The Role of Kinesin and Other Soluble Factors in Organelle Movement along Microtubules

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Abstract. Kinesin is a force-generating ATPase that drives the sliding movement of microtubules on glass coverslips and the movement of plastic beads along microtubules. Although kinesin is suspected to participate in microtubule-based organelle transport, the exact role it plays in this process is unclear. To address this question, we have developed a quantitative assay that allows us to determine the ability of soluble factors to promote organelle movement. Salt-washed organelles from squid axoplasm exhibited a nearly undetectable level of movement on purified microtubules. Their frequency of movement could be increased >20-fold by the addition of a high speed axoplasmic supernatant. Immunoadsorption of kinesin from this supernatant decreased the frequency of organelle movement by more than 70%; organelle movements in both directions were markedly reduced. Surprisingly, antibody purified kinesin did not promote organelle movement either by itself or when it was added back to the kinesin-depleted supernatant. This result suggested that other soluble factors necessary for organelle movement were removed along with kinesin during immunoadsorption of the supernatant. A high level of organelle motor activity was recovered in a high salt eluate of the immunoadsorbent that contained only little kinesin. On the basis of these results we propose that organelle movement on microtubules involves other soluble axoplasmic factors in addition to kinesin.

DIRECTED transport of vesicular organelles on microtubules is an important pathway for the movement of materials within eukaryotic cells (Hayden et al., 1983; Schliwa, 1984; Koonce and Schliwa, 1985). Organelles move from the cell body to the synapse and back in the axons of nerve cells. This process is called fast axonal transport, and the two directions of movement are known as anterograde and retrograde transport, respectively (reviewed by Grafstein and Forman, 1980; Schwartz, 1979). The squid giant axon has been used in many recent studies of organelle movement because its axoplasm is easily removed from the axonal membrane (Brady et al., 1982) and is a rich source of organelles and soluble motor activities (Gilbert and Sloboda, 1984; Vale et al., 1985*a*, *b*, *d*). Extruded axoplasm can be dissociated so that movement of organelles on individual microtubules can be observed directly using video-enhanced light microscopy (Allen et al., 1985; Schnapp et al., 1985; Vale et al., 1985*c*). Studies of dissociated axoplasm have revealed that individual organelles move in specific directions along microtubules (Sheetz et al., 1986*b*). Because almost all of the microtubules in an axon are oriented in the same direction (Heidemann et al., 1981), an organelle will be transported to its destination as long as it moves in a constant direction relative to the polarity of the microtubule.

Previous studies using squid axoplasm in vitro led to the discovery and purification of kinesin (Vale et al., 1985*a*, *d*), a soluble protein that causes the gliding of microtubules on glass coverslips and the movement of plastic beads on microtubules. The movements of plastic beads are directed toward the plus end of microtubules, a direction analogous to anterograde transport in neurons (Vale et al., 1985*b*). A second, distinct protein in axoplasm was described that promotes the translocation of beads in the opposite (retrograde) direction (Vale et al., 1985*b*). The soluble retrograde motor has been recently shown to be cytoplasmic dynein (Paschal et al., 1987; Paschal and Vallee, 1987; Vallee et al., 1988; Schroer, T. A., E. R. Steuer, and M. P. Sheetz, manuscript submitted for publication).

Both kinesin and cytoplasmic dynein were identified as force-generating enzymes on the basis of their ability to promote the movement of inert substances (i.e., glass or plastic) on microtubules. It is not clear what role they play in biological processes such as organelle movement. To address this question, it is necessary to assay organelle movement directly. The transport of membranous organelles was previously reconstituted in vitro from fractions of squid axoplasm, and the results of these studies suggested that soluble factors were involved (Vale et al., 1985*d*). However, isolated axoplasmic organelles showed a variable frequency of movement on microtubules before the addition of any soluble axoplasmic components (Gilbert et al., 1985; Vale et al., 1985*d*). The

frequency of movement was increased to a variable degree by the addition of a high speed axoplasmic supernatant or purified kinesin (Vale et al., 1985*a*, *d*). In contrast, purified kinesin did not promote the movement of highly purified synaptic vesicles (Schroer and Sheetz, 1988). These conflicting results could be explained if a low level of factors required for movement were present in the axoplasmic organelle preparations but had been removed from the more extensively purified synaptic vesicles.

