Plus-end Motors Override Minus-end Motors during Transport of Squid Axon Vesicles on Microtubules

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Abstract. Plus- and minus-end vesicle populations from squid axoplasm were isolated from each other by selective extraction of the minus-end vesicle motor followed by 5′-adenylyl imidodiphosphate (AMP-PNP)–induced microtubule affinity purification of the plus-end vesicles. In the presence of cytosol containing both plus- and minus-end motors, the isolated populations moved strictly in opposite directions along microtubules in vitro. Remarkably, when treated with trypsin before incubation with cytosol, purified plus-end vesicles moved exclusively to microtubule minus ends instead of moving in the normal plus-end direction. This reversal in the direction of movement of trypsinized plus-end vesicles, in light of further observation that cytosol promotes primarily minus-end movement of liposomes, suggests that the machinery for cytoplasmic dynein-driven, minus-end vesicle movement can establish a functional interaction with the lipid bilayers of both vesicle populations. The additional finding that kinesin overrides cytoplasmic dynein when both are bound to bead surfaces indicates that the direction of vesicle movement could be regulated simply by the presence or absence of a tightly bound, plus-end kinesin motor; being processive and tightly bound, the kinesin motor would override the activity of cytoplasmic dynein because the latter is weakly bound to vesicles and less processive. In support of this model, it was found that (*a*) only plus-end vesicles copurified with tightly bound kinesin motors; and (*b*) both plus- and minus-end vesicles bound cytoplasmic dynein from cytosol.

An important goal in the study of organelle trafficking is to understand how distinct membrane compartments are transported along microtubules to their correct intracellular destinations. It is advantageous to consider this question in nerve cell axons because of their simple, linear organization. Because axons, to a large extent, lack protein synthetic machinery and are not engaged in the de novo assembly of membrane compartments, most vesicles in axons are fully assembled and in transit, moving along microtubules between the cell body and the nerve terminals. Membrane compartments carrying synaptic vesicle precursors and components of the axonal membrane are derived from the Golgi and move in the anterograde direction, while endocytic compartments carrying trophic factors (16) and recycling membrane proteins move in the retrograde direction (14, 61). Because axonal microtubules are oriented with their plus ends pointed away from the cell body (25), anterograde and retrograde membrane compartments move toward microtubule plus and minus ends, respectively.

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While it is clear that microtubule motors are involved in the transport and positioning of membrane compartments, two key questions remain unresolved: (*1*) how are motors docked on vesicles, and (*2*) which mechanisms regulate the direction of vesicle movement? To address these questions, we have concentrated on the squid giant axon, the same system that led to the discovery of microtubule motor proteins and their involvement in vesicle transport (6, 54, 64). This system has the advantage that the axon itself can be cleanly isolated from the cell body and the axon terminals. In theory, the organelles recovered in low to moderate speed supernatants from extruded axoplasm should consist only of smooth ER (33) and anterograde and retrograde vesicle populations (61). There should be no contamination from the plasma membrane, mitochondria, or compartments involved in vesicle biogenesis such as the Golgi or early and late endosomes (33, 46). Most important, extruded squid axoplasm exhibits robust bidirectional organelle movement (8, 54) which is recovered in purified vesicle fractions (53, 67). This enrichment in moving vesicles is a great advantage for biochemical studies.

Kinesin and its many relatives transport organelles toward microtubule plus ends, i.e., anterogradely in nerve cell axons (12, 31, 41, 44, 45, 65), while the minus end–directed motor, cytoplasmic dynein, is thought to trans-

port organelles retrogradely (47, 52, 56). It is likely that vesicle trafficking in axons is regulated by mechanisms that cause cytoplasmic dynein to direct the movement of retrograde vesicles, and kinesin-related motors to direct the movement of anterograde vesicles. The situation is complicated because microtubule motors, while associated with vesicle membranes, are also abundant in the cytosol, where they are available for interaction with all organelles (28, 65, 67). These motor proteins bind inert surfaces nonspecifically and can promote processive movement along microtubules (5, 30, 65). Thus, we are likely to encounter not only mechanisms that cause the correct motor to be active on an organelle, but also mechanisms that prevent the activity of motors with the wrong polarity from directing movement.

Although the mechanisms for targeting motors of the correct polarity to organelles have yet to be elucidated, two general models have been proposed (23, 68). In one model, vesicles might bind only a single type of motor; e.g., either a kinesin or cytoplasmic dynein, depending on whether transport is to be anterograde or retrograde. Alternatively, vesicles might bind both plus- and minus-end motors, but the motor activity might be regulated to ensure the correct direction of transport. The latter model is supported by several studies which claim or imply that both kinesin and cytoplasmic dynein are present on certain organelles (26, 27, 43, 73). This conclusion, however, is still not firmly established.

The present study was initiated with the aim of distinguishing between the two models mentioned above. Our strategy was to purify plus and minus end–directed vesicle populations from squid axons and investigate their interaction with plus and minus end–directed motors (52, 53, 64). The method reported here for separating and purifying plus- and minus-end vesicles from squid axoplasm was based on the previous finding that extraction of vesicles with 600 mM potassium iodide (KI)[1] reversibly inactivated the minus-end motor (presumably by stripping cytoplasmic dynein), but did not release or inhibit the plus-end vesicle motors (53). Here, KI is used to selectively inactivate minus-end vesicles, leaving only plus-end vesicles available for purification by AMP-PNP–induced microtubule affinity. The isolated plus- and minus-end vesicle populations, in the presence of cytosol, move in opposite directions on microtubules, as would be predicted from previous observations that individual squid axon vesicles seldom, if ever, reverse, and saltatory movements are rarely seen (54).

We present evidence that the minus-end motor, cytoplasmic dynein, interacts with the lipid bilayers (36) of both plus- and minus-end vesicles, and in both cases, the dynein is capable of driving vesicle movement to microtubule minus ends. The results are consistent with the idea that the direction of vesicle movement could be regulated simply by the acquisition or loss of a tightly bound kinesin motor: because plus-end kinesin motors are processive (5, 22, 30) and tightly bound to vesicles, they can overcome the activity of cytoplasmic dynein when both motors are present on a bead or organelle surface.

1. *Abbreviations used in this paper*: AMP- PNP, 5′-adenylyl imidodiphosphate; KI, potassium iodide; V_i, vanadate ion.

Materials and Methods

Preparation of Vesicles and Cytosol from Squid Axoplasm

Squid (*Loligo pealeii*) axons were obtained from Calamari Inc. (Woods Hole, MA), and stored in liquid nitrogen. Axoplasm was extruded from thawed axons and homogenized 1:5 in "1/2 ×" motility buffer: 10 mM Hepes-KOH (pH 7.2), 175 mM L-aspartic acid, 65 mM taurine, 85 mM betaine, 25 mM glycine, 6.5 mM $MgCl_2$, 5 mM EGTA, 0.5 mM D+glucose, 1.5 mM $CaCl_2$, 1 mM DTT, 1 mM ATP, and protease inhibitors (66). To obtain cytosol and unextracted vesicles, a low speed (12,000 *g* for 8 min) S1 supernatant (67) derived from the homogenized axoplasm was layered onto a 45, 15, and 12% (wt/vol with motility buffer) sucrose density gradient and centrifuged for 90 min at 120,000 *g* (53, 55). Unextracted vesicles were recovered by side puncture of the tube. The lower half of the 15% sucrose layer and the 45/15% interface were collected. The cytosol was collected as five 100-μl fractions from the top of the tube ($S2_1$-$S2_3$ from the supernatant; $S2_4$ and $S2_5$ from the 12% sucrose layer). Unless otherwise stated, "cytosol" was a 1:1 mixture of $S2_3$ and $S2_4$, which together contained the peak activity for minus-end vesicle movement (52) (see Fig. 8 *B*).

KI-extracted vesicles were prepared (53) by first homogenizing axoplasm in motility buffer containing 600 mM KI. After 10 min, the homogenate was diluted 1:1 with motility buffer and layered onto a sucrose gradient similar to the one described above for preparing cytosol and unextracted vesicles. After centrifugation, the gradient was collected in five fractions. KI-extracted vesicles appeared as two narrow bands at the 45/15% interface and were collected together in fraction 4.

Isolation of Plus- and Minus-end Vesicles

KI-extracted vesicles were further fractionated by microtubule affinity purification to separate vesicles with active motors attached to their surfaces (plus-end vesicles) from vesicles devoid of motor activity (minus-end vesicles). The KI-extracted vesicle fraction was diluted with 1 vol motility buffer supplemented with 40 μM taxol and 10 mM AMP-PNP. Taxol-stabilized microtubules (assembled from phosphocellulose-purified bovine brain tubulin) were added to a final protein concentration of 100 μg/ml. After a 30-min incubation at room temperature, microtubules appeared saturated with bound vesicles, as determined by video-DIC microscopy. The mixture was loaded onto a 45/15/12% sucrose density gradient (made with motility buffer supplemented with taxol and AMP-PNP), and was centrifuged at 120,000 *g* for 90 min at 10°C in an ultracentrifuge (Sorvall RC 70; DuPont Co., Biotechnology Systems, Wilmington, DE). 5 × 41-mm Ultra-Clear centrifuge tubes fitted with adapters into the rotor (SW55Ti; Beckman Instruments, Inc., Palo Alto, CA) were used. The microtubule–vesicle complexes appeared as a compact band below the 45/15% interface, while free (minus-end) vesicles formed a diffuse layer above this interface. The two layers were collected separately by side puncture. To release the microtubule-bound (plus-end) vesicles, microtubule–vesicle complexes were diluted 1:10 in motility buffer containing 5 mM ATP and, to solubilize the microtubules and enhance the release of vesicles, 150–300 mM KI. The pellet was triturated 50× with a 30-gauge needle, and incubated on ice until no microtubules were detected by DIC microscopy (~30 min). Released vesicles, collected as a pellet after centrifugation (in Beckman polyallomer centrifuge tubes, 5 × 20 mm), for 45 min at 200,000 *g*) were resuspended either in motility buffer, to about one-fourth the volume of the starting KI-extracted vesicle fraction, or in sample buffer for SDS-PAGE. Some vesicle clumps could not be entirely dispersed by trituration in motility buffer, thus leading to a variable efficiency of recovery of the vesicles in the purified plus-end vesicle fraction. The free (minus-end) vesicles that failed to bind to microtubules in AMP-PNP were diluted in motility buffer, collected as a pellet after centrifugation, and resuspended in SDS-PAGE sample buffer or motility buffer as described for plus-end vesicles.

Preparation of Liposomes

The following phospholipids were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL): 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (PC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (PE), 1-palmi-

toyl-2-oleoyl-sn-glycero-3-[phospho-L-serine] (sodium salt; PS), L-α-phosphatidylinositol (liver-sodium salt; PI), and 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt; PG). A brain extract (Folch fraction I from bovine brain) containing 10–20% phosphatidylinositides, 50–60% phosphatidylserine, as well as other brain lipids, was obtained from Sigma Chemical Co. (St. Louis, MO). Liposomes were prepared as follows: 10 mg of bovine brain lipids, PC, PG, or 1:1 mixtures of PS/PC, PI/PC, and PE/PC were dissolved in 0.4 ml chloroform. After evaporation of the solvent in a stream of nitrogen gas, the dried lipids were rehydrated by vortexing in 0.4 ml motility buffer. This was followed by 10 cycles of freeze-thawing. Liposomes were produced by passing the lipid suspension 21 times through a 100-nm polycarbonate filter in a LiposoFast unit (Avestin, Inc., Ottawa, Canada).

Experimental Manipulations of the Cytosol and Vesicles

Antibody Inactivation of Kinesin. We have used the mAb CG39 (32), which is capable to bind to native squid kinesin heavy chain (65), but which has no effect on minus-end motor activity (52), to block the activity of kinesin in the bead movement assay. The antibody (ascitic fluid) was added to the cytosol nondiluted or diluted 1:10 in motility buffer (final concentration-1:4 or 1:40) before mixing with a bead suspension containing either carboxylated latex (Polysciences, Inc., Warrington, PA) or silica beads (Bangs Laboratories, Inc., Carmel, IN). At both antibody concentrations, plus-end–directed bead movement was inhibited. Similar experiments have been conducted with an affinity-purified polyclonal antibody to the squid kinesin motor domain, SK-394 (see below), and yielded comparable results.

