Kinesin Is Bound with High Affinity to Squid Axon Organelles That Move to the Plus-end of Microtubules

Bruce J. Schnapp,*‡ Thomas S. Reese,§ and Ruth Bechtold*‡

*Department of Physiology, Boston University Medical School, Boston, Massachusetts 02118; ‡Department of Cellular and Molecular Physiology, Harvard Medical School, Boston, Massachusetts 02115; and §Laboratory of Neurobiology, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20894

Abstract. This paper addresses the question of whether microtubule-directed transport of vesicular organelles depends on the presence of a pool of cytosolic factors, including soluble motor proteins and accessory factors. Earlier studies with squid axon organelles (Schroer et al., 1988) suggested that the presence of cytosol induces a >20-fold increase in the number of organelles moving per unit time on microtubules in vitro. These earlier studies, however, did not consider that cytosol might nonspecifically increase the numbers of moving organelles, i.e., by blocking adsorption of organelles to the coverglass. Here we report that treatment of the coverglass with casein, in the absence of cytosol, blocks adsorption of organelles to the coverglass and results in vigorous movement of vesicular organelles in the complete absence of soluble proteins. This technical improvement makes it possible, for the first time, to perform quantitative studies of organelle movement in the absence of cytosol. These new studies show that organelle movement activity (numbers of moving organelles/min/μm microtubule) of unextracted organelles is not increased by cytosol. Unextracted organelles move in single directions, approximately two thirds toward the plus-end and one third toward the minus-end of microtubules. Extraction of organelles with 600 mM KI completely inhibits minus-end, but not plus-end directed organelle movement. Upon addition of cytosol, minus-end directed movement of KI organelles is restored, while plus-end directed movement is unaffected. Biochemical studies indicate that KI-extracted organelles attach to microtubules in the presence of AMP-PNP and copurify with tightly bound kinesin. The bound kinesin is not extracted from organelles by 1 M KI, 1 M NaCl or carbonate (pH 11.3). These results suggest that kinesin is irreversibly bound to organelles that move to the plus-end of microtubules and that the presence of soluble kinesin and accessory factors is not required for movement of plus-end organelles in squid axons.

MICROTUBULE-based vesicular transport occurs in virtually all eukaryotic cells (Schliwa, 1984), but is particularly well organized in nerve cell axons because of the uniform orientation of microtubules (Heidemann et al., 1981). Golgi-derived organelles are transported toward the plus-ends of microtubules, a direction anterograde with respect to the nucleus, while prelysosomal organelles are simultaneously transported in the opposite, or retrograde direction (Tsukita and Ishikawa, 1980). A cell-free organelle transport system from squid axons preserves the specificity evident in the intact axon to the extent that single microtubules support simultaneous movement of organelles in both directions, while individual organelles move in only one direction (Brady et al., 1982; Schnapp et al., 1985; Schnapp and Reese, 1989).

The microtubule motor proteins kinesin and dynein were identified in the cytosol of squid axoplasm by their ability to translocate polystyrene beads along microtubules (Vale et al., 1985*a*,*b*,*c*; Schnapp and Reese, 1989). The superficial similarity between bead and organelle movement in vitro led to the expectation that kinesin, a plus-end motor (Vale et al., 1985*c*), and dynein, a minus-end motor (Vallee et al., 1988), interact with organelles to promote their movement in the appropriate direction along microtubules. While circumstantial evidence has supported this model, the identity of the motors for organelle movement has still not been firmly established.

Despite their superficial similarity, bead movement can be distinguished from movement of squid axon organelles by several criteria which together suggest that motor proteins interact with squid axon organelles by a distinct, and presumably specific mechanism. For example, bead movement driven by motor proteins has a V_{max} of 0.5 μm s^{-1} while organelles from squid axoplasm are transported with a V_{max} of 2.2 μm s^{-1} (Vale et al., 1985*a*). Most importantly, beads adsorb motor proteins nonspecifically, as evident from bidirectional movement of individual beads in cytosol containing both kinesin and dynein (Vale et al., 1985*c*). Organelles, on the other hand, must interact with motor proteins by a specific mechanism since individual organelles move in only

The present address of Bruce J. Schnapp and Ruth Bechtold is Department of Cellular and Molecular Physiology, Harvard Medical School, Boston, MA 02115.

one direction, even in the presence of cytosol containing both plus- and minus-end directed motors (Vale et al., 1985*a*; Schroer et al., 1988; Schnapp and Reese, 1989).

The mechanism by which motor proteins are targeted to the appropriate population of organelles is of interest because this ultimately determines the fate of all membrane compartments that interact with microtubules, including the Golgi and ER (Dabora and Sheetz, 1988*b*; Cooper et al., 1990). An understanding of how motor proteins are targeted to organelles requires an understanding of the biochemical mechanism by which motor proteins bind to organelles. The prevailing view, that motor proteins on organelles freely exchange with a large soluble pool, is based on the finding that the number of salt-stripped organelles moving per unit time was increased 20-fold in the presence of cytosol (Schroer et al., 1988). However, these measurements of organelle movement did not consider that organelles adsorb avidly to the coverglass in the absence of cytosol. Thus, the previously observed dependence of organelle movement on the presence of cytosol could have been due to cytosolic proteins blocking sites on the glass that would otherwise adsorb organelles and thereby deplete the pool available for movement on microtubules. Although many blocking proteins and procedures were screened, most proved ineffective at preventing adsorption of organelles to the coverglass in the absence of cytosol. We now report that one protein, casein, prevents adsorption of organelles to the coverglass as effectively as cytosol. It now becomes feasible to investigate organelle movement in the absence of cytosol, to make a valid comparison between organelle movement in the presence and absence of cytosol, and to evaluate how tightly motors are bound to organelles.

The most interesting outcome is that the presence of cytosol and soluble motors is not required for organelle movement in vitro. Even after extraction of organelles with 600 mM KI, plus-end directed organelle movement is maximal in the absence of cytosol. These KI-extracted, plus-end directed organelles are recovered as a purified population by attachment to microtubules in the presence of AMP-PNP, and immunological analysis of these organelles indicates they copurify with kinesin that is resistant to extraction with 1M NaCl, 1M KI, or pH 11.3. While these results are consistent with the view that kinesin is a motor for plus-end organelle movement, they question whether exchange of soluble and organelle-bound factors is necessary for movement of these organelles.

Materials and Methods

Axoplasmic Cytosol and Organelles

Squid (*Loligo pealeii*) axons were dissected and stored in liquid nitrogen. After thawing, the axoplasm was extruded and homogenized in motility buffer ("1/2 X") containing protease inhibitors and 1 mM DTT as previously described (Vale et al., 1985*a*). A typical experiment utilized 100–200 μl of homogenized axoplasm (from 50–70 cm of axons) for preparation of cytosol and unextracted organelles and 100–200 μl of homogenate for preparation of KI organelles. All assays were completed within two days of extruding the axoplasm.

Cytosol (S2) was prepared from the homogenate by 120,000 g·h centrifugation on discontinuous sucrose gradients as described (Schroer et al., 1988; Schnapp and Reese, 1989). Unextracted organelles were prepared by previously described methods (Schroer et al., 1988) with minor modifications. Briefly, the 45 and 15% layers of the same gradient used to prepare cytosol were pooled, and the organelles concentrated ~10-fold by centrifugation to a pellet before resuspension in motility buffer. KI organelles were prepared (Schroer et al., 1988; Schnapp and Reese, 1989) by incubating homogenized axoplasm with 600 mM KI for 10 min followed by 1:1 dilution with motility buffer. KI extracted homogenates were centrifuged on discontinuous sucrose gradients for 180,000 g·h. Elution of the gradients and concentration of the organelles was performed as described above for unextracted organelles. For experiments that determined the concentration of KI required to extract the minus-end motor from organelles, unextracted organelles were collected from the sucrose gradient, pooled, and divided into four aliquots which were adjusted to 0, 100, 300, and 600 mM KI before centrifugation and resuspension in motility buffer. Total time in the KI was 45 min. For experiments which compared organelles prepared in 600 mM KI with organelles prepared in 900 mM KI, one homogenate of axoplasm was divided into two aliquots. One aliquot was extracted for 10 min with 600 mM KI and the other with 900 mM KI before 1:1 dilution with motility buffer and centrifugation on sucrose gradients.

Purification of Microtubules and Kinesin

Optic lobes (30–100 g) from squid were homogenized in 1.5 vol of motility buffer containing protease inhibitors and 1 mM DTT. Microtubules and kinesin were purified from the supernatant of a high-speed centrifugation (185,000 *g* for 75 min) as described elsewhere (Vale et al., 1985*a*,*c*; Schnapp and Reese, 1989). A brief description follows.

