuser, J. T. Yang, and L. S. R. Goldstein. 1989. Identification of globular mechanochemical heads of kinesin. *Nature (Lond.).* 338:355–357.

Schroer, T. A., E. R. Steuer, and M. P. Sheetz. 1989. Cytoplasmic dynein is a minus end-directed motor for membranous organelles. *Cell.* 56:937–946.

Smith, D. S. 1971. On the significance of cross-bridges between microtubules and synaptic vesicles. *Phil. Trans. R. Soc. Lond. Biol. Sci.* 261:395–405.

Smith, R. S. 1980. The short term accumulation of axonally transported organelles in the region of localized lesions of single myelinated axons. *J. Neurocytol.* 9:39–65.

Smith, R. S. 1988. Studies on the mechanism of the reversal of rapid organelle transport in myelinated axons of Xenopus laevis. *Cell Motil. Cytoskeleton.* 10:296–308.

Tokuyasu, K. T. 1980. Immunochemistry on ultrathin frozen sections. *Histochem. J.* 12:381–403.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some application. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

Tsukita, S., and H. Ishikawa. 1980. The movement of membranous organelles in axons electron microscopic identification of anterogradely and retrogradely transported organelles. *J. Cell Biol.* 85:512–530.

Vale, R. D., T. S. Reese, and M. P. Sheetz. 1985a. Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell.* 42:39–50.

Vale, R. D., B. J. Schnapp, T. Mitchison, E. Steuer, T. S. Reese, and M. P. Sheetz. 1985b. Different axoplasmic proteins generate movement in opposite directions along microtubules in vitro. *Cell.* 43:623–632.

Vallee, R. B., J. S. Wall, B. M. Paschal, and H. S. Shpetner. 1988. Microtubule-associated protein 1C from brain is a two-headed cytosolic dynein. *Nature (Lond.).* 332:561–563.

Walker, R. A., E. D. Salmon, and S. A. Endow. 1990. The Drosophila claret segregation protein is a minus-end directed motor molecule. *Nature (Lond.).* 347:780–782.