UV-Vanadate Photocleavage of Dynein Heavy Chain. Cytosol was irradiated at 365 nm with a Spectroline lamp (model EN-280L; Spectronics, Westbury, NY) for 3 h on ice in the presence of 2 mM ATP and 20 μM vanadate (V_i; prepared from sodium orthovanadate; 19, 21, 52). After irradiation, V_i was reduced with 2 mM norepinephrine. For control cytosol, V_i was added together with the norepinephrine after UV irradiation.

Protease Treatment of Vesicles. Vesicle fractions were incubated for 30 min at room temperature with either 50 μg/ml trypsin or 330 μg/ml proteinase K. The proteolysis was stopped by the addition of soybean trypsin inhibitor (2 mg/ml) or 1 mM PMSF, respectively. After a 20-min incubation on ice, protease-treated vesicles were used in motility assays. Control samples were incubated either without proteases or with proteases and their inhibitors simultaneously.

Binding of Motor Proteins to Liposomes, Vesicles, and Beads

Flotation experiments were performed to measure the binding of kinesin and cytoplasmic dynein to vesicles and liposomes. Suspensions of liposomes or vesicles were incubated with equal volumes of axoplasmic cytosol for 1 h either on ice or at room temperature. At the end of the incubation, mixtures of cytosol with liposomes or vesicles were brought to 1.8 M sucrose in a total volume of 200 μl. Vesicles were overlaid with 200 μl of 1.4 M sucrose and 200 μl of 0.4 M sucrose. Liposomes were overlaid with 200 μl of 0.8 M sucrose and 200 μl of motility buffer. All sucrose solutions were in motility buffer. After centrifugation for 90 min at 120,000 *g*, the bottom, middle, and top layers were collected and analyzed for in vitro motility or processed for SDS-PAGE. Membranes were contained in the top fraction, which included the material that banded at the interface of the two uppermost layers. In some experiments, unextracted vesicles alone (i.e., without cytosol) were subjected to flotation, collected from the top of the tube, and diluted with motility buffer to the volume of the starting vesicle sample before being used in motility assays.

The binding of motor molecules to carboxylated silica beads was assessed as follows: 20 μl of packed beads were resuspended in 80 μl motility buffer and supplemented with 25 μl axoplasmic cytosol, with or without 10 μl CG 39 antibody (ascitic fluid). After a 30-min incubation at 4°C, beads were pelleted by centrifugation in a microfuge, washed once with motility buffer, and successively extracted with 1 M NaCl and 2× SDS sample buffer. For SDS-PAGE analysis and immunoblotting, salt and detergent extracts of beads were combined.

Motility Assays

Vesicles. Flow cells holding 2–4 μl were assembled as described (53). Taxol-stabilized, salt-washed squid microtubules (67) were introduced into the flow cell, where they adhered to the coverglass at a density of four to eight microtubules per field, as viewed by video-enhanced DIC microscopy (51). The microscope field was typically 400 μm^2 and the total length of the microtubules per field was 70–150 μm. To prevent adsorption of vesicles to the coverglass (53), the flow cell was then incubated for 5 min with 5 mg/ml casein in motility buffer containing 10 μM taxol (casein/taxol buffer). Samples of vesicle fractions were mixed with 1 vol of either casein/taxol buffer or cytosol, incubated for 3 min at room temperature, then supplemented with 2.5 mM ATP and introduced into the flow cell. Vesicle movement in one microscope field was recorded for 10 min. The flow cell was then washed with casein/taxol buffer (2 × 10 μl), and kinesin-coated beads were infused into the flow cell in the presence of ATP. These moved unidirectionally toward microtubule plus ends and so defined the polarity of the microtubules.

Beads and Liposomes. Carboxylated latex beads (0.23 μm in diameter) from a 2.5% (wt/wt) solution were diluted 1:25 in casein/taxol buffer and then 1:10 in cytosol supplemented with 2.5 mM ATP. Silica beads (0.2 μm) from a 10% (wt/wt) solution were used at final dilutions of 1:200–1:500. Liposomes, prepared as detailed above, were diluted 1:100 in casein/taxol buffer. Motility assays using beads and liposomes were performed using the procedure described above for the vesicle movement assays. For experiments that measured cytosol-dependent liposome binding to microtubules (see Fig. 4), liposomes in the presence of cytosol and 4 mM AMP-PNP were perfused into a flow cell that had been preincubated with microtubules as described above for the vesicle motility assays. After 30 min, the flow cell was washed with casein/taxol buffer and examined under the microscope to count the numbers of liposomes bound to the microtubules. At the end of the experiment, liposome movement was observed after the infusion of 2.5 mM ATP. The polarity of the microtubules was determined with kinesin-coated beads as detailed above for vesicle movement assays. In control experiments, liposomes were incubated with the microtubules in the flow cell in the presence of cytosol and ATP.

Measurements. For quantifying vesicle, liposome, or bead movement activity, or for determining the numbers of vesicles or liposomes bound to microtubules, microtubule length was measured from digitized images using image analysis software (Image 1; Universal Imaging, Media, PA). Movement activity was calculated by dividing the number of moving particles by the observation time and total microtubule length in the microscope field. Velocities were calculated from the measured distance and time between two points of uninterrupted movement on the microtubule.

The numbers of plus-end vesicles in the KI-extracted vesicle fractions were determined by counting the numbers of vesicles bound to microtubules in the presence of AMP-PNP (no cytosol). Numbers of minus-end vesicles were determined in a similar manner by incubating the unbound (minus-end) vesicles (i.e., KI-extracted vesicles depleted of plus-end vesicles) with microtubules in the presence of cytosol and AMP-PNP. Total microtubule length was computed from the known tubulin concentration added to the vesicle fractions, assuming that 1 μm of a 13-protofilament microtubule contained 3.4×10^{-21} mol of tubulin (assuming a tubulin monomer molecular mass of 55,000 D, and a center-to-center distance between monomers of 4 nm; 53). More than 90% of vesicles that bound to microtubules in AMP-PNP moved upon addition of ATP.

Immunoblotting

Samples were subjected to SDS-PAGE using 5–15% gradient gels. For general protein detection, gels were double stained with Coomassie (brilliant blue G-colloidal concentrate; Sigma Chemical Co.) and silver (40). Proteins were transferred for 3 h at room temperature onto a 0.2-μm polyvinyldene difluoride membrane using the TE70 SemiPhor Semi-Dry Blotter (Hoefer Scientific Instruments, San Francisco, CA) set at a constant current of 85 mA. Transfers were processed by antibody overlay using alkaline phosphatase–coupled secondary antibodies (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) at a dilution of 1:5,000. Antibody binding was visualized with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate. The following primary antibodies were used: CG39 is a mAb to squid kinesin heavy chain (32, 65); SK-394 (affinity purified, 0.8 μg/ml) is a polyclonal antibody raised to the NH_2-terminal 394 amino acids of squid kinesin heavy chain; JR61, provided by Dr. D.J. Asai (Purdue University, West Lafayette, IN) is a rabbit polyclonal antibody raised against a synthetic peptide derived from a conserved sequence motif of cytoplasmic dynein heavy chain (2); Cl 10.1, provided by Dr. R. Jahn (Boyer Center for Molecular Medicine, Yale University, New Haven, CT), is a monoclonal anti-synaptobrevin (VAMP) antibody (3); antibody No. 354 (address 59), raised in rabbit to a *Drosophila* α-spectrin

fusion protein (10), was provided by Dr. D. Branton (Harvard University, Cambridge, MA); anti-HIPYR antibody (polyclonal; affinity purified), raised to a synthetic peptide corresponding to a conserved region of the kinesin motor domain (50), was provided by Dr. T.J. Mitchison (University of California at San Francisco). The concentration of proteins in the vesicle fractions was determined either in motility buffer or SDS-PAGE sample buffer with the dotMETRIC protein assay (Geno Technology, Inc., St. Louis, MO).

Results

Isolation of Plus- and Minus-end Vesicle Populations

The selective inhibition of minus-end vesicle motility (53) by KI extraction suggested a strategy for isolating plus- and minus-end vesicle populations (Fig. 1). The putative plus-end vesicles were purified by incubation of KI-extracted vesicles with microtubules and AMP-PNP. The expectation was that plus-end vesicles would bind to the microtubules via AMP-PNP–induced kinesin-microtubule rigor bonds (64), while minus-end vesicles, because they had presumably been stripped of cytoplasmic dynein, would not attach to the microtubules. The resulting microtubule–vesicle complexes were separated on a sucrose density gradient from free vesicles. After collecting the vesicle-microtubule complexes, the putative plus-end vesicles were released from the microtubules by resuspension of the vesicle-microtubule complexes in buffer containing ATP and KI. The released vesicles were collected as a pellet by centrifugation and resuspended in motility buffer. In motility assays, these vesicles moved in the absence of cytosol to the plus ends of microtubules (Table I and Fig. 2). Remarkably, in the presence of cytosol capable of reconstituting the minus-end movement of the KI-extracted vesicles (53), the microtubule affinity-purified vesicles still moved exclusively in the plus-end direction (Fig. 2). We refer to these vesicles as "purified plus-end vesicles;" they are likely to constitute the anterograde vesicle population in the squid giant axon. Using a quantitative assay of vesicle movement activity (53), we estimated that up to 50% of the starting plus-end vesicle movement activity in the KI-extracted vesicles was recovered in the purified plus-end vesicle fraction (Table I).

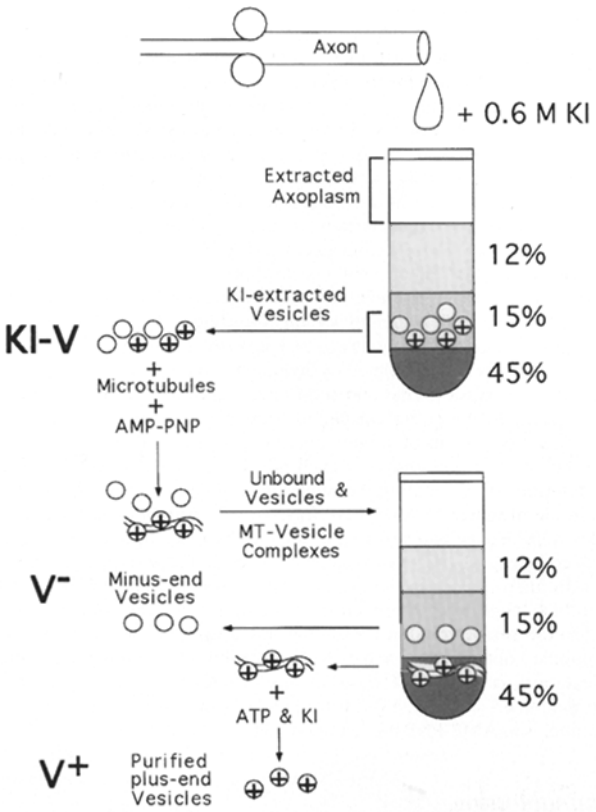

Figure 1. Diagram showing the isolation of plus- and minus–end vesicle populations from squid axons. A vesicle fraction (*KI-V*), which in the absence of cytosol showed plus end but not minus end–directed motility, was obtained from KI-extracted axoplasm by sedimentation on a sucrose density gradient (53). Incubation of KI-V with microtubules and AMP-PNP induces formation of complexes between microtubules and vesicles having active plus end–directed motors. The complexes were separated from free, nonbound vesicles by sucrose density fractionation. The microtubule-bound vesicles were released by extraction of the microtubule–vesicle complexes with ATP and KI. These purified plus-end vesicles (V^+) moved in the plus-end direction either in the presence or absence of cytosol. KI-V, which in the presence of AMP-PNP did not bind to microtubules, were completely nonmotile in the absence of cytosol, moved in the minus-end direction upon addition of cytosol, and were called minus-end vesicles (V^-). The concentration of the sucrose layers (wt/vol) is indicated on the right.