Microtubules

The tubulin in the supernatant was assembled into microtubules by addition of 20 μM taxol, and the microtubules were then separated by centrifugation, resuspended in 1 M NaCl to remove microtubule-associated proteins, centrifuged again, and finally resuspended in BRB80 (80 mM Pipes, 1 mM $MgCl_2$, and 1 mM EGTA) with taxol. Aliquots were stored as drops in liquid nitrogen. Taxol polymerized microtubules (from PC-purified bovine tubulin prepared by cycling and phosphocellulose chromatography using standard techniques) were used for the formation of KI organelle microtubule complexes.

Kinesin

AMP-PNP (5–10 mM) was added with the taxol to the high-speed supernatant. The microtubule pellet was resuspended in BRB80 containing taxol and 100–200 mM KCl to release microtubule-associated proteins. Kinesin and dynein were released from the salt-washed microtubule pellet by resuspension in 1–2 ml of BRB80 containing taxol, 100–200 mM KCl, and 5–10 mM ATP. Microtubules and released proteins were separated by centrifugation, and kinesin in the released fraction was purified by velocity sedimentation on 5–20% (wt/wt) sucrose gradients.

Experimental Manipulation of Axoplasmic Cytosol Immunoadsorption

The kinesin in axoplasmic cytosol was immunoadsorbed with a mAb (CG39) that recognizes the tail domain of the kinesin heavy chain (Kosik et al., 1990). An affinity resin was prepared with this antibody by conjugating a purified IgG fraction to CNBr-activated Sepharose (Sigma Chemical Co., St. Louis, MO) as described previously (Vale et al., 1985*c*). Immunoadsorption of kinesin was accomplished by combining one part resin with three to four parts cytosol for 2–12 h at 4°C. Control resin was prepared identically using nonspecific mouse IgG (Sigma Chemical Co., St. Louis, MO).

Microtubule Affinity Depletion of Kinesin

AMP-PNP (5 mM) and squid microtubules (100 μg ml^{-1}) were added to immunoadsorbed cytosol for 60 min at room temperature, followed by separation of the microtubules by centrifugation at 100,000 *g* for 30 min. AMP-PNP was removed from the depleted supernatant by spin dialysis (Neal and Florini, 1973) into motility buffer. The amount of kinesin remaining in the supernatant was determined by two methods: (*a*) Immunoblotting according to established procedures (Towbin et al., 1979) using a rabbit antisquid kinesin heavy chain antiserum and 2) observation of microtubule gliding on glass precoated with blocking protein (Howard et al., 1989). The latter method detected kinesin in normal cytosol diluted by a factor of 1,000 (<0.5 nM).

UV-Vi Photocleavage

Cytoplasmic dynein in cytosol was inactivated by UV irradiation in the

presence of 20 μM Vi and 2 mM ATP, as described (Gibbons et al., 1987; Schnapp and Reese, 1989). After irradiation, the Vi was reduced by addition of 2 mM norepinephrine (Gibbons et al., 1978). As shown previously (Schnapp and Reese, 1989), the identical treatment without UV irradiation has no effect on plus-end or minus-end organelle movement activity, or on the ability of cytosol to promote bead movement toward either the plus or minus-ends of microtubules.

Organelle Movement Assays

Organelle movement assays were assembled on coverglasses in 3.8-μl wells that were formed by punching a standard-sized hole in a 200-μm silicon rubber sheet. Assays in the presence of cytosol were assembled by incubation of the well with 1 μl squid microtubules (50 μg ml^{-1} stock solution in BRB80 containing 20 μM taxol), 2 μl cytosol, and 1 μl of organelles. The solution was mixed 5 × by drawing it into a P-20 pipette tip before sealing the chamber with a second coverglass. In the absence of cytosol, the assay was assembled by incubating the well with 2 μl squid microtubules (~90 μg ml^{-1}) for 2 min and then with 2 μl casein (5 mg ml^{-1} in motility buffer) for 2 min. After removal of 2 μl of this mixture, 1 μl motility buffer and 1 μl of organelles were added and the solution was mixed by drawing and expelling with a P-20 pipette tip. The microtubules were added first because casein prevented their adsorption to the coverglass. The final concentration of ATP in the assay was 2 mM. For each assay, a representative 400-μm^2 field was observed for 10 min by video microscopy (Schnapp, 1986), and the numbers of organelles moving on microtubules attached to the coverglass were counted. The total length of microtubule surface adsorbed to the coverglass was measured at 2-min intervals using an image processing program (Image 1; Universal Imaging, Media, PA), and the average value over the five 2-min intervals was used to normalize the numbers of moving organelles to microtubule length.

Organelle movement was assayed in a flow cell to determine the relative numbers of organelles moving in each direction on single microtubules. Flow cells (2–4 μl), 22 nm long × 1 mm wide with a height determined by the thickness of double-stick Scotch tape were constructed using 22 × 22 mm and 60 × 24 mm coverglasses. The cell was first incubated with squid microtubules, then with casein in motility buffer, followed by motility buffer alone. Single microtubules were identified, and the numbers of organelles moving in the two directions along a single microtubule were counted for arbitrary time intervals. The data was expressed as the percentage of moves in the predominant direction (see Table II). When organelle movement was bidirectional, it was not essential to know whether plus or minus-end movements predominated, and the polarity of the microtubules was not determined (see Table II; the "polarity of predominant direction" was marked NA in these cases). When minus-end movement was inhibited and organelle movement was unidirectional, the polarity of the microtubules was determined before introducing organelles, as follows: 0.4-μm beads precoated with <1 kinesin per bead, on average, were infused into the flow cell and manipulated onto microtubules with optical tweezers until movement was observed (Block et al., 1990). The beads were then washed out before infusion of organelles.

Biochemical Studies with KI Extracted Organelles

Immunoblotting of KI organelle fractions was performed using a chemiluminescent procedure (Gillespie and Hudspeth, 1991) and a mAb (CG39) to the tail domain of the kinesin heavy chain (Kosik et al., 1990).

For extraction experiments, KI organelles (from 50–100 cm of axons) were collected from sucrose gradients and centrifuged, and the pellet of organelles was resuspended in 20 μl of either 1 M NaCl, 1 M KI, or 100 mM sodium carbonate (pH 11.3). The organelles were incubated for 30 min, centrifuged, and the organelle pellet was resuspended in 25 μl of PAGE sample buffer. Equivalent volumes of organelle pellets and released proteins were immunoblotted. Triton X-114 extraction of the KI organelle pellets was carried out exactly as specified (Bordier, 1981).

KI organelle-microtubule complexes were formed by incubating the KI vesicle fraction with 1 vol of motility buffer plus taxol, 100 μg/ml taxol-stabilized bovine microtubules (from PC purified tubulin), and 10 mM AMP-PNP. Control incubations were KI vesicles + microtubules + ATP, microtubules + AMP-PNP, and KI vesicles + AMP-PNP. The complexes were viewed at this stage by video microscopy, or were centrifuged over 25% (wt/vol) sucrose for 90 min at 120,000 *g* to separate microtubule-bound organelles from other material in the KI organelle fraction, including unbound vesicular organelles. The pellet of KI organelle–microtubule complexes was analyzed by immunoblotting and by negative stain and thin section EM. Unbound organelles, i.e., those remaining in the supernatant after sedimenting the organelle-microtubule complexes, were pelleted by centrifugation (150,000 *g* for 45 min), and resuspended in either motility buffer for organelle movement assays, or in PAGE sample buffer for immunoblotting.

For extracting kinesin from KI vesicles, KI organelle-microtubule complexes (from 300 cm of squid axons) were centrifuged over 25% (wt/vol) sucrose for 90 min at 120,000 *g* and the pellet resuspended in 2% Triton X-100 in BRB80 buffer containing taxol, protease inhibitors and 1 mM AMP-PNP. After 10 min the sample was centrifuged to pellet the microtubules and attached vesicular motors, and the microtubule pellet was then resuspended in 80 μl of 100 mM KCl in BRB80 plus taxol. After 1 h at room temperature the microtubules were again sedimented, the supernatant ("KCl release") was saved for motility analysis, and the microtubules were resuspended in 80 μl of the same buffer now containing 5 mM ATP. After 1 h the microtubules were sedimented again and the supernatant ("ATP release") was saved for motility analysis.