To definitively identify and characterize the soluble factors that power organelle movement it was necessary to improve upon the existing assay by making it more reproducible and quantitative. In this report, we describe a procedure for the purification of organelles from squid axoplasm that renders them unable to move by themselves but preserves their ability to bind soluble proteins in a high speed axoplasmic supernatant that drive their transport along microtubules. If kinesin is removed from the supernatant by immunoadsorption, the frequency of organelle movement is significantly reduced, but purified kinesin alone does not promote organelle movement. We show here that other axoplasmic factors in addition to kinesin are required for organelle movement in vitro.

Materials and Methods

Materials

Squid (*Loligo pealeii*) were obtained from the Marine Resources Department at the Marine Biological Laboratory at Woods Hole, MA. Optic lobes and axons were dissected and stored in liquid nitrogen as previously described (Vale et al., 1985*c*). Centricon 30 microconcentrators were obtained from Amicon Corp. (Danvers, MA). Polystyrene latex beads (0.15 μm diam, 2.5% solids) were obtained from Polysciences Inc. (Warrington, PA). Spectra/mesh 52-μm nylon filter was obtained from Spectrum Medical (Los Angeles, CA). Taxol was a gift of Dr. Matthew Suffness, National Cancer Institute. All other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO).

Preparation of Axoplasmic S2 Supernatant and Organelles

S2 supernatant was prepared as described in Vale et al. (1985*d*) with minor modifications. Axons were thawed in motility buffer (175 mM potassium aspartate, 65 mM taurine, 85 mM betaine, 25 mM glycine, 10 mM Hepes, 6.4 mM magnesium chloride, 5 mM EGTA, 2 mM ATP, 1 mM dithiothreitol (DTT), pH 7.2) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml p-tosyl-L-arginine methyl ester). Axon lengths were measured and the axoplasm from ~60 cm of axon was extruded into 60 μl of motility buffer on Parafilm and placed on ice. Motility buffer (150 μl) was added and the axoplasm was homogenized by triturating 50 times with a P-200 Pipetman. The homogenate was centrifuged at 12,000 *g* for 4 min at 4°C. The supernatant (S1; 150–200 μl) was set aside on ice. S1 was used to prepare untreated organelles and axoplasmic S2 supernatant as described below. Salt-washed organelles were prepared from the remaining pellet. The pellet (bulk axoplasm) was rehomogenized with 10 triturations in 150 μl motility buffer containing 0.6 M potassium iodide (KI)[1]. The 0.6 M KI homogenate was centrifuged as above and the supernatant (KI-S1; ~200 μl) was removed. S1 and KI-S1 were immediately diluted 1:1 in motility buffer and each layered onto two 600-μl discontinuous sucrose gradients. Sucrose solutions were made in motility buffer and the gradients were composed of: 100 μl 10% sucrose, 100 μl 12% sucrose, 140 μl 15% sucrose, and 60 μl 45% sucrose. The gradients were centrifuged at 114,000 *g* for 90 min (4°C) in a SW50.1 rotor. The final supernatant (S2 or KI-S2; 140–190 μl) was collected from the top of each gradient and the remaining gradient was collected from the bottom in a total of four fractions. Organelles were present in the 15% sucrose layer (fractions 2 and 3). The organelles were concentrated by diluting 125 μl of fraction 2 with an equal volume of motility buffer and centrifuging at 190,000 *g* for 35 min. The organelle pellet was resuspended in 5 μl of motility buffer.

1. *Abbreviation used in this paper*: KI, potassium iodide.

Movement Assays

All samples were analyzed for movement using video-enhanced differential interference contrast microscopy as described by Schnapp (1986). Still photographs were made directly from the video monitor.