Those KI-extracted vesicles that did not bind to microtubules in the presence of AMP-PNP and that hypothetically comprised the minus-end or retrograde population were collected by centrifugation, resuspended in motility buffer containing ATP, and tested for their ability to move on microtubules in vitro. These vesicles did not move on microtubules in the absence of cytosol (Table I and Fig. 2). In the presence of cytosol, these vesicles moved exclusively toward the minus ends of microtubules (Table I and Fig. 2) despite the presence of active plus-end motors in the cytosol (see Figs. 5 and 8). Quantitative measurements of vesicle movement activity indicated that up to 75% of the minus-end vesicle movement activity detected in the KI-extracted vesicle fraction was reconstituted in the minus-end vesicle fraction obtained after the depletion of plus-end vesicles (Table I).

Table I. Effect of Cytosol on Vesicle Movement Activity

	Movement activity × 10^3 (moves per minute per micrometer microtubule)			
	No cytosol		Plus cytosol	
Vesicle fraction	Plus end	Minus end	Plus end	Minus end
KI-V	45.8	0.7	40.8	30.8
V^+	20.7	0.2	18.3	0.2
V^-	0.5	0.3	0.3	23.4

Vesicle movement activity was determined from in vitro motility assays as described under Materials and Methods. Data are from a typical experiment in which purified plus-end vesicles (V^+), minus-end vesicles (V^-), and vesicles from the parental fraction of KI-extracted vesicles (KI-V) were assayed under identical conditions. Values are adjusted to the volume of the KI-V fraction to compensate for the different resuspension volume of the purified vesicles (see Materials and Methods). The apparent reduction of plus end–directed movement activity of V^+ compared to that of KI-V is caused by the unavoidable clumping of vesicles in the pellet during the last centrifugation step of the purification procedure.

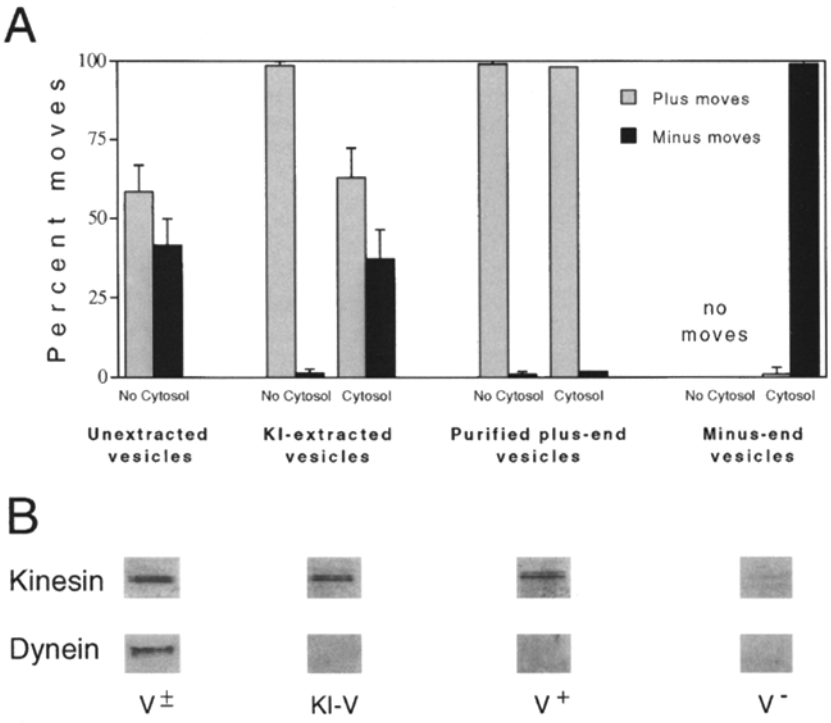

Figure 2. (*A*) Vesicles in the two isolated populations move in opposite directions on microtubules. Vesicle motility on microtubules in vitro was tested in the presence or absence of cytosol. Polarity of microtubules was determined at the end of each experiment with kinesin-coated latex beads that moved only towards the plus ends of microtubules. Plus-end and minus-end moves were expressed as the percentage of total moves. Data were derived from at least six experiments where each used an independent preparation of homogenized squid axoplasm. Error bars indicate SEM (two samples were tested in each experiment). Previous studies (53) showed that cytosol has no effect on the movement of unextracted bidirectional vesicles. (*B*) Association of kinesin and cytoplasmic dynein with the different vesicle fractions shown in *A*. Unextracted bidirectional vesicles ($V^{\pm}$), KI-extracted vesicles (*KI-V*), purified plus-end vesicles (V^{+}), and minus-end vesicles (V^{-}) were analyzed by Western blotting with anti-kinesin antibody (SK-394, *upper row*) and anti-cytoplasmic dynein antibody (JR61, *lower row*). At equal protein loads, the kinesin heavy chain doublet is detected in the V^{+} fraction, but not in the V^{-} fraction. Note that $V^{\pm}$ vesicles as a population contain both kinesin and cytoplasmic dynein, while KI-V vesicles do not contain dynein.

These isolated vesicles represent a significant fraction of the total vesicle population observed to move in extruded intact squid axoplasm. In intact squid axoplasm, 100–400 moving vesicles (anterograde and retrograde combined) are observed in a typical video field (20 μm × 20 μm × 0.1 μm, where 0.1 μm represents the depth of focus), i.e., there are 2.5–10 moving vesicles per cubic micrometer (54). From three independent preparations of axoplasm, 0.5–1.6 plus-end vesicles per cubic micrometer of axoplasm were recovered by microtubule affinity (see Materials and Methods), and >90% of these moved upon addition of ATP (see also reference 53). Similar numbers of minus-end vesicles were recovered. Therefore, at least 10% of all moving vesicles in undiluted squid axoplasm were recovered in the purified vesicle fractions.

These experiments confirm that there are distinct populations of vesicles in the axon programmed to move in opposite directions on microtubules, even in the presence of cytosol containing both plus- and minus-end motors. Somehow, the plus-end vesicles can avoid being moved by the cytosolic minus-end motor, and the minus-end vesicles can somehow discriminate between the soluble plus- and minus-end motors in the cytosol, and select only minus-end motors.

A Minus-end Motor in the Cytosol Interacts Functionally with Both Plus- and Minus-end Vesicles

One explanation for why the two vesicle populations move in opposite directions might have been that they each have receptors specific for either plus- or minus-end motors. To test whether such receptor proteins on the vesicle surface are important, we first treated the vesicles with protease and subsequently measured their ability to move on microtubules in the presence of cytosol (Fig. 3). Trypsin treatment had no significant effect on the minus-end vesicle movement activity, but completely blocked plus end–directed vesicle movement. The most remarkable result was that plus-end vesicles, treated with either trypsin (Fig. 3 *A*) or proteinase K (data not shown), now moved exclusively and with high activity to the minus ends of microtubules in the presence of cytosol. This result is striking because native, i.e., not protease-treated, purified plus-end vesicles do not normally move toward the minus ends of microtubules (Fig. 2 *A*). The ability of protease treatment to reverse the direction of movement of purified plus-end vesicles in the presence of cytosol, and the fact that cytosol-dependent, minus-end vesicle movement is insensitive to trypsin treatment, suggests that the minus-end motor, likely to be cytoplasmic dynein (see reference 52 and below), normally interacts with both plus- and minus-end vesicle populations by a mechanism that may not involve a receptor protein. It is not possible, however, to rule out alternative explanations, e.g., that protease treatment of plus-end vesicles unmasks a cryptic binding site for cytoplasmic dynein on the vesicle. This alternative interpretation is considered in more detail in the Discussion.

To explore further the possibility that a minus-end motor in squid axoplasmic cytosol can interact with lipid bilayers, we prepared liposomes of various phospholipid compositions and tested the ability of cytosol to promote their movement on microtubules in vitro. In the presence of cytosol, liposomes, regardless of their composition, moved mostly toward the minus ends of microtubules (Fig.

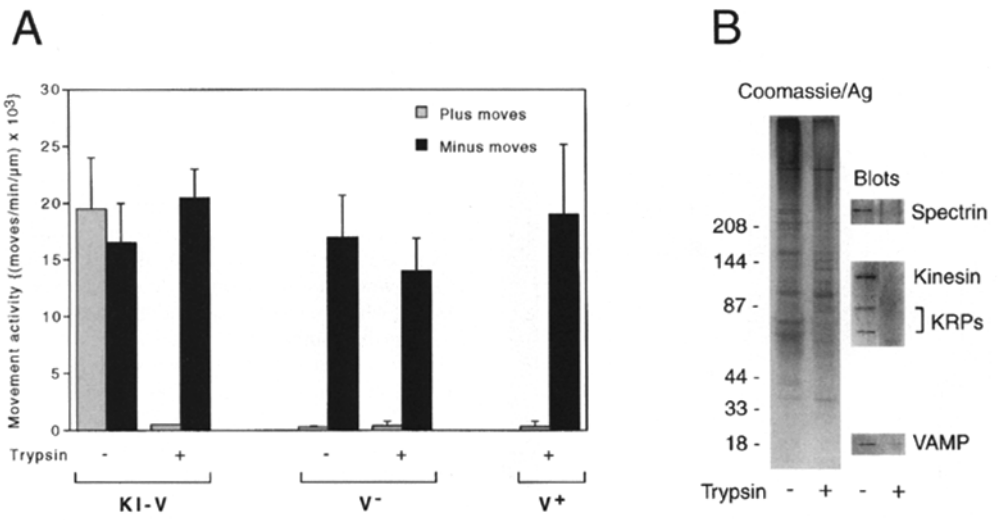

Figure 3. (*A*) Protease treatment of vesicles blocks plus-end but not minus-end movement and causes purified plus-end vesicles to move toward the minus ends of microtubules in the presence of cytosol. KI-extracted vesicles (*KI-V*), purified plus-end vesicles (V^+), or minus-end vesicles (V^-) were treated with trypsin, as described in Materials and Methods. The motility of trypsin-treated and nontreated vesicles was tested in vitro on microtubules of known polarity in the presence of cytosol. Data were derived from two (for KI-V and V^+) or four (for V^-) experiments using independent preparations of homogenized squid axoplasm. Error bars indicate SEM (two samples were tested in each experiment). (*B*) Trypsin cleaves vesicle-associated proteins. Trypsin-treated and nontreated KI-V were recovered by centrifugation and analyzed by SDS-PAGE (*Coomassie/Ag, left lanes*) and Western blotting (*Blots, right lanes*) with antibodies to α-spectrin, kinesin and kinesin-related proteins (*KRPs*, with anti-HIPYR antibody), and synaptobrevin (*VAMP*). Note that the protein profile of trypsinized vesicles, as compared to that of nonproteolyzed vesicles, shows fewer high molecular mass proteins. Among the cleaved proteins are the integral membrane protein synaptobrevin and a protein that cross-reacted with an anti-spectrin antibody. As expected from the total loss of plus-end directed motility, trypsin treatment also cleaved kinesin heavy chain as well as other vesicle-associated, kinesin-related proteins. Positions of molecular mass markers (in kilodaltons) are shown on the left.

4 *A* and Table II). Yet cytosol promoted primarily plus-end movement of latex beads (see Figs. 5 and 8 and references 52, 65). We conclude that liposomes prefer to interact with a cytosolic minus-end motor over cytosolic plus-end motors. This conclusion was further supported by the finding that purified kinesin capable of promoting robust movement of latex beads failed to promote significant movement of liposomes (data not shown).