Microtubule gliding assays were performed by the procedure of Howard et al. (1989) with minor changes. Briefly, a closed chamber (5 μl) was used instead of a flow cell. Test solutions consisted of 1 μl of sample (either KCl or ATP releases or purified squid optic lobe kinesin of known concentration determined by video densitometry of Coomassie-stained gels), 3 μl of buffer (final concentrations in the assay were 80 mM Pipes, 1 mM EGTA, 0.1 mg/ml casein, 5 μM taxol, 2 mM ATP, 2 mM $MgCl_2$, and a phosphocreatine–creatine phosphokinase ATP regeneration system) and 1 μl of 1.5 mg/ml taxol polymerized microtubules made from phosphocellulose-purified bovine tubulin. The microtubules were sheared by sucking them in and out of a 30-guage syringe needle for five cycles immediately before use. The assay was incubated for 10 min before observing by video microscopy. For each sample, the numbers of moving microtubules was counted for 2-min intervals in five video fields measuring 30 × 20 μm.

Calculation of Kinesin/Vesicle Ratio

KI organelle–microtubule complexes were prepared as described above and the average number of organelles per μm of microtubule (usually 1–2/μm) was determined by video microscopy. The total number of KI organelles bound to microtubules was then calculated from the total microtubule length in the mixture. Total microtubule length was calculated from the known tubulin concentration (determined by a colorimetric protein assay from Biorad Laboratories [Palo Alto, CA], using BSA as the standard), assuming 1 μm of a 13 protofilament microtubule contains 3.4 × 10^{-21} moles of tubulin [assumes a molecular mass of 55,000 Daltons per tubulin monomer, and a 4-nm center to center distance between monomers (Amos and Klug, 1974)]. Kinesin in the KI organelle–microtubule complexes was quantified by immunoblotting the complexes with the CG39 monoclonal to the kinesin heavy chain, and comparing the intensity of these blots with those from purified optic kinesin of known concentration (previously calibrated by gel densitometry of the 110-kD band using BSA as the standard).

Results

Organelles Are Transported on Microtubules in the Absence of Cytosol

Organelles were derived from extruded axoplasm that had been homogenized in motility buffer (unextracted organelles) or motility buffer containing 600 mM KI (KI organelles). The homogenate was then centrifuged on sucrose gradients to separate organelles from cytosol. The KI organelles consisted entirely of vesicular structures, while unextracted organelles were contaminated with intermediate filaments (Schroer et al., 1988). The KI organelle fraction showed the full range of sizes and shapes expected of both Golgi-derived anterograde organelles and prelysosomal retrograde organelles (see Fig. 5 *c*). Both unextracted and KI extracted organelles moved at ~2 μm s^{-1}, fourfold faster than microtubule gliding or bead movement driven by cytosolic kinesin (Vale et al., 1985*a*). The velocity of organelle movement was not obviously different in the presence and absence of cytosol.

Organelle movement activity, defined here as the numbers of organelles moving per min per μm microtubule in a 400-

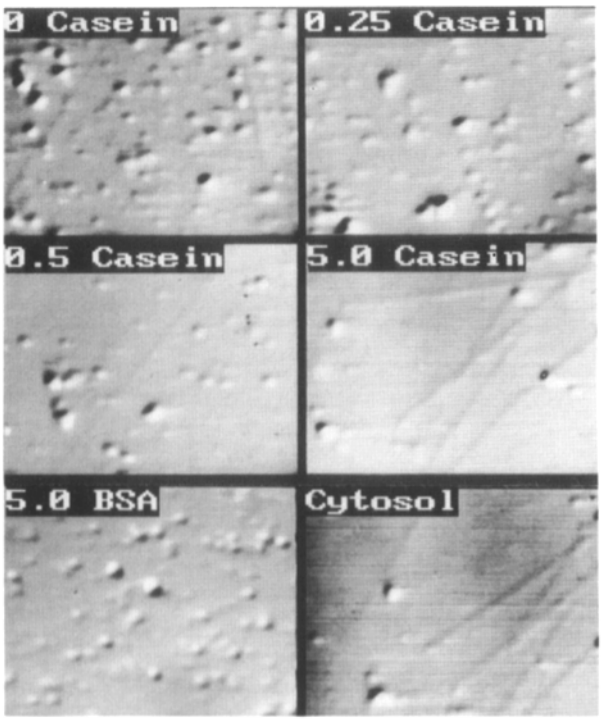

Figure 1. Adsorption of organelles to the coverglass is prevented by casein or cytosol, but not by BSA. In the absence of cytosol or casein, many particles adsorb to the coverglass (*upper left*). Concentrations of casein or BSA (mg ml^{-1}) are indicated; the final concentration in the assay is 1/4 of that used to coat the glass. In the absence of cytosol, adsorption of organelles is reduced as the concentration of casein used to coat the coverglass is increased. Incubation with 5.0 mg ml^{-1} casein is about as effective as cytosol in preventing adsorption of particles to the glass, while BSA in the absence of cytosol has little effect. Adsorption of particles was inversely correlated with the measurements of organelle movement activity in the same assays (Fig. 2). Each field corresponds to ~7 × 7 μm.

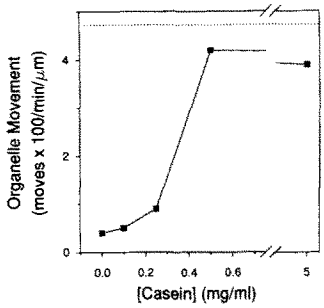

Figure 2. Incubation of the coverglass with increasing concentrations of casein increases organelle movement activity in the absence of cytosol. In the absence of casein, organelle movement activity in cytosol is increased >20-fold compared to motility buffer (dotted line: average organelle movement activity in the presence of cytosol for this axoplasmic homogenate; $n = 5$ assays). In contrast, the cytosol-induced increase is <25% if organelles are assayed on a coverglass incubated with 5 mg ml^{-1} casein. Comparison of this plot with the corresponding micrographs of the assays (Fig. 1) shows that adsorption of particles to the coverglass correlates inversely with the organelle movement activity. All data points are from single assays which used organelles from the same homogenate of axoplasm. Assays were performed by adsorbing microtubules (1 μl) to sample chambers and then adding the indicated concentration of casein in motility buffer (1 μl), followed by 1 μl KI organelles and 2 μl motility buffer or cytosol (*dotted line*). Organelle movement activity was determined by counting the number of organelles moving in 10 min in a 400-μm^2 field and dividing by the total length of microtubules on the coverglass in the field. All of the organelle movement in this paper occurred at the rate of ~2 μm/s, which is fourfold faster than the rate of kinesin or dynein driven bead movement (Vale et al., 1985*a*).

μm^2 field, was measured in the presence and absence of cytosol. The assay was performed on a coverglass by combining 2 μl cytosol, motility buffer, or blocking protein with 1 μl microtubules and 1 μl organelles. The coverglass was heavily decorated with adsorbed particles in the absence, but not the presence of cytosol (Fig. 1). These particles were derived from the organelle fraction, as determined by imaging each component of the assay alone. The coverglass became coated with particles rapidly, within the 1–2 min which was needed to set up the assay on the microscope.

In the absence of cytosol, treatment of the coverglass with siliconizing reagents, nitrocellulose, BSA (Fig. 1), ovalbumin, tubulin, or cytochrome c neither prevented adsorption of organelles to the coverglass nor led to significant organelle movement activity. Treatment with casein, however, dramatically reduced the number of particles decorating the coverglass (Fig. 1). Increasing concentrations of casein correlated with both a reduction in particle binding to the glass and an increase in organelle movement activity (Figs. 1 and 2). To test whether these effects were due to casein adsorbed to the coverglass, as opposed to soluble casein, organelle movement assays were performed in a flow cell as follows: taxol-stabilized microtubules from squid optic lobe were infused and allowed to attach to the glass; then 1–5 mg ml^{-1} of casein in motility buffer was infused for 2–3 min, followed by continuous perfusion of motility buffer for 5 min to wash out the free casein. When organelles were then introduced into the flow cell, vigorous organelle movement was observed without significant adsorption of particles to the glass. These flow cell experiments indicate that casein need not be free in solution to increase organelle movement activity. The implication is that casein increased organelle movement activity by adsorbing to the coverglass, thereby blocking the adsorption of organelles.

In addition to using casein, the reconstituted system was modified in the following ways to permit its use as a quantitative assay: (*a*) the volume and geometry of each assay was standardized (Materials and Methods); (*b*) the concentration of microtubules in the assay was decreased 10-fold compared to previous studies (Schroer et al., 1989; Schnapp and Reese, 1989), resulting in only 3–12 microtubules on the glass in a 400-μm^2 field, and allowing measurement of microtubule length; (*c*) the number of organelle moves counted per unit time was normalized to the length of microtubules on the glass. The data was normalized because kinesin-containing cytosol increased the number of microtubules attached to the coverglass by up to fourfold. With these technical improvements, we could now make a valid comparison between organelle movement activity in the presence and absence of cytosol.