Microtubules on glass: the movement of purified (1 M NaCl washed, microtubule-associated protein-free, taxol-stabilized) optic lobe microtubules on glass coverslips was assayed as described in Vale et al. (1985*d*). Briefly, 1 μl of microtubules (0.16 mg/ml protein) was mixed with 3 μl of a test sample, placed on a glass coverslip, and examined for movement.

Beads on centrosomes: the movement of 0.15 μm polystyrene latex beads was assayed as described in Vale et al. (1985*b*), except that 5 μl of each test sample and 1 μl beads (1:20 dilution) were used.

Organelles on bulk microtubules: 1 μl of organelles, 1 μl of purified optic lobe microtubules (as above; 0.8 mg/ml protein), and 3 μl of a test sample were mixed then placed on a glass coverslip and examined for movement. Video fields were selected that contained a mat of microtubules on the glass coverslip. Organelles in solution diffused into the vicinity of the microtubules and moved along them if a supernatant containing organelle motor activity was used. The same volume of organelles was used in every assay so that the number of organelles in each video field was fairly constant, although it was not possible to determine the absolute number of organelles present. Large aggregates of organelles were occasionally observed in the assays.

Organelles on centrosomes: 1 μl of organelles and 3 μl of a test sample were added to a centrosome preparation as previously described (Vale et al., 1985*b*).

Quantitative Measurement of Organelle Movement

Organelle motor activity (frequency of organelle movement): a field containing a mat of 50–100 microtubules was selected and a 1–4-min video recording was made. The organelle movements that occurred in the video field were counted and normalized with respect to time to give the frequency of organelle movements (number of movements per minute). An event was considered to be a movement only if the organelle traveled in a single direction over a distance of at least 3 μm. Each moving organelle was counted only once regardless of the distance moved. At least three separate video fields were analyzed per sample, for an overall total of 10 min. In a given experiment the organelle motor activity of a particular sample was calculated to be the mean of the frequencies determined for all video fields. Data analysis was performed after the entire experiment was completed. Velocities of organelle movement were measured using the computer program described in Sheetz et al. (1986*a*).

Adsorption of Supernatants with Antikinesin Antibody

MCK-1 IgG (antikinesin monoclonal antibody) or control mouse IgG was prepared and coupled to CNBr-activated Sepharose CL-4B as described by Vale et al. (1985*b*; 5 mg IgG/ml resin). The resin was equilibrated with fresh motility buffer immediately before use. S2 supernatant was incubated with an equal volume of resin on an end-over-end rotator for 2 h at 4°C; 30 μl of control resin and 150–250 μl of antikinesin resin were used. Mock-adsorbed S2 supernatants were recovered by centrifuging the Sepharose slurry through a 52-μm Spectra/mesh nylon filter to allow recovery of all the fluid from the resin. Kinesin-depleted S2 supernatants were obtained by pelleting the resin and removing the supernatant.

The high salt eluates of the antikinesin resin were obtained by resuspending the resin in an equal volume of high salt elution buffer (motility buffer containing 0.5 M potassium chloride and 10 mM ATP). The resin was mixed by rotation for 45 min at 4°C and the high salt eluate supernatant recovered by centrifugation through a nylon filter. The high salt eluate was desalted by spin dialysis through Sephadex G-25 resin preequilibrated with motility buffer (Neal and Florini, 1973) and concentrated to 50 μl using a Centricon 30 microconcentrator. The desalted, concentrated high salt eluate (50 μl) was assayed for microtubule, bead, and organelle movement. Rinse fractions were prepared by the same method except that normal motility buffer was used instead of high salt elution buffer.

Kinesin Purification

Microtubule-affinity purification: kinesin was purified from squid optic lobes by microtubule affinity as previously described (Vale et al., 1985*a*).