The interaction of the minus-end motor with liposomes was highly dependent on lipid composition (Fig. 4, *B* and *C*). This dependence was investigated in a quantitative microtubule binding and motility assay. Microtubules were perfused into a microscopy flow cell, where they adhered to the coverglass. Subsequently, liposomes were incubated with cytosol and perfused into the flow cell in the presence of 4 mM AMP-PNP, conditions that cause the formation of a rigor bond between squid cytoplasmic dynein, the likely minus-end vesicle motor, and microtubules (52). The number of liposomes bound per length of microtubule was determined. Liposomes composed of more acidic phospholipids interacted more strongly with cytoplasmic dynein (Fig. 4, *B* and *C*). The strongest interaction occurred with the acidic lipid phosphatidylglycerol; however, this lipid is not normally present in eukaryotic membranes. Significant interactions were also observed with liposomes made from a mixture of phosphatidylserine and phosphatidylcholine, both of which are normal constituents of eukaryotic membranes. Liposomes prepared from a crude fraction of endogenous bovine brain lipids (containing high amounts of phosphatidylserine and phosphatidylinositides) showed high microtubule binding activity. Liposomes composed of neutral phospholipids, including phosphatidylcholine and phosphatidylethanolamine, were the least effective at promoting a functional interaction.

Two observations indicated that liposome binding to microtubules in this assay involved motors and not other types of microtubule-binding proteins. First, perfusion of ATP-containing buffer into the flow cell after liposome capture caused the liposomes to detach or move to the minus ends of microtubules. Second, liposome binding to microtubules in the presence of cytosol was nucleotide dependent; addition of 2.5 mM ATP instead of AMP-PNP to the incubation mixture dramatically reduced the accumulation of liposomes on the microtubules (Fig. 4 *B*, *right image*). Under these conditions, liposomes bound to, moved along, and dissociated from microtubules, but the encounter frequency was low.

Beads Carrying Both Kinesin and Cytoplasmic Dynein Move Primarily to the Plus Ends of Microtubules

The vesicle proteolysis studies and the liposome motility studies suggested that plus-end vesicles in the presence of cytosol carry both a tightly bound plus-end motor as well as a minus-end motor, presumably cytoplasmic dynein, whose attachment to the vesicle is salt labile. These observations raise the question of why these vesicles are normally observed to move in the plus-end, but not the minus-end direction (Fig. 2). One possibility is that the activity of the minus-end motor is normally inhibited by a posttranslational modification to the vesicle or the motor. A second possibility is that the kinesin motor predominates because it is processive (5, 22, 30, 37); i.e., it pulls for most of its mechanochemical cycle. If the minus-end motor were endowed with a longer dwell time in a weakly bound state, then it might be possible for the plus-end kinesin motor to take over the movement of these vesicles by overriding the minus-end motor.

To test whether such an override mechanism is even possible, bead movement along microtubules of known

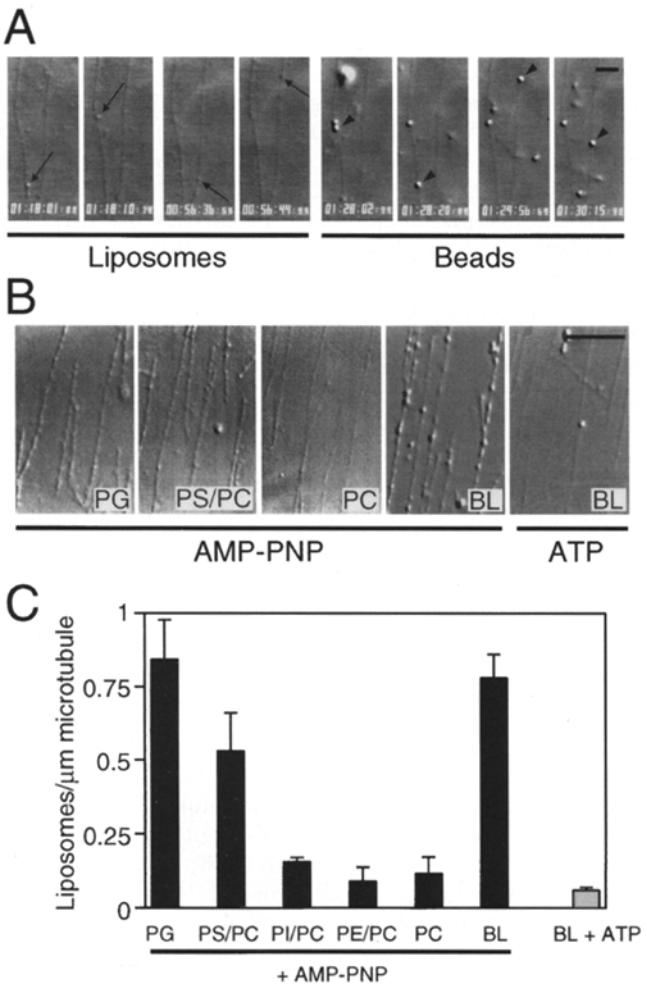

Figure 4. The movement of liposomes is driven primarily by the cytosolic minus-end motor. (*A*) Motility of phosphatidylglycerol liposomes (*arrows*, *left images*) was opposite in direction to the plus-end movement of kinesin-coated latex beads (*arrowheads*, *right images*). (*B* and *C*) Liposomes bind to microtubules in the presence of cytosol and AMP-PNP, but not ATP. Liposomes of uniform phospholipid composition (*PG*, *PS/PC*, *PI/PC*, *PE/PC*, *PC*) or prepared from bovine brain lipids (*BL*) were incubated in the flow cell with cytosol and either AMP-PNP (to induce motor protein–dependent binding of liposomes to microtubules) or ATP. *B* shows video-DIC images of liposomes bound to microtubules. Liposomes detached or moved to the minus ends of microtubules after the application of ATP. Note that, when incubated in the presence of ATP, liposomes did not bind to microtubules (right image in *B*, right column in *C*). Bars: (*A*) 2 μm; (*B*) 5 μm. PS, PI, and PE liposomes were made from a 1:1 mixture with PC.

polarity was observed in the presence of axoplasmic cytosol (pooled $S2_1$ and $S2_2$ fractions; see Materials and Methods). Previous studies have established that in axoplasmic cytosol, plus- and minus-end bead movement is driven exclusively by classical kinesin and cytoplasmic dynein, respectively (52, 65). In the present experiment, bead movement was observed to be entirely plus-end directed for both latex and silica beads (Fig. 5 *A*). It was clear, however, that the beads carried active minus-end motors because upon inhibition of the kinesin-driven movement using an mAb to squid kinesin heavy chain (32, 65), the bead movement became predominantly minus-end directed. By

Table II. Liposomes in the Presence of Cytosol Move to Microtubule Minus Ends

Liposome composition	Moves in minus-end direction	Range	Number of experiments
	%	%	
PG	94.4	93–96.1	3
PS/PC	100	–	2
PI/PC	100	–	2
PE/PC	100	–	2
PC	95.8	91.5–100	3
BL	93.6	92.9–94.7	2

The cytosol-dependent motility of liposomes of different phospholipid composition was tested on microtubules of known polarity. Each experiment was performed in duplicate using different preparations of liposomes and cytosol. While cytosol translocated latex beads primarily toward the plus ends of microtubules (see Figs. 5 and 8), liposome movement was primarily minus end directed.
PG, phosphatidylglycerol; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; BL, bovine brain lipids. PS, PI, and PE liposomes were made from a 1:1 mixture with PC.

several criteria, including sensitivity to UV-V_i, sedimentation properties, and velocity, this minus end–directed bead movement activity appeared to be driven by cytoplasmic dynein (Fig. 8 and reference 52). Thus, the cytosolic plus-end motor, kinesin, takes over bead movement activity in the presence of cytoplasmic dynein competent to drive minus-end bead movement.

This observation that kinesin takes over the direction of bead movement could have been explained in one of two ways. One possibility was that kinesin and cytoplasmic dynein compete for a limited number of binding sites on the bead surface, but kinesin binds with higher affinity; in this scenario, the anti-kinesin antibody might have impaired kinesin binding to the bead, thereby making sites available for cytoplasmic dynein. Such an explanation would neither support nor rule out an override mechanism. A second possibility was that kinesin and cytoplasmic dynein might bind independently to the bead surface; in this scenario, the presence of the anti-kinesin antibody would not affect the binding of cytoplasmic dynein to the bead. Evidence for this second possibility was obtained from measurements of the amount of kinesin and cytoplasmic dynein bound to the beads in the presence and absence of anti-kinesin antibody. Fig. 5 *B* clearly shows that the binding of kinesin to beads was unaffected by the presence of the antibody; this indicates that the effect of the antibody was to inactivate bead-bound kinesin rather than to prevent binding of kinesin to the beads. Furthermore, the presence of the anti-kinesin antibody did not affect the amount of cytoplasmic dynein bound to the bead surface (Fig. 5 *B*). Thus, it can be concluded that when both cytoplasmic dynein and kinesin are present on the same bead surface under conditions when both are available for driving bead movement, movement is taken over by kinesin. A simple explanation is that the activity of kinesin overrides dynein because of differences in their duty cycles.

We attempted to reproduce this antibody inhibition experiment using purified plus-end vesicles, asking whether such vesicles would reverse their direction of movement in the presence of cytosol and a function-blocking anti-kinesin antibody. Unfortunately, it was not possible to perform this critical experiment because existing antibodies did not inhibit the movement of purified plus-end vesicles in vitro.

A

	Movement activity x 10^3 (Moves per min per μm microtubule)	
	Cytosol	Cytosol + anti-Kinesin
Plus-end moves	107.8 (28.3)	14.8 (4.0)
Minus-end moves	0 (0)	99.4 (23.5)

B

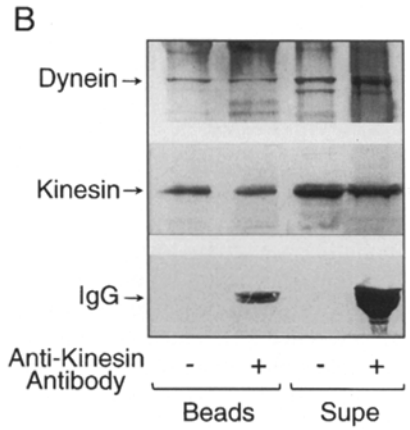

Figure 5. Kinesin overrides dynein when both motors are bound to latex or silica beads. (*A*) Direction of bead movement promoted by cytosol with and without anti-kinesin antibody. The direction of movement of carboxylated latex or glass (values in parentheses) beads was tested on microtubules of known polarity in the presence of axoplasmic supernatant (combined top cytosol fractions $S2_1$ and $S2_2$; see Materials and Methods) with or without a monoclonal anti-kinesin antibody, CG39, which binds to native kinesin heavy chain from squid (32, 65). In the absence of antibody, 100% of the beads moved toward microtubule plus ends; in the presence of antibody, beads moved primarily to microtubule minus ends. (*B*) The anti-kinesin antibody CG39 does not affect the amount of dynein or kinesin bound to beads. Beads were incubated with cytosol plus or minus CG39 antibody, and were subsequently pelleted and washed with motility buffer. Proteins adsorbed to the beads were solubilized successively in high salt and detergent, and were analyzed for cytoplasmic dynein by SDS-PAGE (*upper lane*; see Fig. 6) and for kinesin by Western blotting with a polyclonal anti-kinesin antibody (SK-394, *middle lane*). Mouse IgG was detected with an alkaline phosphatase–labeled goat anti–mouse IgG + IgM (H + L) antibody (*lower lane*). Unbound cytosolic motors and mouse IgG are shown at right (*Supe*). The presence of the CG39 antibody (*IgG*) on the beads is consistent with the idea that this antibody inhibits plus-end bead movement by binding to bead-associated kinesin.

Additional antibodies to the squid kinesin motor domain were raised, but these also failed to inhibit vesicle movement in a convincing way.

Kinesin Is Preferentially Associated with Plus-end Vesicles

The results presented thus far, based mainly on motility assays of the purified vesicle populations, are consistent with the idea that a plus-end kinesin motor binds tightly to only plus-end vesicles, while the minus-end motor binds to both vesicle populations via an interaction with acidic phospholipids. It was logical to ask whether the localization or binding of any known axoplasmic motor proteins to the two vesicle populations is consistent with this interpretation of the results. Using a biochemical approach, we tested the hypothesis that kinesin is tightly bound only to plus-end vesicles, while cytoplasmic dynein binds to both vesicle populations. The experiments were focused on kinesin and cytoplasmic dynein because they are the most abundant and best characterized microtubule motors in squid axoplasm.