Unextracted organelles and KI organelles were affected differently by the presence of cytosol. Organelle movement activity of unextracted organelles in the presence of cytosol (3.3 ± 1.4; mean ± SD; $n = 6$ assays from 1 homogenate of axoplasm) was not significantly different from that in motility buffer plus casein (3.1 ± 2.5; $n = 6$ assays using the same organelle fraction). On the other hand, KI organelle

Table I. Effects of Normal Cytosol and Motor-depleted Cytosol on Organelle Movement Activity of KI-extracted Organelles

Treatment of cytosol	Soluble factors compared	Mean ratio of organelle movement activities	Standard deviation of ratios	*Significance	Number of experiments (*n*)
None	Casein / Normal cytosol	0.48	0.22	<0.001	24
‡ Vanadate + UV	Dynein-inhibited cytosol / Control cytosol	0.67	0.1	<0.02	4
§ Immunoprecipitation	Kinesin-depleted cytosol / Control cytosol	1.03	0.38	NS	13
‖ Immunoprecipitation and microtubule affinity	Kinesin-depleted cytosol / Control cytosol	0.98	0.18	NS	4

The organelle movement activity (number of moves per minute per μm microtubule) was determined under standard conditions as described in Materials and Methods. Each experiment utilized an independent homogenate of axoplasm which provided KI-extracted organelles and cytosol. In each experiment, two to six assays of organelle movement activity were performed for each of the two soluble factors being compared. The mean organelle movement activity was calculated for each set of two to six assays, and the ratio of the means for the two solbule factors was determined. The mean ratio and SD of the ratios (over *n* experiments) is given in the table. Control treatments of cytosol are detailed in Materials and Methods.

* A paired *t* test was used to test whether the organelle movement activity promoted by the two soluble factors was different.

‡ The UV-Vi experiment confirms a more extensive earlier report that UV-Vi treatment of cytosol blocks minus-end organelle movements without significantly decreasing the numbers of plus-end organelle movements (Schnapp and Reese, 1989).

§ Cytosol was immunoadsorbed with CG39 monoclonal to the tail domain of the kinesin heavy chain (Kosik et al., 1990).

‖ Cytosol was first immunoadsorbed with CG39 monoclonal to the kinesin heavy chain, and then incubated with microtubules and AMP-PNP, followed by centrifugation and desalting of the supernatant (see Materials and Methods).

NS, Not significant.

movement activity was increased in the presence of cytosol by an amount that varied considerably between experiments (Table I). This variability appeared to be inherent in the measurement of organelle movement activity itself, rather than a result of differences in the way the individual assays were set up, since large fluctuations in organelle movement activity were evident between successive 2-min intervals in a single, 10-min assay. The difference in KI organelle movement activity between assays with and without cytosol was significant at the 0.1% confidence level in a paired *t* test of $n = 24$ independent homogenates of axoplasm, each of which provided a mean organelle movement activity (from two to six assays) in the presence and absence of cytosol. Averaged over all 24 experiments, cytosol increased KI organelle movement activity by a factor of 2.07 ± 0.94 (mean $\pm$ SD). This increase was not limited by the concentration of cytosolic proteins in the assay, since organelle movement activity was not increased further by concentrating the cytosol fivefold by ultrafiltration. As discussed below, the cytosol-induced increase in KI organelle movement activity depends largely, and perhaps exclusively, on the reactivation of minus-end organelle movement by dynein in the cytosol.

The Minus-end Motor Is Extracted from Organelles by 300 mM KI

In the absence of cytosol, unextracted organelles moved bidirectionally, while KI organelles moved exclusively toward the plus-end of microtubules (Table II), suggesting that 600 mM KI strips off the minus-end, but not the plus-end organelle motor (in the context of the experiments discussed in this paper, bidirectional refers to the population of organelles, not to individual organelles; fewer than 0.5% of organelles reversed their direction). In the presence of cytosol, KI organelles moved bidirectionally (Table II). This cytosol

Table II. Effects of KI Extraction and Soluble Factors on the Direction of Organelle Movement

Soluble factor	Organelle fraction	Moves in predominant direction*	Range‡	Polarity of predominant direction§	Organelle moves	Microtubules	Experiments
		%			*n*	*n*	*n*
None	Unextracted	57	51–65	Plus	122	3	2
None	Unextracted	67	51–78	NA	613	6	3
None	KI-extracted	100	–	Plus	217	3	3
Cytosol	KI-extracted	73	56–86	NA	147	10	3
UV-Vi Cytosol	KI-extracted	100	–	Plus	21	1	1

The numbers of organelles moving in the two directions specified by single microtubules were determined using fixed cell or flow cell assays, as described in Materials and Methods. KI organelles were derived from axoplasmic homogenates extracted with 600 mM KI, as described in Materials and Methods. Each experiment used an independent preparation of homogenized axoplasm from squid. For each expermental condition, the total number of observed organelle movements and microtubules are indicated.Organelle movement was determined to be unidirectional on a given microtubule if >17 moves were counted in only one direction. The effect of UV-Vi treatment of cytosol on the direction of KI organelles confirms an earlier, move extensive report (see Schnapp and Reese, 1989), in which UV-Vi treatment of cytosol selectively blocked only minus-end directed movement of KI organelles.

* Means of percentages of moves in the predominant direction calculated separately for each microtubule.

‡ Ranges in percentages of moves in the predominant direction calculated separately for each microtubule.

§ The polarity of each microtubule tested was determined by movement of kinesin-coated beads (see Materials and Methods), except in certian cases (NA) when both directions of organelle movement were observed. In the latter cases, the polarity of the microtubules was not determined because we were primarily concerned with whether or not minus-end movements occurred and not with the relative numbers of plus and minus-end moves.

dependent restoration of minus-end movement of KI organelles was blocked by prior exposure of the cytosol to UV light in the presence of 20 μM vanadate and ATP (Gibbons et al., 1987). UV-vanadate specifically cleaves and inactivates the heavy chain of cytoplasmic dynein in squid axoplasmic cytosol (Schnapp and Reese, 1989).

These experiments suggest that cytoplasmic dynein is normally bound to organelles but can be stripped off by high salt. The strength of binding was evaluated by first extracting organelles with KI concentrations ranging from 100 to 600 mM for ~45 min and then counting the numbers of organelles moving in each direction on single microtubules. Minus-end movement was completely inhibited by extraction with 300 mM KI but not with 100 mM KI.

The Presence of Cytosolic Kinesin Is Not Required for Plus-end Directed Organelle Movement

The above results suggest that the restoration of minus-end organelle movement accounted for a large part of the cytosol induced increase in KI organelle movement activity. UV-irradiation of cytosol in the presence of ATP and 20 μM vanadate (which inactivates minus-end movement of KI organelles but has no effect on plus-end movement; Schnapp and Reese, 1989) decreased total KI organelle movement activity (Table I) by an average of 33% compared to untreated cytosol. Since the total cytosol-induced increase in KI organelle movement activity was only twofold (on average), movement of plus-end organelles is stimulated by no more than 67%.

Although this putative increase in plus-end organelle movement activity is not dramatic, it is worthwhile establishing whether cytosolic kinesin has a role in it (Table I). Cytosol was immunoadsorbed using a mAb (CG39) known to bind to the carboxy-terminal half of the kinesin heavy chain (Kosik et al., 1990). Although this procedure depleted >95% of the cytosolic kinesin, as detected by immunoblotting (Fig. 3), there was no statistical difference in organelle movement activity between immunoadsorbed and control cytosol (n = 13 separate axoplasmic homogenates; Table I). In a second set of experiments, immunoadsorbed cytosol was further depleted of kinesin by incubation with microtubules and AMP-PNP, followed by centrifugation to remove the microtubules. Although kinesin could not be detected in the depleted cytosol by immunoblotting (Fig. 3) or by microtubule gliding assays which detected <0.5 nM kinesin (Howard et al., 1989), there was no significant difference between untreated cytosol and kinesin-depleted cytosol in the stimulation of KI organelle movement activity (Table I). These results indicate that no part of the cytosol-induced increase in organelle movement activity was due to cytosolic kinesin.