Antibody-affinity purification: kinesin was purified from squid optic lobes using MCK-1 antikinesin Sepharose resin (described by Vale et al., 1985*b*). S3 supernatant (a high speed supernatant that is depleted of tubulin by taxol-induced polymerization followed by centrifugation) was prepared from 40 g of squid optic lobes as previously described (Vale et al., 1985*a*). The S3 (~50 ml) was passed over a 2-ml antikinesin column (prepared as in Vale et al., 1985*b*) at a flow rate of ≤0.5 ml/min. The resin was rinsed with 20 ml of motility buffer (without ATP), followed by 10 ml of 1 M KCl in motility buffer (without ATP). Kinesin was eluted from the antikinesin column with 10–20 ml of 1 M KCl and 100 mM diethylamine, pH 11.5. The kinesin (final protein concentration ≅0.1–0.2 mg/ml) was immediately desalted into 80 mM Pipes, 1 mM EGTA, 1 mM EDTA, pH 6.8, then stored on ice and used within 48 h of preparation.

Calculation of Kinesin/Organelle Mass Ratio

We have estimated the concentration of kinesin by quantitative immunoblotting in the high salt eluate to be <5 μg/ml and in S2 to be >50 μg/ml, and the KI-washed organelle preparation contains ~500 μg/ml protein (data not shown). A standard video microscope assay sample contains a 3:1 ratio (by volume) of supernatant to organelles, so the mass ratio of kinesin/organelles is <1:33 in the high salt eluate and >1.0:3.3 in S2.

SDS-PAGE and Immunoblots

Discontinuous SDS-PAGE was performed as described by Laemmli (1970). Silver staining was performed as described by Merril et al. (1981). Immunoblotting was performed as described by Towbin et al. (1979) using a rabbit antisquid kinesin antiserum. The 110,000-M_r kinesin polypeptide was the only species that reacted with the antikinesin antiserum in the samples described here.

Quantitative comparison of mock-adsorbed and kinesin-depleted S2: varying concentrations of each of the two supernatants were run on SDS-PAGE and immunoblotted. The blot was analyzed by video densitometry. The ratio of kinesin-depleted S2:mock-adsorbed S2 required for an equivalent amount of kinesin immunoreactivity was ~1:10, indicating that 90% of the kinesin had been removed from S2 by the antikinesin resin.

Video Densitometry

Video densitometry was performed using a system developed by Stephen G. Turney (Sheetz laboratory). Measurements were made directly from the immunoblots. Scans of individual lanes were extracted from digitized, RS-170 format video pictures generated by a SONY AVC-D1 CCD camera and stored in an Imaging Technology Inc. IP-512 image processor. Variations in background intensity due to nonuniform illumination and gel imperfections were corrected by subtracting the optical density profile of a protein-free lane from the profile of the sample lane. Background subtraction, integration of peaks and lane comparisons were performed by a FORTRAN program running on a Digital PDP-11/73 computer to which the image processor memory was tied.

The kinesin concentrations of different supernatants were determined from immunoblots. The total staining intensity of each band was determined by multiplying the average staining intensity per scan line times the width of the band.

Results

In Vitro Assay for Organelle Movement

We have improved upon our previous reconstituted system for organelle movement (Vale et al., 1985*d*) by further purifying and concentrating the organelles. Organelles and supernatant fractions were prepared from squid axoplasm as depicted in Fig. 1. A cytosol fraction containing factors that promote organelle movement was prepared from squid axoplasm extruded and homogenized in motility buffer (Vale et al., 1985*d*; see Materials and Methods). To prepare KI-extracted organelles, a second sample of axoplasm was homogenized in motility buffer containing 0.6 M KI. The axoplasmic homogenates (with and without KI) were diluted twofold, layered onto discontinuous sucrose gradients, and