It was reported previously that kinesin copurified with KI-extracted vesicles (53). Here, it was asked whether this tightly bound kinesin is present only in the plus-end vesicle population or in both the plus- and minus-end populations. When these two vesicle fractions are loaded on a gel at equal protein concentrations, kinesin is associated primarily with the purified plus-end vesicle fraction, not the minus-end vesicle fraction (Fig. 2 *B*). The interpretation of this result depends, however, on whether the two fractions contained similar amounts of functional vesicles, not just protein. We therefore determined the relative amounts of vesicles in the two fractions by incubating each at the same protein concentration with microtubules and AMP-PNP. Incubations were performed in the presence of cytosol, which had no effect on plus-end vesicle binding, but was required for minus-end vesicle binding to microtubules. Using video-DIC microscopy, we found that the numbers of vesicles bound per length of microtubule was greater for the minus-end vesicle fractions than for the purified plus-end vesicle fractions (data not shown) confirming that only the plus-end vesicles contain tightly bound kinesin. The possibility that the particles that decorate the microtubules were not vesicles but protein aggregates was ruled out by the observation that these particles were solubilized by 1% Triton X-100, and by the finding that the tightly bound kinesin copurifies with the vesicles during flotation experiments (see below and Fig. 7 *A*).

Classical kinesin is unlikely to be the only plus-end motor in squid axoplasm. In addition, it is possible that those vesicles that carry tightly bound kinesin represent only a subpopulation of the purified plus-end vesicle fraction. Recently, we identified several kinesin-related proteins, defined by cross-reactivity with pan-kinesin antibodies and nucleotide-dependent binding to microtubules (Muresan, V., and B.J. Schnapp, unpublished results), which like classical kinesin, remain associated with vesicles upon KI extraction (see Fig. 3 *B*). The question of whether these other kinesin motors are likewise restricted to the purified plus-end vesicle population is under investigation.

In summary, these experiments suggest that minus-end vesicles lack a tightly bound kinesin, consistent with models that invoke specific targeting of plus-end motors to anterograde but not retrograde vesicles. The next question we wished to address was whether the presumed minus-end vesicle motor, cytoplasmic dynein, interacts preferentially with the minus-end vesicles or equally well with both plus- and minus-end populations.

Binding of Cytoplasmic Dynein to Isolated Vesicles and Liposomes

Initial experiments indicated that Western blotting was

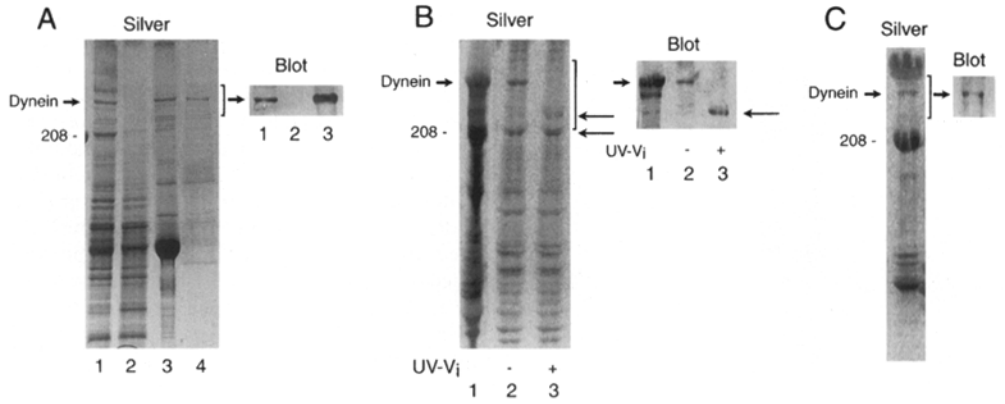

Figure 6. Use of Coomassie and silver staining to detect cytoplasmic dynein in squid axoplasmic cytosol and unextracted vesicles. The protein band presumed to be cytoplasmic dynein heavy chain (*short arrows*) in axoplasmic cytosol (*A* and *B*) and unextracted vesicle fractions (*C*) binds to microtubules in a nucleotide-dependent manner (*A*), is cleaved by UV-V_i treatment (*B*), and cross-reacts with a polyclonal antibody to a conserved domain of cytoplasmic dynein heavy chain (JR61; *A–C*). (*A*) Nucleotide-dependent microtubule binding of cytoplasmic dynein. Axoplasmic cytosol (combined fractions $S2_3$ and $S2_4$; lane 1) was incubated with microtubules in the presence of AMP-PNP (52). Microtubules with bound proteins (lane *3*) were separated from unbound proteins (lane *2*) by centrifugation, and were extracted with ATP. Released proteins are shown in lane *4*. The blot at right, probed with an anti-cytoplasmic dynein antibody (JR61), identifies immunologically the high molecular mass protein that binds to microtubules as cytoplasmic dynein heavy chain. (*B*) UV-V_i cleavage of cytoplasmic dynein. Axoplasmic cytosol was UV-irradiated in the absence (lane *2*) or presence (lane *3*) of 20 μM V_i and analyzed by SDS-PAGE (Coomassie and silver staining of the transferred gel; *left lanes*) or Western blotting for cytoplasmic dynein (JR61 antibody, *right lanes*). An ATP release of bovine brain proteins bound to microtubules in the absence of nucleotides, containing cytoplasmic dynein, is shown in lane *1*. Note that squid cytoplasmic dynein heavy chain is cleaved into two fragments (*long arrows*; see also Fig. 7 *D*), the larger fragment being still recognized by the anti-dynein antibody (*long arrow in blot*). The smaller cleavage fragment is obscured by a comigrating protein of molecular mass ~200 kD. (*C*) Cytoplasmic dynein heavy chain is detected in gels stained with Coomassie and silver (*left lane*) and immunoblot (*right lane*, JR61 antibody) of unextracted vesicles. In all figures, the immunoblots correspond to the marked area (]) of the gel. The position of the molecular mass marker of 208 kD is shown on the left.

not sufficiently sensitive to measure the amount of cytoplasmic dynein that rebinds to vesicles. It was possible, however, to definitively identify cytoplasmic dynein heavy chain directly in SDS-polyacrylamide gels stained with Coomassie and silver (Fig. 6).

Since purified cytoplasmic dynein is unable to reconstitute minus-end vesicle movement in squid axons (52), presumably because additional cytosolic factors, e.g., the dynactin complex (20), are required, it was necessary to examine the binding of cytoplasmic dynein from the cytosol to the vesicle fractions. Vesicles were incubated with cytosol and then separated from soluble, unbound proteins by flotation on sucrose gradients.

This method was first tested with the mixed population of KI-extracted vesicles (Fig. 7 *A*). Vesicle-associated kinesin and the vesicle marker synaptobrevin, i.e., VAMP (3, 58), were recovered in the upper fraction, indicating that the flotation assay worked as expected. In addition, cytoplasmic dynein was detected in the upper fraction (Fig. 7 *A*). Because cytoplasmic dynein was not detected in the upper fractions when cytosol alone (i.e., in the absence of KI-extracted vesicles) was centrifuged (Fig. 7 *C*), it can be concluded that some KI-extracted vesicles had rebound this motor from the cytosol and carried it to the upper layer. To determine which population(s) in the KI-extracted vesicle fraction bound cytoplasmic dynein from cytosol, similar flotation experiments were performed on the purified vesicle populations (Fig. 7 *B*). Cytoplasmic dynein was carried into the upper layer of the gradients by flotation when either plus- or minus-end populations were incubated in the presence of cytosol, indicating that cytoplasmic dynein binds to both purified plus-end and minus-end vesicles. To determine whether this ability of KI-extracted vesicles to rebind cytoplasmic dynein from cytosol involved membrane proteins, trypsin-treated, KI-extracted vesicles were incubated with cytosol and subjected to flotation (Fig. 7 *C*). Again, cytoplasmic dynein cofractionated with the vesicles in the upper fraction (Fig. 7 *C*), indicating that binding of the cytoplasmic dynein from cytosol is insensitive to protease treatment. Taken together, the results of these biochemical experiments are consistent with the data from in vitro motility assays; both suggest that cytoplasmic dynein interacts with plus- and minus-end vesicles by a lipid-binding mechanism.

Similar biochemical experiments were performed to determine directly whether cytoplasmic dynein from cytosol binds to liposomes. Liposomes prepared from bovine brain lipids (Fig. 7 *C*) or from phosphatidylglycerol (data not shown) were incubated with cytosol and subsequently separated from unbound proteins by flotation. A substantial fraction of cytoplasmic dynein was recovered with the liposomes (Fig. 7 *C*). These experiments are consistent with earlier reports that purified cytoplasmic dynein binds to acidic liposomes (36). In the present case, however, preliminary experiments (Muresan, V., and B.J. Schnapp, unpublished observations) indicate that other cytosolic factors, including components of the dynactin complex, also bind to the liposomes. The participation of these and other cytosolic components in the docking of cytoplasmic dynein to vesicles and liposomes is currently under investigation, but is outside the scope of the present report.

Several lines of evidence indicated that cytoplasmic dynein is the motor that drives the minus end–directed movement of liposomes, minus-end vesicles, and protease-treated plus-end vesicles. First, such minus-end motility was blocked by UV-V_i treatment of the cytosol (Fig. 8 *A*),

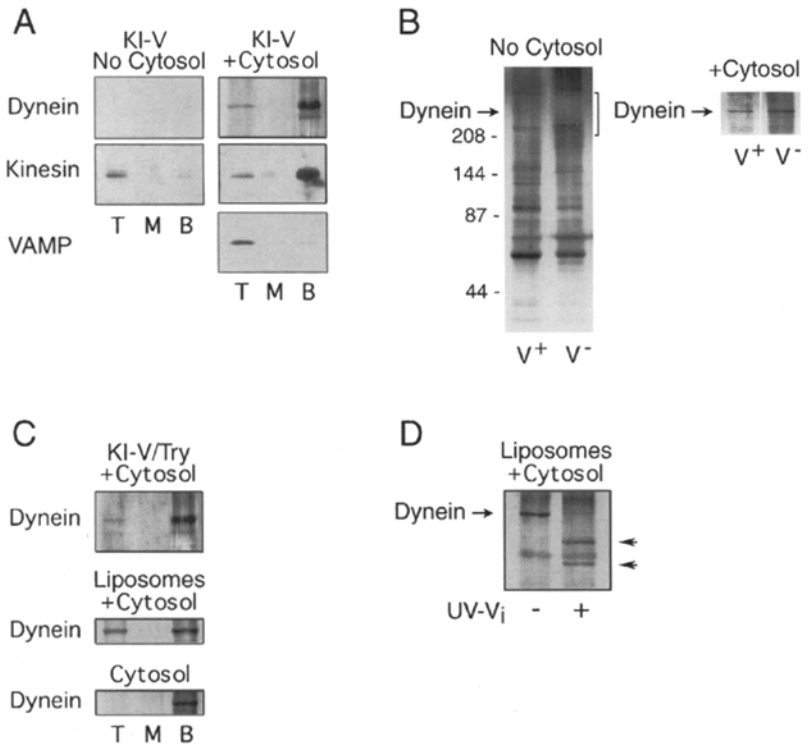

Figure 7. The minus end–directed motor, cytoplasmic dynein, binds to plus- and minus-end vesicles, trypsin-treated vesicles, and liposomes. (*A*) Interaction of kinesin and dynein with KI-extracted vesicles (*KI-V*). KI-extracted vesicles were incubated without and with cytosol before separation of vesicles from unbound proteins by flotation on sucrose density gradients. Top (*T*), middle (*M*), and bottom (*B*) fractions were collected. In each fraction, cytoplasmic dynein was detected in gels stained with Coomassie and silver, as shown in Fig. 6; kinesin heavy chain was detected by Western blotting using a polyclonal antibody (SK-394) to squid kinesin heavy chain; vesicles were identified by Western blotting using an antibody against the integral membrane protein synaptobrevin (*VAMP*). In the absence of cytosol, cytoplasmic dynein is not evident on KI-extracted vesicles, while kinesin heavy chain in the KI-extracted vesicle fraction is carried quantitatively to the upper fraction of the gradient, indicating that it is membrane bound. When flotation is performed on KI-extracted vesicles incubated with cytosol, some dynein is carried to the top layer in association with the vesicles. VAMP staining indicates that secretory vesicles are quantitatively recovered in the upper fraction by this method. (*B*) Plus (V^+) and minus-end (V^-) vesicles, which differ in their general protein composition and do not contain dynein in the absence of cytosol (*left lanes*), bind cytoplasmic dynein from the cytosol (*right lanes*). The gel at right corresponds to the marked area (]) of the gel at the left. Only the top fractions (*T*) are shown. Positions of molecular mass markers (in kilodaltons) are indicated on the left. (*C*) Cytoplasmic dynein from cytosol cofractionates with trypsin-treated KI-extracted vesicles (*top panel*) and liposomes prepared from bovine brain lipids (*middle panel*). Cytoplasmic dynein remains in the bottom fraction when flotation is performed on cytosol in the absence of vesicles or liposomes (*lower panel*). (*D*) Liposomes were separated by flotation after incubation with cytosol that was UV-irradiated in the presence or absence of V_i. Note that the liposome-associated protein presumed to be cytoplasmic dynein is cleaved by UV-V_i treatment into two fragments (*arrowheads*), confirming its identity as cytoplasmic dynein heavy chain. Only the top fractions (*T*) are shown.