Organelles were exposed to harsher extraction conditions with the aim of extracting the plus-end organelle motor and then restoring plus-end organelle movement by addition of cytosol. Axoplasmic homogenates were extracted with 900 mM KI before purifying organelles by density gradient centrifugation. Compared to extraction of the same homogenate with the usual 600 mM KI, extraction with 900 mM KI did not significantly decrease organelle movement activity in the absence of cytosol, and there was no difference in the cytosol-induced increase in organelle movement activity of the two populations of organelles (not shown).

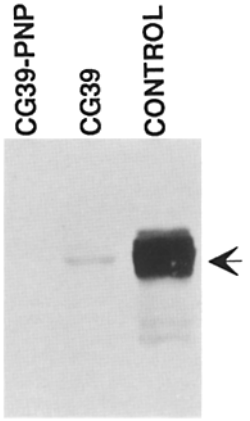

Figure 3. Cytosolic kinesin is not detected after immunoadsorption followed by microtubule affinity depletion. Immunoblots show 110 kD kinesin heavy chain (*arrow*) in control cytosol, CG39-adsorbed cytosol, and cytosol incubated with microtubules and AMP-PNP after immunoadsorption (CG39 + PNP). Note that ~5% of the kinesin remains after immunoadsorption alone, but none is detected after microtubule affinity depletion. Transfers were exposed to a polyclonal serum to the squid kinesin heavy chain. Control cytosol was incubated with a nonspecific mouse IgG resin. Quantitative immunoblots showed that the concentration of kinesin in normal cytosol is 0.2–0.5 μM.

Experiments were performed to determine whether purified squid optic lobe kinesin increased organelle movement activity. Purified optic lobe kinesin (~0.2 μM in the assay) together with casein did not increase organelle movement activity compared to casein alone (n = two independent organelle fractions, two to five assays each condition). Addition of purified optic lobe kinesin to kinesin-depleted axoplasmic cytosol did not increase organelle movement activity compared to the kinesin-depleted cytosol alone (not shown).

Kinesin Is Tightly Bound to Organelles That Move to the Plus-end of Microtubules

The experiments detailed above, in negating the previous conclusion that the presence of soluble kinesin is required for organelle movement (Vale et al., 1985*b*; Schroer et al., 1988) also questioned one of the most important pieces of evidence in favor of kinesin being the plus-end organelle motor. If kinesin is the plus-end organelle motor, it must be

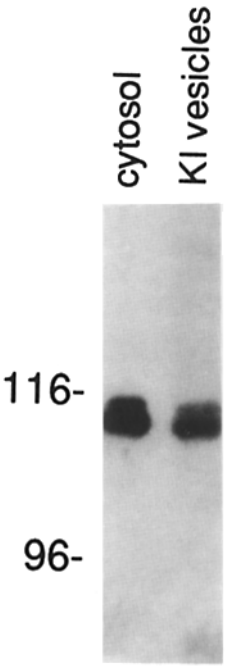

Figure 4. The kinesin heavy chain is associated with the KI organelle fraction. A lane of axoplasmic cytosol is shown for comparison. The electrophoretic mobility of organelle bound kinesin is slightly but consistently greater than that of cytosolic kinesin. The cytosol is diluted 1,000-fold. Transfers were stained with a mAb (CG39) to the squid kinesin heavy chain tail domain (Kosik et al., 1990).

bound with unexpectedly high affinity. To determine whether kinesin is bound to KI organelles, the KI organelle fraction was immunoblotted with the CG39 monoclonal to the kinesin heavy chain. The only reactive polypeptide in the KI organelle fraction was a doublet centered at ~112 kD which migrated slightly ahead of a similar doublet in the cytosolic fraction (Fig. 4).

With the aim of determining whether the kinesin in the KI organelle fraction was deployed on the surfaces of organelles in a configuration capable of promoting movement on microtubules (as opposed, for instance, to being within inside-out vesicles), we asked whether AMP-PNP would cause KI organelles to attach to microtubules. Incubation of KI organelles and microtubules in the presence of AMP-PNP resulted in the formation of extensive KI organelle–microtubule complexes (Fig. 5). These complexes were subsequently separated from unbound KI organelles (i.e., those organelles that did not attach to microtubules in the presence of AMP-PNP) by centrifugation (see Materials and Methods).

Experiments were performed to determine whether the AMP-PNP–induced attachment of KI organelles to microtubules could be reversed by addition of ATP. KI organelle–microtubule complexes were perfused into a laminar flow cell in the presence of 4 mM AMP-PNP and allowed to bind to the coverglass. Any free organelles and other material that did not bind to the microtubules in the presence of AMP-PNP was washed out of the flow cell by perfusion of taxol-containing motility buffer in the absence of nucleotide. The organelles remained immotile until the flow cell was perfused with 2 mM ATP in motility buffer containing taxol; during a period of ~1 min following the perfusion of ATP, the majority of organelles moved to the ends of the microtubule (Fig. 6). These experiments indicated that the AMP-PNP induced attachment of KI organelles to microtubules could be reversed by ATP.

Immunoblots of both the organelle-microtubule complexes (Fig. 7) and the unbound organelles (following concentration of the latter by centrifugation to a pellet and resuspension in PAGE sample buffer) indicated that most, if not all, of the kinesin in the KI organelle fraction copurified with the organelle–microtubule complexes, and no kinesin was detected in the pellet of unbound organelles.

AMP-PNP was required for both the formation of organelle–microtubule complexes with high densities of organelles, and the enrichment of the microtubule pellet with kinesin, since in the presence of ATP, less than one organelle was observed per 5 μm of microtubule (Fig. 5), and only trace amounts of kinesin were detected in the microtubule pellet (Fig. 7).

With the aim of determining whether the kinesin that copurifies with KI organelles is active, we asked whether kinesin-like microtubule gliding activity could be recovered from AMP-PNP–induced organelle–microtubule complexes. We extracted KI–organelle microtubule complexes with the nonionic detergent Triton X-100, separated the microtubules from the Triton extract by centrifugation, and subsequently extracted the microtubule pellet first with motility buffer containing 100 mM KCl (KCl release), and subsequently with 100 mM KCl plus 5 mM ATP (ATP release). The microtubule gliding activity of the KCl and the ATP releases was then quantified using a sensitive microtubule gliding as-

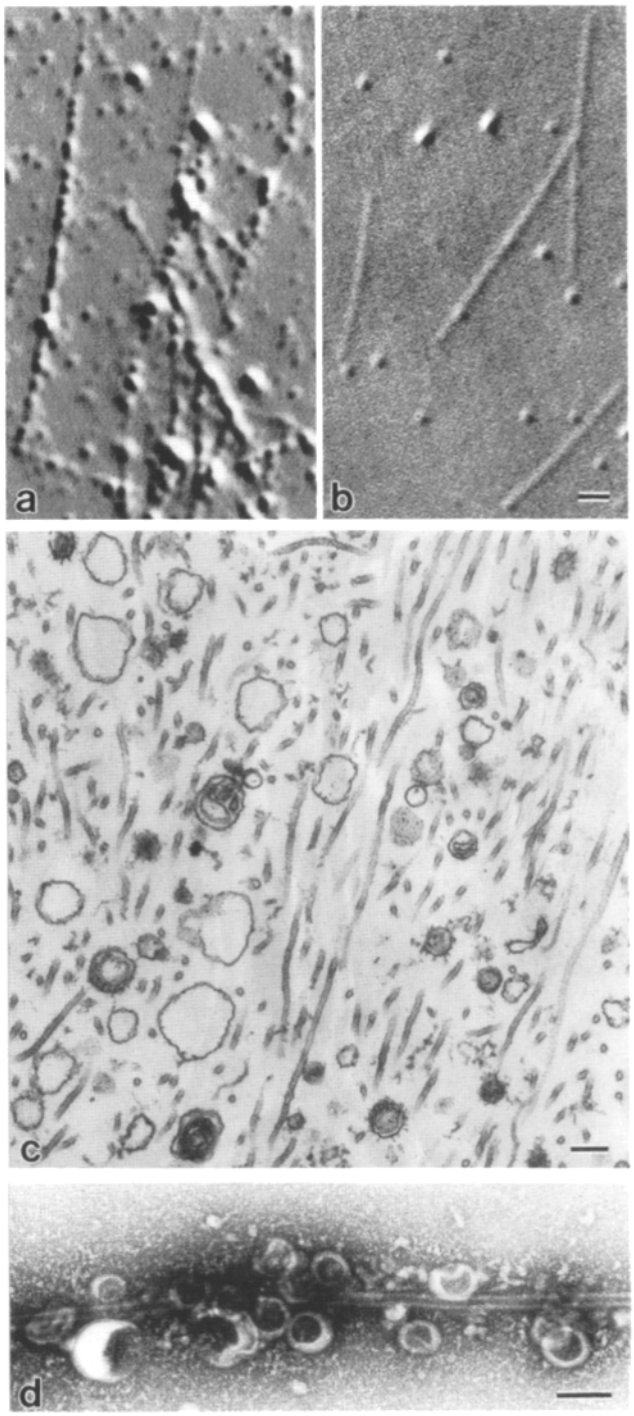

Figure 5. Attachment of KI organelles to microtubules is enhanced in the presence of AMP-PNP (*A*), compared to ATP (*B*). (*A*) Video micrograph of KI organelles + microtubules + AMP-PNP. Coverglass was not initially blocked with casein in order to cause adsorption of organelle-microtubule complexes. (*B*) KI organelles + microtubules + ATP. (*C*) Thin section EM of KI organelle-microtubule complexes showing that the particles which cosediment with the microtubules are membrane delimited organelles. Attachments to microtubules are seen more clearly after negative staining (*below*). (*D*) Negative stain EM of KI organelle–microtubule complexes formed in the presence of AMP-PNP showing attachments of organelles to microtubule. Bars: (*B*) 2 μm; (*C* and *D*) 0.1 μm.