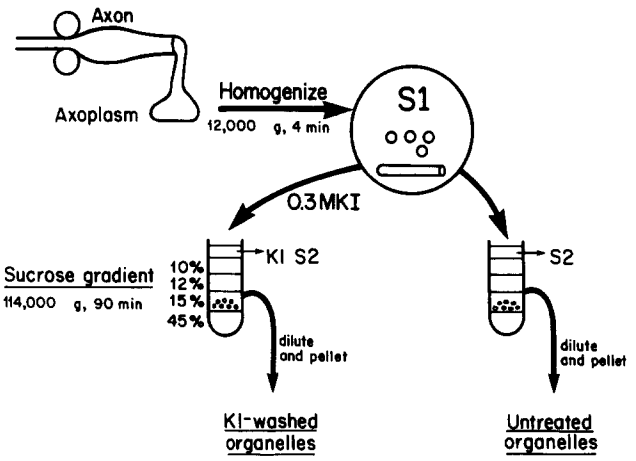

Figure 1. Schematic representation of the organelle preparation. KI-washed organelles, untreated organelles, and S2 supernatant are prepared on discontinuous sucrose gradients. Extruded axoplasm is homogenized in motility buffer or motility buffer containing 0.6 M KI, and a low speed supernatant is applied to the sucrose gradient. After ultracentrifugation, organelles have sedimented into the 15% sucrose layer and organelle motors remain in the final supernatant (S2). The experimental protocol is described in detail in Materials and Methods.

subjected to ultracentrifugation. The supernatant from the control gradient (S2 supernatant) was used as the source of the soluble factors required for organelle movement. Organelles were collected from the 15% sucrose layer of each gradient, diluted, and concentrated by pelleting and resuspension. Their movement on purified squid optic lobe microtubules was assayed by video-enhanced microscopy.

The qualitative features of the organelle movements in the assays to be described here were indistinguishable from those in dissociated axoplasm or the original organelle motility assay (Vale et al., 1985*c*, *d*). Organelle movements on microtubules could be easily distinguished from the Brownian motion of organelles free in solution. Organelles were clearly observed to bind to and move along microtubules traveling 3–20 μm before moving out of the field or dissociating into the buffer. Most organelles moved in continuous paths, often switching between intersecting microtubules. The isolated organelles moved unidirectionally, like those in intact and dissociated axoplasm (Brady et al., 1982; Vale et al., 1985*c*, *d*).

Organelle motor activity was defined as the frequency of organelle movements observed under standardized experimental conditions (described in Materials and Methods). The mean activity was determined for a 10-min period during which at least three different video fields were sampled. Fig. 2 is a representative video field indicating the density of microtubules and organelles present in samples used for quantitative analysis. As reported previously (Vale et al., 1985*d*), organelles from the control gradient (untreated organelles) moved occasionally on microtubules in the absence of S2 supernatant (~0.5 movements per minute), and the frequency of movement was increased three- to fivefold (to 2.3 ± 0.7 movements per minute; mean ± SD) when S2 was added. In contrast, organelles prepared in the presence of 0.6 M KI (KI-washed organelles) moved very infrequently

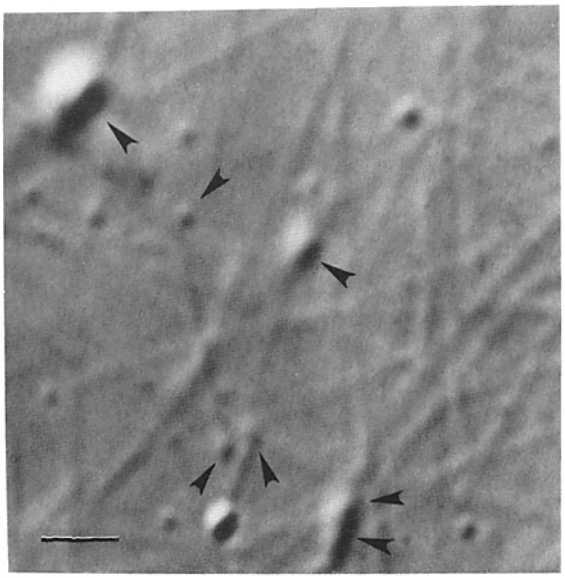

Figure 2. Typical microscopic field used to determine the frequency of organelle movement in vitro. KI-washed organelles were mixed with S2 supernatant and optic lobe microtubules then viewed by video-enhanced microscopy. The frequency of organelle movements was determined in a region of the sample containing a standard density of microtubules and organelles equivalent to what is shown here. In this field, 7 organelles (*arrowheads*) were moving on microtubules. Bar, 1 μm.