a procedure that cleaves and inactivates cytoplasmic dynein (19) and blocks minus-end motility of KI-extracted vesicles (52). UV-V_i treatment had no effect on plus end–directed bead movement (Fig. 8 *A*), suggesting that this treatment was specific for the minus-end motor activity (52). Second, a direct correlation was found between the movement activity of liposomes and the amount of dynein that sediments into the upper and lower cytosol fractions (Fig. 8 *B*; see Materials and Methods and reference 52). Third, all minus end–moving objects discussed in this paper moved at the same velocity of 1.2–1.5 μm/s (data not shown), suggesting that all are powered by a minus-end motor with the same velocity as cytoplasmic dynein (52).

The flotation experiments with KI-extracted vesicles and liposomes showed that some cytoplasmic dynein copurifies with the membranes (Fig. 7). The following experiments indicated that vesicle-associated cytoplasmic dynein rapidly exchanges with free, unbound dynein, and that the constant presence of cytosolic factors is necessary to sustain movement.

KI-extracted vesicles and liposomes recovered by flotation after incubation in cytosol (Fig. 7) had significantly less minus-end movement activity than they had in the presence of cytosol before flotation (data not shown). A similar reduction in minus-end movement activity after flotation was observed for unextracted vesicles (Table III), which contain cytoplasmic dynein and move potently to microtubule minus ends in the absence of cytosol (Fig. 2). These reductions in the minus-end vesicle movement activity are unlikely to have been caused by loss or inhibition of vesicles during flotation, since (*a*) the activity was restored by the addition of cytosol to the vesicles after flotation (Table III); and (*b*) plus-end vesicle movement activity of vesicles that were collected in upper fractions after flotation was not significantly diminished (Table III). These results indicate that the lifetime of the dynein—vesicle complex is short compared to the interaction of vesicles with the plus-end motor.

Discussion

This paper reports the isolation of plus- and minus-end vesicle populations from squid axoplasm, as well as analyses of their motile properties and their interactions with motors. The strategy used to isolate these vesicle populations emerged from the recognition (53) that only minus-end, but not plus-end, vesicle movement depended on the continuous presence of cytosol. In the present paper, this difference in cytosol dependency was exploited to purify the plus-end vesicle population by microtubule affinity. The results indicate that the isolated populations retain their directional specificity in vitro. Thus, one population

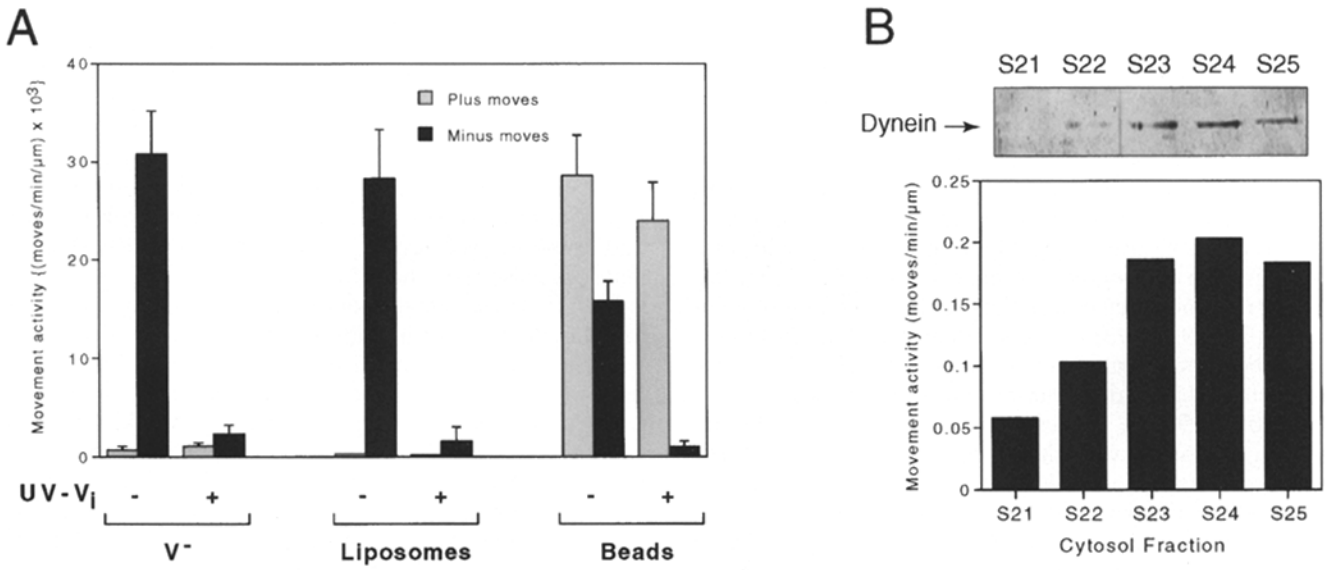

Figure 8. Evidence that cytoplasmic dynein is the main motor for minus-end movement of vesicles and liposomes. (*A*) UV-V_i treatment of cytosol blocks motility of minus-end vesicles, liposomes, and latex beads. Motility was tested in vitro on microtubules of known polarity in the presence of cytosol that had been UV-irradiated with or without V_i. Minus-end movement of vesicles, liposomes, and beads was inhibited by UV-V_i treatment of cytosol, but plus-end directed motility of beads was not significantly changed. Data were derived from two experiments using independent preparations of homogenized squid axoplasm. Error bars indicate SEM (two samples were tested in each experiment). (*B*) Cytosolic fractions containing increasing amounts of cytoplasmic dynein promote increasing levels of liposome movement activity. Squid axoplasm was homogenized and fractionated by centrifugation over a sucrose density gradient. Fractions ($S2_1$–$S2_5$; see Materials and Methods) were collected from the top of the tube down to the 15% sucrose layer of the gradient, and were analyzed by Western blotting for the presence of cytoplasmic dynein (JR61 antibody, *upper lane*) and for their ability to promote in vitro motility of phosphatidylglycerol liposomes (histogram). Only minus-end movement is shown. In no case were plus-end moves >5% of the total.

moves exclusively to the plus ends of microtubules and the other goes to the minus ends, even in the presence of cytosol containing soluble motors of both polarities. Because microtubules in squid axons are uniformly oriented (71), it is likely that the plus- and minus-end vesicles correspond to anterograde and retrograde vesicle populations in situ. The availability of these two vesicle fractions enables the identification of factors that regulate the direction of vesicle movement.

Table III. Minus End–directed Motility Is Reversibly Lost during Flotation of Squid Axon Vesicles

Movement activity × 10^3 (moves per minute per micrometer microtubule)					
Before flotation		After flotation			
No cytosol		No cytosol		Plus cytosol	
Plus end	Minus end*	Plus end	Minus end	Plus end	Minus end
69 ± 10	132 ± 8	48 ± 13	21 ± 5	47 ± 11	93 ± 24

Unextracted vesicles ($V^{\pm}$ in Fig. 2) obtained by sedimentation on a sucrose density gradient were separated by flotation from weakly bound proteins that cosedimented with the vesicles during the first centrifugation. The motility of vesicles before and after flotation was tested in vitro on microtubules of known polarity in the absence or presence of cytosol. The minus end–directed motility of the vesicles was markedly reduced after flotation, and this reduction correlated with a loss of vesicle-bound cytoplasmic dynein (data not shown). Minus-end vesicle motility was largely restored by the addition of cytosol. Data were derived from two experiments using independent preparations of homogenized squid axoplasm. Values show mean ± SEM (two samples were tested in each experiment). Differences in plus end–directed motility between the three experimental conditions are not significant.

*Note that in these experiments, the preferred direction of movement of unextracted vesicles was toward microtubule minus ends. In most cases, plus-end moves were predominant (see Fig. 2 *A*).

Evidence is presented that plus-end vesicles from squid axons have tightly bound kinesin motors, but also associate with cytoplasmic dynein via a weak, salt labile interaction that allows for free exchange with the soluble pool of dynein. This interaction of plus-end vesicles with cytoplasmic dynein was documented in two independent approaches: (*a*) in motility assays by the observation that after proteolysis, plus-end vesicles, in cytosol, move to the minus ends of microtubules (Fig. 3); and (*b*) in biochemical experiments that directly demonstrate the binding of dynein from cytosol to plus- and minus-end vesicles (Fig. 7). The additional observation that kinesin overrides cytoplasmic dynein (Fig. 5) when both motors are bound to beads raises the possibility that a similar override mechanism might explain why minus-end movement of plus-end vesicles is not normally observed (Fig. 2 and Table I). This idea would suggest that for squid axoplasmic vesicles, the direction of vesicle movement may be regulated by the presence or absence of a functional plus-end kinesin motor.

Tight Binding of Plus-end Kinesin Motors to Plus-end Vesicles

It was shown previously that a small fraction of the total axoplasmic kinesin heavy chain copurified with KI-extracted vesicles (53) and was not extracted with either carbonate buffer at pH 11.3, 1 M KI, or 1 M NaCl. Here, it is shown that this tightly bound kinesin is associated exclusively

with the plus-end vesicles (Fig. 2 *B*). Preliminary results indicate that in addition to kinesin heavy chain, other kinesin-related proteins cosediment with KI-extracted vesicles. This suggests that the purified plus-end vesicle fraction might consist of subpopulations of vesicles, each of which carries a distinct, tightly bound, kinesin-related motor. The idea that different kinesin motors interact with distinct vesicle populations has been suggested from recent studies in the mouse of novel kinesin-related proteins that participate in anterograde axonal transport (31, 41, 44, 45).

Several groups have investigated the rebinding of soluble native (49, 57, 73) or recombinant (57) kinesin to salt- or carbonate-extracted microsomal membrane fractions. Such studies indicate saturable binding with a K_d in the range of 10–50 nM, suggesting that the membrane-bound kinesin exchanges with the soluble pool. How can these measurements be reconciled with the tight binding we observe with squid axon vesicles? It is possible that there are two modes of binding. The exchangeable binding measured in the rebinding experiments may serve to recruit kinesin motors to the vesicle surface, while a separate mechanism makes the interaction permanent. A second possibility is that the different conclusions reflect differences in the vesicle fractions that were investigated. The rebinding studies examined crude membrane fractions that were likely to contain ER, Golgi, constitutive and regulated secretory vesicles, and compartments of the endocytic pathway. Our studies focus on two relatively homogenous vesicle populations. A third possibility is suggested by the fact that the kinesin heavy chain can presumably interact with a diverse population of alternatively spliced light chain isoforms (4, 13). Thus, there potentially exists a large number of different kinesin heavy chain motors, each distinguished by a specific set of light chain isoforms. It is possible that the tight-binding mechanism applies to only a subset of the native kinesin motors in the cell. Consistent with this proposal is that the kinesin which we observe to be tightly associated with the purified plus-end vesicles is a small fraction (~1%) of the total axoplasmic kinesin heavy chain, most of which is soluble (53).