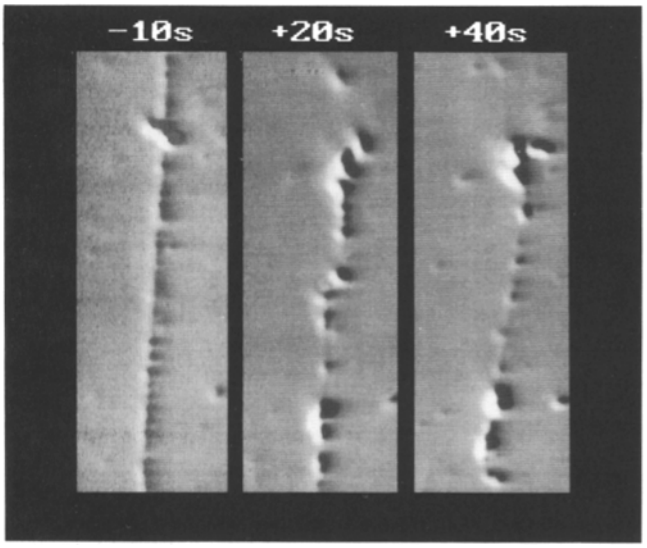

Figure 6. Sequence of video frames showing that AMP-PNP induced rigor linkage of KI organelles to microtubules is reversed in the presence of ATP. The first (*left*) video frame in this series shows a single microtubule decorated with KI organelles in the presence of 4 mM AMP-PNP in taxol containing motility buffer. The organelles in this frame were not moving along the microtubule. This frame was taken 10 s before perfusion of 2 mM ATP into the flow cell. The next two frames show the same microtubule 20 and 40 s after a 15-s perfusion of 2 mM ATP in taxol containing motility buffer. Perfusion of ATP causes most of the organelles to move. All organelle movement was unidirectional, toward the bottom of the picture. All of the single organelles in the center of the microtubule at the +40-s time point were moving. The large clumps are organelles that aggregated while moving along the microtubule; some of these aggregates became immotile, presumably because they attach to the glass. Casein was used to block the coverglass; cytosol was not present.

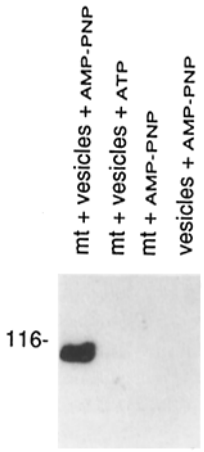

Figure 7. Kinesin copurifies with KI vesicle–microtubule complexes formed in the presence of AMP-PNP. Bovine microtubules (taxol-polymerized from PC-purified bovine brain tubulin; 100 μg/ml) were incubated with KI organelles in the presence of 5 mM AMP-PNP; microtubules and attached organelles (but not unbound organelles) were collected as a pellet after centrifuging through sucrose cushions. Immunoblots of this microtubule pellet indicate the presence of kinesin (approximately one kinesin per organelle). Kinesin was not detected in immunoblots of the organelles left in the supernatant after sedimentation of the organelle-microtubule complexes (not shown). Kinesin is barely detected in the microtubule pellet formed after incubation of microtubules and KI organelles with 2 mM ATP, consistent with the much lower steady-state numbers of attached organelles (Fig. 5 *B*). Organelles attached to microtubules in the presence of ATP were moving at ~2 μm/s. Kinesin is not detected in pellets when either the vesicles or the microtubules are left out of the incubation.

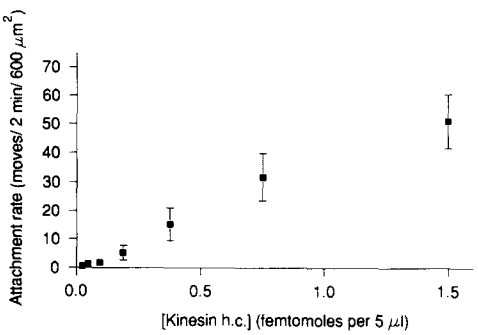

Figure 8. Typical standard curve relating attachment rate of gliding microtubules to known amounts of kinesin heavy chain (purified from squid optic lobe). The rate of attachment of microtubules to the coverglass is roughly proportional to the concentration of kinesin heavy chain in the assay. This assay system, based on that of Howard et al. (1989), can reproducibly detect and quantify <1 femtomole of kinesin and was used in this paper to quantify the amount of kinesin extracted from KI organelle–microtubule complexes.

say (Howard et al., 1989) that detected gliding driven by single kinesin molecules. In this assay, the concentration of kinesin could be predicted with reasonable accuracy from the rate of attachment of microtubules (Fig. 8). The KCl release showed only four microtubules moving in a total of 10 min, corresponding to only trace amounts of kinesin activity. The ATP release showed an average of >30 moving microtubules per 2-min counting interval, indicating the presence of 0.75 femtomoles of kinesin heavy chain per μl of ATP release (Fig. 8) or a total of 12 femtomoles of vesicular kinesin from 100 cm of squid axons. The velocity of the microtubule gliding activity extracted from KI vesicle microtubule complexes was compared with that of purified cytosolic kinesin from squid optic lobe. The velocity of microtubule gliding was measured under the same conditions, i.e., movement was driven by single motors as indicated by nodal point pivoting and dissociation from the glass when the end of the microtubule reached the nodal pivot point (see Howard et al., 1989). The velocity of microtubule gliding driven by the vesicular motor, 0.65 ± 0.08 μm s^{-1} (mean ± SD; n = 14), was similar to the velocity of gliding driven by squid optic lobe kinesin purified from cytosol, 0.56 ± 0.10 μm s^{-1} (n = 13). The kinesin-like gliding activity extracted from the KI vesicle microtubule complexes was ~1% of the total amount of kinesin detected by quantitative immunoblotting of the complexes (77–212 femtomoles of vesicular kinesin per 100 cm of axons).

The molar ratio of kinesins to organelles in the AMP-PNP–induced KI organelle–microtubule complexes was determined by quantitative immunoblotting of the complexes and counting the numbers of organelles per length of microtubules, as detailed in Materials and Methods. In two experiments using different axoplasmic homogenates, we calculated molar ratios of 1.3 and 1.1 kinesins per organelle, assuming no loss of kinesin during gel analysis and immunoblotting, and assuming all of the tubulin was assembled into microtubules. Errors in either of these assumptions would underestimate the numbers of kinesins per organelle.