without S2 (<0.5 movements per minute) and much more frequently when S2 supernatant was added (11.1 ± 2.3 movements per minute; mean ± SD; Table I). Thus, the movement of KI-washed organelles was increased more than 20-fold by the addition of S2 supernatant. When the behavior of untreated and KI-washed organelles was compared in the same experiment, KI-washed organelles moved with a two- to fivefold higher frequency in the presence of S2 supernatant (Table I).

The protein composition of KI-washed organelles was less complex than that of the untreated organelles (Fig. 3). The most notable difference in the KI-washed preparation was the absence of polypeptides of approximate M_r of 220,000, 74,000, 65,000, and 60,000 kD that were the major proteins present in the untreated axoplasmic organelles (Fig. 3, lane *B*). These are likely to be neurofilament proteins as judged

Table I. Comparison of the Movement of Untreated and KI-washed Organelles

Type of organelle	S2 supernatant	Relative organelle movement (in percent) Experiment 1	2	3	4	5	Average
KI-washed	−	ND	ND	<5	<5	<5	<5
KI-washed	+	100	100	100	100	100	100
Untreated	+	50	20	30	ND	ND	33

The movement of KI-washed and untreated organelles are compared in columns 1-5. To make comparisons between experiments, the frequency of organelle movement was normalized relative to the frequency observed for KI-washed organelles in each experiment. The average of all experiments is given in the right hand column.

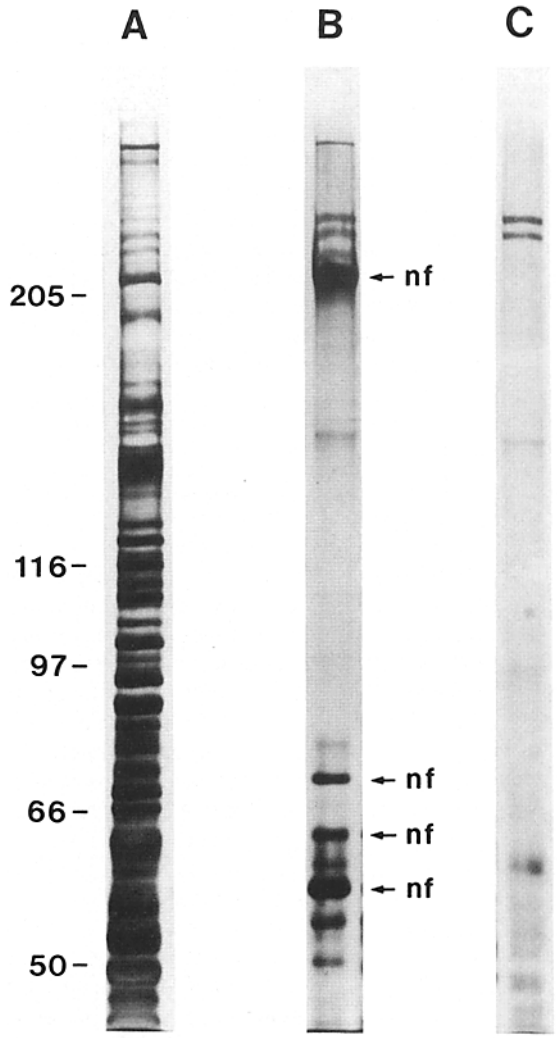

Figure 3. Polypeptide compositions of S2 supernatant, untreated, and KI-washed organelles. SDS-PAGE of organelles prepared in the absence (untreated) or presence (KI-washed) of 0.6 M KI. The 6% polyacrylamide gel was stained with silver nitrate. Lane *A*, S2 supernatant. Lane *B*, untreated organelles. Lane *C*, KI-washed organelles. Equal volumes of organelles were loaded in lanes *B* and *C*. The electrophoretic positions of squid neurofilament proteins are indicated in lane *B*.

by several criteria. They have the same molecular masses as squid axon neurofilament proteins (Lasek et al., 1979; Gallant et al., 1986), and the 220,000-M_r protein is recognized by an antineurofilament antibody in immunoblots (data not shown). Structures resembling neurofilaments in electron micrographs are observed to be associated with untreated organelles (Vale et al., 1985*d*; see also Gilbert and Sloboda, 1984). Finally, squid neurofilaments are known to be disassembled by high salt treatment (Zackroff and Goldman, 1980).