The Interaction of Cytoplasmic Dynein with Lipid Bilayers

The evidence presented in this and previous papers (11, 47, 52, 56, 62) suggests that cytoplasmic dynein is the main motor for minus-end vesicle movement, although the limitations of in vitro motility assays and the lack of specific, high affinity antibodies still leave open the question of whether cytoplasmic dyneins are the only minus-end vesicle motors. Recent studies (35, 36) provided evidence that purified cytoplasmic dynein exhibits saturable binding, with a K_d in the range of 20–80 nM, to liposomes containing acidic phospholipids or to isolated synaptic vesicles that have been stripped of their membrane proteins. This binding involves electrostatic interactions, as determined by its sensitivity to NaCl, and by the dependence of binding on the acidity of the phospholipid head group. These earlier studies, however, did not resolve two key questions: Does dynein promote movement of liposomes? Is this interaction of physiological significance, i.e., does it occur within the natural environment of the cell?

The results reported here address these two issues by showing that cytosol promotes the minus end–directed movement of acidic liposomes (Fig. 4 and Table II) or protease-treated plus- and minus-end vesicles (Fig. 3). Although the possibility still exists that another, yet undiscovered minus-end motor(s) is present in squid axoplasm, the results of several experiments are consistent with the idea that most of the minus-end motility of both vesicles and liposomes is caused by cytoplasmic dynein(s) (Figs. 7 and 8; see also reference 52).

Motility assays suggesting that cytoplasmic dynein interacts functionally with both populations of vesicles and with liposomes were confirmed by biochemical measurements (Fig. 7). These biochemical experiments also indicated that dynein binding to vesicles is weak. Previous studies have shown that the dynein–lipid interaction is salt labile (36). Since the squid motility buffer used in our experiments contains >250 mM salt, it is not surprising that significant amounts of both the vesicle-associated cytoplasmic dynein and the minus-end vesicle movement activity were lost during flotation of unextracted vesicles. It can be inferred that under the high ionic strength conditions that are present in squid axoplasm, soluble dynein, and possibly other components of the minus-end motor machinery (e.g., the dynactin complex; 20), exchange rapidly with the vesicle-bound motor, and the constant presence of the cytosolic minus-end motor is necessary to sustain movement.

The in vitro studies reported here indicate that the strength of the interaction between cytoplasmic dynein and lipid membranes depends on the acidity of the phospholipid head group. It is possible that this dependency could act in vivo to selectively target dynein or its docking partners to specific membrane compartments. For example, in the mammalian liver, the acidic lipid phosphatidylserine is located on the cytoplasmic surface of the membrane bilayer (70) and is concentrated in the plasma membrane, Golgi, and endocytic organelles, but less so in the ER (70). It is also notable that lipids synthesized in phosphatidylinositol 3-kinase signaling pathways, e.g., PIP, PIP_2 and PIP_3, are among the most highly charged lipids in the cell, and that activation of this pathway induces the retrograde delivery of membranes (9, 29, 39).

While there is now substantial data that certain motors, e.g., cytoplasmic dynein and myosin-I (17, 36), bind directly to lipid bilayers in vitro, it is still unresolved whether these interactions are physiologically relevant or whether these interactions are one component in a hierarchy of interactions that dock the motor to membranes. In the case of cytoplasmic dynein, it will be particularly important to establish whether components of the dynactin complex also bind, directly or indirectly, to lipid bilayers and to proteolyzed vesicles. The experiments reported here leave open the possibility that the observed interactions of cytoplasmic dynein with plus-end vesicles, proteolyzed vesicles, and liposomes involve other soluble components, including the dynactin complex.

Regulation of the Direction of Vesicle Movement in the Squid Giant Axon

Evidence presented here indicates that the minus-end mo-

tor, cytoplasmic dynein, does not discriminate between plus- and minus-end vesicles and binds to both vesicle populations in an active form. The focus of regulation appears to be the plus-end kinesin motor. This idea is generally consistent with other studies. Data of ultrastructural cytochemistry from axons of rat and mouse peripheral neurons have shown that cytoplasmic dynein is localized on both anterogradely and retrogradely transported organelles in vivo (27; see also reference 15), while kinesin is associated only with anterogradely moving organelles (26). These findings, like those reported here, suggest that the membrane association of kinesin is regulated. In contrast, other studies have shown that in fish melanophores, where pigment granules are transported in unison, both dynein and kinesin were associated with the granules during anterograde and retrograde transport cycles (43), and it was proposed that the activity of the permanently bound kinesin is regulated (24). Immunocytochemical studies of vesicles cycling constitutively and bidirectionally between the ER and Golgi complex in mammalian cells in culture showed that these vesicles are always associated with kinesin (38), implying that kinesin must be inactivated during organelle movement toward microtubule minus ends.

One way that the presence of a tightly bound kinesin motor could determine the direction of vesicle movement is by overriding the activity of vesicle-associated dynein. There are two factors, discussed below, that could enable kinesin to take over the movement of vesicles by overriding the activity of cytoplasmic dynein: (*a*) differences in the duty ratios of these two motors; and (*b*) differences in the affinity of these two motors for vesicle membranes.

Bead movement experiments (Fig. 5) indicate that under certain conditions, the kinesin motor can override the dynein motor when both are present on the same surface. A reasonable explanation for this behavior is that compared to dynein, the kinesin motor has a higher duty ratio; i.e., it spends a greater fraction of its cycle time in a strongly bound, force-generating state. Although the high processivity of the kinesin motor is well established (5, 22, 30, 37), the mechanics of dynein-driven movement is largely unknown. Certain properties of dynein-driven movement suggest that it is less processive than kinesin. First, vesicles moving to the plus-ends of microtubules virtually never pause or dissociate before reaching the end of the microtubule; minus end–directed movement of organelles and liposomes is jittery, and dissociation from the microtubule is common. Second, cytoplasmic dynein wanders over the surface of the microtubule (72) while kinesin tracks along single protofilaments (18, 48). Although cytoplasmic dynein at limiting dilution was reported to promote continuous movement of microtubules for long distances (63), the small diffusion constant of microtubules could have obscured the existence of prolonged release events during movement (5, 30). Previous studies using a microtubule gliding assay showed that a single kinesin can overcome as many as 10 dyneins (63). On a 0.5-μm vesicle or bead, it would not be possible for more than 10 motors to interact simultaneously with the microtubule surface (7); these geometric constraints would therefore prevent dyneins from overcoming kinesins if both motors were uniformly bound to the bead at comparable densities.

Unlike kinesin motors, which are bound tightly to squid axon plus-end vesicles (Figs. 2 and 3 and reference 53), the binding of cytoplasmic dynein to vesicles or liposomes is weak and salt labile (Fig. 7 and Table III). This weak binding implies a substantial off-rate, and this is confirmed by flotation studies that show that vesicles lose up to 80% of their minus-end movement activity during a 90-min flotation experiment (Table III). This short lifetime of the dynein–vesicle complex would be expected to decrease further the processivity of dynein-driven vesicle movement, and may contribute to the ability of plus-end motors to control the movement of plus-end vesicles in squid axons despite the presence of cytoplasmic dynein on these vesicles.

An override mechanism is only one of several explanations that could account for the observation that protease treatment of purified plus-end vesicles from squid axons causes these vesicles to move toward microtubule minus ends in the presence of cytosol. The most obvious alternative possibility is that the cytoplasmic dynein that rebinds to purified plus-end vesicles (Fig. 7) is inactive; trypsin treatment may expose a cryptic dynein-binding site and/or a vesicle-associated, dynein-activating mechanism that is normally masked on the surfaces of native plus-end vesicles. Such an explanation accommodates better the expectation that membrane binding and activation will be mediated by posttranslational events (such as phosphorylation) of the motors themselves, of their membrane receptors, or of accessory factors (1). There is evidence that phosphorylation of cytoplasmic dynein subunits modulates the association of this motor with membranes in a cell cycle–dependent manner (42). In other studies, differential phosphorylation of dynein polypeptides was observed between the total cellular pool of cytoplasmic dynein and the pool that was being transported anterogradely with organelles in rat optic nerves. It was proposed that this differential phosphorylation inactivates organelle-bound dynein during fast anterograde axonal transport (15).

At the present time, there is insufficient data to distinguish between these different models that have been proposed to explain how the direction of organelle movement is regulated. It is also possible that different mechanisms will operate in different situations. The override model proposed here, while just a hypothesis, makes clear predictions that can be tested. In future work, it will be necessary to verify at an ultrastructural level the dual presence of cytoplasmic dynein and kinesin on individual organelles in vivo and in vitro. High resolution tracking of vesicle motility will be required to detect whether a vesicle moving in the plus-end direction also contains an active minus-end motor (59). Elucidating the nature of the linkage between motors and membranes is also a clear priority. Although the interaction of kinesin and cytoplasmic dynein with target membranes is thought to be mediated by specific motor-docking proteins (69), there is only one candidate for such a protein, kinectin (34, 60), and its function is still uncertain.