The mechanism of attachment of kinesin to organelles was explored by resuspending KI organelles in the following extraction solutions: 1M NaCl, 1M KI, 100 mM carbonate (pH

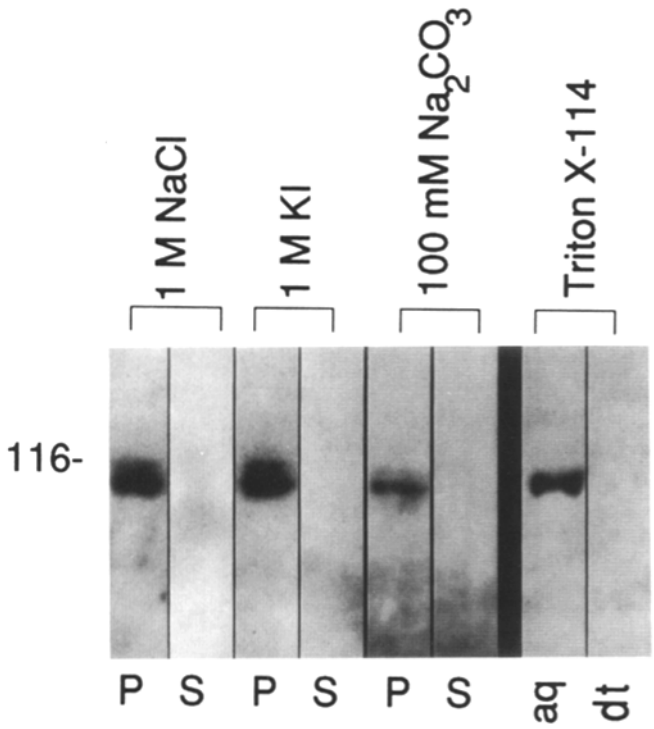

Figure 9. (*A*) Kinesin is not extracted from KI organelles by either 1 M NaCl, 1 M KI, or 100 mM carbonate (pH 11.3). KI organelles, collected as pellets after centrifuging the organelle fraction from sucrose density gradients, were resuspended and incubated for 30 min in one of the three extraction solutions. The bound and free kinesin was separated by centrifugation of the organelles. Immunoblots of the supernatants (*S*) and organelle pellets (*P*), loaded at equivalent volumes for each experiment, indicate that the kinesin is not released from the organelles by any of these procedures. Organelle fraction for the carbonate extraction was derived from 50 cm of axons, while organelles for the other extractions were derived from 100 cm of axons. (*B*) Organelle bound kinesin partitions into the aqueous phase (*a*) but not the detergent phase (*d*) after extraction of organelles with Triton X-114.

11.2), or Triton X-114 (Fig. 9). The organelle-bound kinesin was not extracted by either 1M NaCl, 1M KI, or 100 mM carbonate, indicating that electrostatic interactions alone can not account for the attachment of kinesin to the organelle membrane. However, when KI organelles were solubilized in Triton X-114, and the soluble material was subjected to phase separation, the kinesin partitioned in the aqueous phase, not the detergent phase, indicating that the organelle bound kinesin is not an integral membrane protein in the usual sense (Bordier, 1981).

Discussion

The Presence of Cytosol Is Not Required for Movement of Squid Axon Organelles

Cytosolic microtubule motor proteins were first discovered in the course of investigating the biochemical mechanism of organelle transport (Vale et al., 1985*a*,*b*,*c*). The large cytosolic pools of kinesin and cytoplasmic dynein suggested that continuous exchange might occur between soluble and organelle bound motors, and this view seemed consistent with earlier reports of cytosol induced enhancement of organelle movement (Vale et al., 1985*a*; Schroer et al., 1988). However, a few observations have contradicted the idea that soluble kinesin must be present for organelle movement: Cell fractionation studies show that kinesin co-purifies with microsomal membrane fractions and cannot be salt-extracted (Schmitz, F., K. T. Wallis, and D. B. Murphy. 1990. *J. Cell Biol.* 111:417*a* [Abstr.]), and low levels of organelle movement in the absence of cytosol have been observed previously (Gilbert et al., 1985; Schroer et al., 1988).

The development of a method for preventing adsorption of organelles to the coverglass in the absence of cytosol provided the novel opportunity to perform quantitative studies of organelle movement in the absence of soluble factors. We now find that cytosol has no effect on organelle movement activity of unextracted organelles from squid axons. These organelles retain their ability to move even after several hours of sedimentation on a sucrose gradient, dilution, pelleting, and resuspension in buffer. These results are inconsistent with the commonly held view of rapid exchange of organelle-bound factors and soluble factors under physiological conditions.

A second consequence of quantifying organelle movement in the absence of cytosol was the discovery that the plus-end and minus-end motors are attached to organelle membranes with different affinities. The minus-end motor, cytoplasmic dynein (Vallee et al., 1988; Schnapp and Reese, 1989; Schroer et al., 1989), is extracted in 300 mM KI, suggesting that ionic interactions play a role in attaching it to a protein receptor or to head groups of membrane lipids. On the other hand, significant numbers of plus-end motors remain bound to and functional on organelles even after extraction with 900 mM KI.

It was particularly surprising that the organelle movement activity of organelles extracted with >600 mM KI was unaffected by cytosol, since it was previously reported that immunoadsorption of kinesin from cytosol resulted in a 72% reduction of total organelle movements per minute (Schroer et al., 1988). However, these earlier studies did not consider that up to fourfold more microtubules attach to the coverglass when kinesin is present. In the present study, where the frequency of organelle movements was normalized to microtubule length, cytosolic kinesin did not contribute to the cytosol-induced increase in organelle movement activity in 16 experiments, each utilizing a separate homogenate of axoplasm. Depletion of cytosolic kinesin to <0.5 nM had no significant effect on this increase, and addition of purified optic lobe kinesin to the assay did not influence organelle movement activity, either in the presence or absence of cytosol.

Our findings with squid axoplasmic organelles conflict with several recent studies reporting that cytosolic factors participate in movement of organelles from other organisms (Dabora and Sheetz, 1988*b*; Allan and Vale, 1991; Schroer and Sheetz, 1991; Urrutia et al., 1991). It is possible that the transport of certain classes of membrane compartments do require the presence of cytosolic factors, while other classes do not. The squid axon organelles studied here represent subclass(es) of membrane compartments specialized for movement between the Golgi and the synaptic terminal; while these classes of organelles are highly enriched in axons, they may represent only a small fraction of the membrane compartments derived from crude membrane frac-

tions used in other investigations (Dabora and Sheetz, 1988; Allan and Vale, 1991; Schroer and Sheetz, 1991). It is also possible that the different results arise from differences in procedure. The use of in vitro organelle motility assays to quantify organelle movement is more complex than commonly appreciated, particularly for determining the role of soluble factors in organelle movement. The main problem is that soluble factors can influence a variety of nonspecific parameters that affect the numbers of organelles observed to move per unit time. As shown here, it is particularly important to: (*a*) determine whether organelles move independently of soluble factors; and (*b*) control for soluble factors influencing the distribution of microtubules in the assay.

Identity of the Plus and Minus-end Organelle Motors

The present results confirm earlier studies that cytoplasmic dynein restores minus-end directed movement of KI-extracted organelles (Schnapp and Reese, 1989; Schroer et al., 1989). On the other hand, the results reported here negate earlier reports (Vale et al., 1985*a*,*b*; Schroer et al., 1988), based on reconstitution experiments, that the presence of cytosolic kinesin is required for plus-end directed movement of organelles. The results reported here also negate the earlier conclusion that kinesin is required for both directions of organelle transport (Schroer et al., 1988). Since these previous reconstitution experiments had provided the main evidence for the identity of kinesin as the plus-end organelle motor, it became necessary to reexamine this question.

Although we found that depletion of kinesin from cytosol had no effect on organelle movement activity, it was still possible that kinesin was bound with high affinity to organelles. Indeed, antibodies to the kinesin heavy chain label objects that look like organelles (Pfister et al., 1989), reduce the velocity of organelle movement in dissociated squid axoplasm (Brady et al., 1990), and collapse tubular lysosomes (Hollenbeck and Swanson, 1990). That kinesin is indeed attached with high affinity was verified here by immunoblotting KI organelles with a mAb to the tail domain of the squid kinesin heavy chain.

It is likely that the kinesin which copurifies with KI organelles is the same gene product as cytosolic kinesin. First, the antibody used for immunoblotting reacts with the heavy chain tail domain (Kosik et al., 1990), which is divergent between different kinesin genes (Enos and Morris, 1990; Meluh and Rose, 1990). Second, genetic analysis of kinesin function in *Drosophila* (Saxton et al., 1991) is consistent with the view that this gene product functions in axonal transport. While there exists a minor difference in the electrophoretic mobility of kinesin in the cytosol and organelle fractions (Fig. 4), this may be due to posttranslational modification, rather than differences in primary structure.

Although we attempted to address the question of whether the kinesin associated with KI organelles is active, the results of these analyses were somewhat inconclusive. Microtubule gliding activity similar to that of purified cytosolic kinesin was released by detergent and ATP extraction of KI organelle–microtubule complexes. However, the amount of activity was <2% of what would have been expected if all of the kinesin associated with KI organelle–microtubule complexes was recovered in the extract in active form. There are several possible reasons for this, including inefficient release of the kinesin from the microtubules (by ATP) or from the organelle (by detergent), poor adsorption of the vesicular kinesin to the coverglass compared to cytosolic kinesin, or inactivity of most of the enzyme.