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References

1. Allan, V. 1995. Membrane traffic motors. *FEBS Lett.* 369:101–106.
2. Asai, D.J., S.M. Beckwith, K.A. Kandl, H.H. Keating, H. Tjandra, and J.D. Forney. 1994. The dynein genes of *Paramecium tetraurelia.* Sequences adjacent to the catalytic P-loop identify cytoplasmic and axonemal heavy chain isoforms. *J. Cell Sci.* 107:839–847.
3. Baumert, M., P.R. Maycox, F. Navone, P. De Camilli, and R. Jahn. 1989. Synaptobrevin: an integral membrane protein of 18,000 daltons present in small synaptic vesicles of rat brain. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:379–384.
4. Beushausen, S., A. Kladakis, and H. Jaffe. 1993. Kinesin light chains: identification and characterization of a family of proteins from the optic lobe of the squid *Loligo pealeii. DNA Cell Biol.* 12:901–909.
5. Block, S.M., L.S. Goldstein, and B.J. Schnapp. 1990. Bead movement by single kinesin molecules studied with optical tweezers [see comments]. *Nature (Lond.).* 348:348–352.
6. Brady, S.T. 1985. A novel brain ATPase with properties expected for the fast axonal transport motor. *Nature (Lond.).* 317:73–75.
7. Brady, S.T. 1991. Molecular motors in the nervous system. *Neuron.* 7:521–533.
8. Brady, S.T., R.J. Lasek, and R.D. Allen. 1982. Fast axonal transport in extruded axoplasm from squid giant axon. *Science (Wash. DC).* 218:1129–1131.
9. Brown, W.J., D.B. DeWald, S.D. Emr, H. Plutner, and W.E. Balch. 1995. Role for phosphatidylinositol 3-kinase in the sorting and transport of newly synthesized lysosomal enzymes in mammalian cells. *J. Cell Biol.* 130:781–796.
10. Byers, T.J., R. Dubreuil, D. Branton, D.P. Kiehart, and L.S.B. Goldstein. 1987. *Drosophila* spectrin. II. Conserved features of the alpha-subunit are revealed by analysis of cDNA clones and fusion proteins. *J. Cell Biol.* 105:2103–2110.
11. Corthesy-Theulaz, I., A. Pauloin, and S. R. Pfeffer. 1992. Cytoplasmic dynein participates in the centrosomal localization of the Golgi complex. *J. Cell Biol.* 118:1333–1345.
12. Coy, D.L., and J. Howard. 1994. Organelle transport and sorting in axons. *Curr. Opin. Neurobiol.* 4:662–667.
13. Cyr, J.L., K.K. Pfister, G.S. Bloom, C.A. Slaughter, and S.T. Brady. 1992. Molecular genetics of kinesin light chains: generation of isoforms by alternative splicing. *Proc. Natl. Acad. Sci. USA.* 88:10114–10118.
14. Dahlstrom, A.B., A.J. Czernik, and J.Y. Li. 1992. Organelles in fast axonal transport. What molecules do they carry in anterograde vs. retrograde directions, as observed in mammalian systems? *Mol. Neurobiol.* 6:157–177.
15. Dillman, J.F.R., and K.K. Pfister. 1994. Differential phosphorylation in vivo of cytoplasmic dynein associated with anterogradely moving organelles. *J. Cell Biol.* 127:1671–1681.
16. DiStefano, P.S., B. Friedman, C. Radziejewski, C. Alexander, P. Boland, C.M. Schick, R.M. Lindsay, and S.J. Wiegand. 1992. The neurotrophins BDNF, NT-3, and NGF display distinct patterns of retrograde axonal transport in peripheral and central neurons. *Neuron.* 8:983–993.
17. Doberstein, S.K., and T.D. Pollard. 1992. Localization and specificity of the phospholipid and actin binding sites on the tail of *Acanthamoeba* myosin IC. *J. Cell Biol.* 117:1241–1249.
18. Gelles, J., B.J. Schnapp, and M.P. Sheetz. 1988. Tracking kinesin-driven movements with nanometre-scale precision. *Nature (Lond.).* 331:450–453.
19. Gibbons, I.R., M.P. Cosson, J.A. Evans, B.H. Gibbons, B. Houck, K.H. Martinson, W.S. Sale, and W.J. Tang. 1978. Potent inhibition of dynein adenosinetriphosphatase and of the motility of cilia and sperm flagella by vanadate. *Proc. Natl. Acad. Sci. USA.* 75:2220–2224.
20. Gill, S.R., T.A. Schroer, I. Szilak, E.R. Steuer, M.P. Sheetz, and D.W. Cleveland. 1991. Dynactin, a conserved, ubiquitously expressed component of an activator of vesicle motility mediated by cytoplasmic dynein. *J. Cell Biol.* 115:1639–1650.
21. Goodno, C.C. 1982. Myosin active-site trapping with vanadate ion. *Methods Enzymol.* 85:116–123.
22. Hackney, D.D. 1995. Highly processive microtubule-stimulated ATP hydrolysis by dimeric kinesin head domains. *Nature (Lond.).* 377:448–450.
23. Haimo, L.T. 1995. Regulation of kinesin directed movements. *Trends Cell Biol.* 5:165–168.
24. Haimo, L.T., and C.D. Thaler. 1994. Regulation of organelle transport: lessons from color change in fish. *BioEssays.* 16:727–733.
25. Heidemann, S.R., J.M. Landers, and M. A. Hamborg. 1981. Polarity orientation of axonal microtubules. *J. Cell Biol.* 91:661–665.
26. Hirokawa, N., R. Sato-Yoshitake, N. Kobayashi, K.K. Pfister, G.S. Bloom, and S.T. Brady. 1991. Kinesin associates with anterogradely transported membranous organelles in vivo. *J. Cell Biol.* 114:295–302.
27. Hirokawa, N., R. Sato-Yoshitake, T. Yoshida, and T. Kawashima. 1990. Brain dynein (MAP1C) localizes on both anterogradely and retrogradely transported membranous organelles in vivo. *J. Cell Biol.* 111:1027–1037.
28. Hollenbeck, P.J. 1989. The distribution, abundance and subcellular localization of kinesin. *J. Cell Biol.* 108:2335–2342.
29. Horazdovsky, B.F., D.B. DeWald, and S.D. Emr. 1995. Protein transport to the yeast vacuole. *Curr. Opin. Cell Biol.* 7:544–551.
30. Howard, J., A.J. Hudspeth, and R.D. Vale. 1989. Movement of microtubules by single kinesin molecules. *Nature (Lond.).* 342:154–158.
31. Kondo, S., R. Sato-Yoshitake, Y. Noda, H. Aizawa, T. Nakata, Y. Matsuura, and N. Hirokawa. 1994. KIF3A is a new microtubule-based anterograde motor in the nerve axon. *J. Cell Biol.* 125:1095–1107.
32. Kosik, K.S., L.D. Orecchio, B.J. Schnapp, H. Inouye, and R.L. Neve. 1990. The primary structure and analysis of the squid kinesin heavy chain. *J. Biol. Chem.* 265:3278–3283.
33. Krijnse-Locker, J., R.G. Parton, S.D. Fuller, G. Griffiths, and C.G. Dotti. 1995. The organization of the endoplasmic reticulum and the intermediate compartment in cultured rat hippocampal neurons. *Mol. Biol. Cell.* 6: 1315–1332.
34. Kumar, J., H. Yu, and M.P. Sheetz. 1995. Kinectin, an essential anchor for kinesin-driven motility. *Science (Wash. DC).* 267:1834–1837.
35. Lacey, M.L., and L.T. Haimo. 1992. Cytoplasmic dynein is a vesicle protein. *J. Biol. Chem.* 267:4793–4798.
36. Lacey, M.L., and L.T. Haimo. 1994. Cytoplasmic dynein binds to phospholipid vesicles. *Cell Motil. Cytoskeleton.* 28:205–212.
37. Leibler, S., and D.A. Huse. 1993. Porters versus rowers: a unified stochastic model of motor proteins. *J. Cell Biol.* 121:1357–1368.
38. Lippincott-Schwartz, J., N.B. Cole, A. Marotta, P.A. Conrad, and G.S. Bloom. 1995. Kinesin is the motor for microtubule-mediated Golgi-to-ER membrane traffic. *J. Cell Biol.* 128:293–306.
39. Liscovitch, M., and L.C. Cantley. 1995. Signal transduction and membrane traffic: the PITP/phosphoinositide connection. *Cell.* 81:659–662.
40. Morrissey, J.H. 1981. Silver staining of SDS gels. *Anal. Biochem.* 117:307–310.
41. Nangaku, M., R. Sato-Yoshitake, Y. Okada, Y. Noda, R. Takemura, H. Yamazaki, and N. Hirokawa. 1994. KIF1B, a novel microtubule plus end-directed monomeric motor protein for transport of mitochondria. *Cell.* 79:1209–1220.
42. Niclas, J., V.J. Allan, and R.D. Vale. 1996. Cell cycle regulation of dynein association with membranes modulates microtubule-based organelle transport. *J. Cell Biol.* 133:585–593.
43. Nilsson, H., M. Rutberg, and M. Wallin. 1996. Localization of kinesin and cytoplasmic dynein in cultured melanophores from atlantic cod, *Gadus morhua. Cell Motil. Cytoskeleton.* 33:183–196.
44. Noda, Y., R. Sato-Yoshitake, S. Kondo, M. Nangaku, and N. Hirokawa. 1995. KIF2 is a new microtubule-based anterograde motor that transports membranous organelles distinct from those carried by kinesin heavy chain or KIF3A/B. *J. Cell Biol.* 129:157–167.
45. Okada, Y., H. Yamazaki, Y. Sekine-Aizawa, and N. Hirokawa. 1995. The neuron-specific kinesin superfamily protein KIF1A is a unique monomeric motor for anterograde axonal transport of synaptic vesicle precursors. *Cell.* 81:769–780.
46. Parton, R.G., K. Simons, and C.G. Dotti. 1992. Axonal and dendritic endocytic pathways in cultured neurons. *J. Cell Biol.* 119:123–137.
47. Paschal, B.M., and R.B. Vallee. 1987. Retrograde transport by the microtubule-associated protein MAP 1C. *Nature (Lond.).* 330:181–183.
48. Ray, S., E. Meyhofer, R.A. Milligan, and J. Howard. 1993. Kinesin follows the microtubule's protofilament axis. *J. Cell Biol.* 121:1083–1093.
49. Sato-Yoshitake, R., H. Yorifuji, M. Inagaki, and N. Hirokawa. 1992. The phosphorylation of kinesin regulates its binding to synaptic vesicles. *J. Biol. Chem.* 267:23930–23936.
50. Sawin, K.E., T.J. Mitchison, and L.G. Wordeman. 1992. Evidence for kinesin-related proteins in the mitotic apparatus using peptide antibodies. *J. Cell Sci.* 101:303–313.
51. Schnapp, B.J. 1986. Viewing single microtubules by video light microscopy. *Methods Enzymol.* 134:561–573.
52. 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.
53. Schnapp, B.J., T.S. Reese, and R. Bechtold. 1992. Kinesin is bound with high affinity to squid axon organelles that move to the plus-end of microtubules. *J. Cell Biol.* 119:389–399.
54. Schnapp, B.J., R.D. Vale, M.P. Sheetz, and T.S. Reese. 1985. Single microtubules from squid axoplasm support bidirectional movement of organelles. *Cell.* 40:455–462.
55. Schroer, T.A., B.J. Schnapp, T.S. Reese, and M.P. Sheetz. 1988. The role of kinesin and other soluble factors in organelle movement along microtubules. *J. Cell Biol.* 107:1785–1792.
56. 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.
57. Skoufias, D.A., D.G. Cole, K.P. Wedaman, and J.M. Scholey. 1994. The carboxyl-terminal domain of kinesin heavy chain is important for membrane binding. *J. Biol. Chem.* 2697:1477–1485.
58. Sudhof, T.C., M. Baumert, M.S. Perin, and R. Jahn. 1989. A synaptic vesicle membrane protein is conserved from mammals to *Drosophila. Neuron.* 2:1475–1481.
59. Svoboda, K., C.F. Schmidt, B.J. Schnapp, and S.M. Block. 1993. Direct observation of kinesin stepping by optical trapping interferometry. *Nature (Lond.).* 365:721–727.
60. Toyoshima I., H. Yu, E.R. Steuer, and M.P. Sheetz. 1992. Kinectin, a major kinesin-binding protein on ER. *J. Cell Biol.* 118:1121–1131.
61. 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.* 84:513–530.

62. Vaisberg, E.A., P.M. Grissom, and J.R. McIntosh. 1996. Mammalian cells express three distinct dynein heavy chains that are localized to different cytoplasmic organelles. *J. Cell Biol.* 133:831–842.
63. Vale, R.D., F. Malik, and D. Brown. 1992. Directional instability of microtubule transport in the presence of kinesin and dynein, two opposite polarity motor proteins. *J. Cell Biol.* 119:1589–1596.
64. Vale, R.D., T.S. Reese, and M.P. Sheetz. 1985. Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell.* 42:39–50.
65. Vale, R.D., B.J. Schnapp, T. Mitchison, E. Steuer, T.S. Reese, and M.P. Sheetz. 1985. Different axoplasmic proteins generate movement in opposite directions along microtubules in vitro. *Cell.* 43:623–632.
66. Vale, R.D., B.J. Schnapp, T.S. Reese, and M.P. Sheetz. 1985. Movement of organelles along filaments dissociated from the axoplasm of the squid giant axon. *Cell.* 40:449–454.
67. Vale, R.D., B.J. Schnapp, T.S. Reese, and M.P. Sheetz. 1985. Organelle, bead, and microtubule translocations promoted by soluble factors from the squid giant axon. *Cell.* 40:559–569.
68. Vallee, R.B., and G.S. Bloom. 1991. Mechanisms of fast and slow axonal transport. *Annu. Rev. of Neurosci.* 14:59–92.
69. Vallee, R.B., and M.P. Sheetz. 1996. Targeting of motor proteins. *Science (Wash. DC).* 271:1539–1544.
70. van Meer, G., and I.L. van Genderen. 1994. Intracellular lipid distribution, transport and sorting. *Subcell. Biochem.* 23:1–24.
71. Viancour, T.A., and D.S. Forman. 1987. Polarity orientation of microtubules in squid and lobster axons. *J. Neurocytol.* 16:69–75.
72. Wang, Z., S. Khan, and M.P. Sheetz. 1995. Single cytoplasmic dynein molecule movements: characterization and comparison with kinesin. *Biophys. J.* 69:2011–2023.
73. Yu, H., I. Toyoshima, E.R. Steuer, and M.P. Sheetz. 1992. Kinesin and cytoplasmic dynein binding to brain microsomes. *J. Biol. Chem.* 267:20457–20464.