Although the existing data suggest that kinesin is the motor for plus-end organelle movement, it cannot be definitively ruled out that the kinesin detected by immunoblotting KI organelles is partially inactive and another protein is the real plus-end organelle motor. If the plus-end organelle motor is not kinesin, the motor is likely to be a member of the kinesin gene family since KI organelles form rigor attachments with microtubules in the presence of AMP-PNP and are released by ATP. These properties are characteristic of the kinesin family of proteins.

Mechanism of Attachment of Kinesin to Organelles

The main finding of this paper is that the plus- and minus-end organelle motors are firmly attached to organelles under physiological conditions. This is particularly true for kinesin, which resists extraction with carbonate (pH 11.3), 1 M KI, or 1 M NaCl. These findings suggest that ionic interactions are not sufficient to explain the interaction of kinesin with its binding site on the organelle membrane. This is distinct from the interaction of myosin I with phospholipids, which involves ionic interactions with acidic phospholipid head groups; this binding is abolished by 500 nM NaCl (Adams and Pollard, 1989). What is the mechanism of such tight binding? One possibility is that organelle-bound kinesin is acyl modified. A second possibility is that strong hydrophobic interactions with a receptor protein account for its attachment, although this interaction would be unusual in withstanding the chaotrophic effects of 1 M KI. It is unlikely that organelle-bound kinesin interacts directly with the hydrophobic interior of the lipid bilayer, since the protein does not partition into Triton X-114 like most amphipathic integral membrane proteins, and the heavy chain primary structure (Kosik et al., 1990) has no obvious transmembrane domain.

The present paper changes our views on how motor proteins interact with vesicular organelles. Previously, it was thought that the soluble pools of kinesin and dynein exchange freely with the organelle-bound motors. Such a model seemed attractive because it implied that masking kinesin receptors and unmasking dynein receptors at the synaptic terminal could accomplish the redirection of membrane compartments which must take place in this region of the cell. If, however, motors are irreversibly attached to organelles, redirection of membrane compartments can not be fully explained by a dynamic interaction of receptors with the soluble pool of motor proteins.

We thank Jing Ping Xie for suggesting the use of "Blotto" to prevent adsorption of organelles to glass, Gretchen Volk for expert technical assistance, Joe Degiorgies for assisting with the EM experiments, and Chris Godek for performing preliminary experiments with organelles released from KI-organelle microtubule complexes. We are indebted to Dan Wolf for performing some of the microtubule gliding experiments.

This work was supported by grants from the Whitaker Health Sciences Fund and the National Institutes of Health (NS26846) to B. J. Schnapp.

Received for publication 31 March 1992 and in revised form 21 June 1992.

References

Adams, R. J., and T. D. Pollard. 1989. Binding of myosin-I to membrane

lipids. *Nature (Lond.)*. 340:565–588.
Allan, V. J., and R. D. Vale. 1991. Cell cycle control of microtubule-based membrane transport and tubule formation in vitro. *J. Cell Biol.* 113:347–359.
Amos, L., and A. Klug. 1974. Arrangement of subunits in flagellar microtubules. *J. Cell Sci.* 14:533–549.
Block, S. M., L. S. B. Goldstein, and B. J. Schnapp. 1990. Bead movement by single kinesin molecules studied with optical tweezers. *Nature (Lond.)*. 348:348–340.
Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Sci.* 256:1604–1607.
Brady, S. T., Lasek, R. J., and Allen, R. D. 1982. Fast axonal transport in extruded axoplasm from the squid giant axon. *Science.* 218:1129–1131.
Brady, S. T., K. K. Pfister, and G. S. Bloom. 1990. A monoclonal antibody against kinesin inhibits both anterograde and retrograde fast axonal transport in squid axoplasm. *Proc. Natl. Acad. Sci. USA.* 87:1061–1065.
Cooper, M. S., A. H. Cornell-Bell, A. Chernjavasky, J. W. Dani, and S. J. Smith. 1990. Tubulovesicular processes emerge from trans-Golgi cisternae, extend along microtubules, and interlink adjacent trans-Golgi elements into a reticulum. *Cell.* 61:135–145.
Dabora, S. L., and M. P. Sheetz. 1988*a*. Cultured cell extracts support organelle movement on microtubules in vitro. *Cell. Motil. Cytoskeleton.* 10: 482–495.
Dabora, S. L., and M. P. Sheetz. 1988*b*. The microtubule dependent formation of a tubulovesicular network with characteristics of the ER from cultured cell extracts. *Cell.* 54:27–35.
Enos, A. P., and N. R. Morris. 1990. Mutation of a gene that encodes a kinesin-like protein blocks nuclear migration in A. nidulans. *Cell.* 60:1019–1027.
Gibbons, I. R., M. P. Cosson, J. A. Evans, B. H. Gibbons, B. Houck, K. H. Martinson, W.S. Sale, and W. J. Y. Tang. 1978. Potent inhibition of dynein adenosinetriophosphatase and of the motility of cilia and sperm flagella by vanadate. *Proc. Natl. Acad. Sci. USA.* 79:2220–2224.
Gibbons, I. R., A. Lee-Eiford, G. Mocz, C. A. Phillipson, W. J. Y. Tang, and B. H. Gibbons. 1987. Photosensitized cleavage of dynein heavy chains. *J. Biol. Chem.* 262:2780–2786.
Gilbert, S. P., R. D. Allen, and R. D. Sloboda. 1985. Translocation of vesicles from squid axoplasm on flagellar microtubules. *Nature (Lond.)*. 315:245–248.
Gillespie, P., and A. J. Hudspeth. 1991. Chemiluminescence detection of proteins from single cells. *Proc. Natl. Acad. Sci. USA.* 88:2563–2567.
Heidemann, S. R., J. M. Landers, and M. A. Hamborg. 1981. Polarity orientation of axonal microtubules. *J. Cell Biol.* 91:661–665.
Hollenbeck, P. J., and J. A. Swanson. 1990. Radial extension of macrophage tubular lysosomes supported by kinesin. *Nature (Lond.)*. 346:864–866.
Howard, J., A. J. Hudspeth, and R. D. Vale. 1989. Movement of microtubules by single kinesin molecules. *Nature (Lond.)*. 324:154–158.
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.
Meluh, P. B., and M. D. Rose. 1990. KAR3, a kinesin-related gene required for yeast nuclear fusion. *Cell.* 60:1029–1041.
Neal, M. W., and J. R. Florini. 1973. A rapid method for desalting small volumes of solution. *Anal. Biochem.* 55:328–330.
Pfister, K. K., M.C. Wagner, D. S. Stenoien, S. T. Brady, and G. S. Bloom. 1989. Monoclonal antibodies to kinesin heavy and light chains stain vesicle-like structures, but not microtubules, in cultured cells. *J. Cell Biol.* 108: 1453–1463.
Saxton, W. M., J. Hicks, L. S. B. Goldstein, and E. C. Raff. 1991. Kinesin heavy chain is essential for viability and neuromuscular functions in Drosophila, but mutants show no defects in mitosis. *Cell.* 64:1093–1102.
Schliwa, M. 1984. Mechanisms of intracellular organelle transport. *Cell Mus. Motil.* 5:1–82.
Schnapp, B. J. 1986. Viewing single microtubules by video light microscopy. *Methods Enzymol.* 134:561–573.
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.
Schnapp, B. J., R. D. Vale, M. P. Sheetz, and T. S. Reese. 1985. Single microtubules from squid axoplasm support the bidirectional movement of organelles. *Cell.* 40:455–462.
Schroer, T. A., and M. P. Sheetz. 1991. Two activators of microtubule-dependent vesicle transport. *J. Cell Biol.* 115:1309–1318.
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.
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.
Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *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.* 84:513–530.
Urrutia, R., M. McNiven, J. P. Albanesi, D. B. Murphy, and B. Kachar. 1991. Purified kinesin promotes vesicle motility and induces active sliding between microtubules in vitro. *Proc. Natl. Acad. Sci. USA.* 88:6701–6705.
Vale, R. D., B. J. Schnapp, T. S. Reese, and M. P. Sheetz. 1985*a*. Organelle, bead, and microtubule translocations promoted by soluble factors from the squid giant axon. *Cell.* 40:559–569.
Vale, R. D., T. S. Reese, and M. P. Sheetz. 1985*b*. 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. 1985*c*. 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.
Yang, J. T., R. A. Laymon, and L. S. B. Goldstein. 1989. A three-domain structure of kinesin heavy chain revealed by DNA sequence and microtubule binding analyses. *Cell.* 56:879–889.