Treatment with KI abolished the tendency of organelles to move in the absence of supernatant and enhanced the effect of S2 without any other demonstrable effect on organelle movement. The movements of untreated and KI-washed organelles were qualitatively (see above) and quantitatively identical. KI-washed organelles moved along microtubules

at the same velocity as untreated organelles or endogenous organelles in intact and dissociated axoplasm (Brady et al., 1982; Vale et al., 1985*c*; mean velocity ± SD = 1.50 ± 0.31 μm/s). KI-washed organelles also behaved indistinguishably from untreated organelles in the directionality assay (Vale et al., 1985*b*). The organelles moved either anterogradely or retrogradely on microtubules regrown from centrosomes (Mitchison and Kirschner, 1984; Vale et al., 1985*b*) and a similar mean velocity was observed for movement in the two directions. A modest (albeit variable) preference for retrograde organelle movement was observed (mean = 65% retrograde movement; a range of 44–87% was observed in four preparations). The KI-washed organelles were used in all the studies described below.

Role of Kinesin in Organelle Movement

As mentioned earlier (Table I), S2 supernatant markedly increased the frequency of KI-washed organelle movement. The S2 supernatant contains the anterograde microtubule-based motor, kinesin, and since S2 supports anterograde movement of KI-washed organelles, it seemed possible that kinesin was the anterograde organelle motor. To determine whether organelle movement was dependent on kinesin present in S2, a monoclonal antikinesin IgG immobilized on Sepharose CL-4B resin (antikinesin resin; see Vale et al., 1985*b*) was used to adsorb kinesin from the supernatant (Fig. 4). A nonspecific mouse IgG Sepharose was used as control resin. S2 was incubated with the antikinesin or control resin, and the kinesin-depleted and mock-adsorbed S2 supernatants were assayed for their kinesin content (by immunoblot) and for the ability to promote organelle movement. As previously observed, the antikinesin resin removed most (~90%) of the kinesin from the S2 supernatant (Fig. 4, *A* and *B*; see Materials and Methods). In the organelle motility assay, the mock-adsorbed S2 had equivalent activity to the unadsorbed S2 (12.3 ± 2.4 movements per minute), supporting approximately equal numbers of organelle movements in the anterograde and retrograde directions. The immunoadsorption procedure per se did not appear to have an inhibitory effect on organelle movement, since the organelle motor activities of the mock-adsorbed and unadsorbed S2 were indistinguishable. Microtubule gliding on the glass coverslip and plastic bead movement (see Vale et al., 1985*a*, *b*) were also unaffected by mock-adsorption.

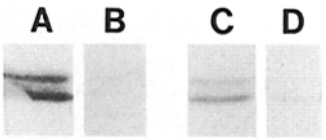

Figure 4. Kinesin content of adsorbed S2 supernatants and high salt eluate. Supernatants used in the organelle movement assay were run on a 7.5% polyacrylamide gel and analyzed by immunoblotting with a rabbit antiserum that recognizes the 110,000-M_r polypeptide of kinesin. No proteins other than kinesin were stained by the antikinesin serum. *A*, mock-adsorbed S2 supernatant (10 μl). *B*, kinesin-depleted S2 supernatant (10 μl). *C*, kinesin-depleted S2 supernatant (25 μl). *D*, high salt eluate of the antikinesin resin after adsorption with S2 supernatant (25 μl). Since the same volume (3 μl) of each sample was used in the motility assay (see Materials and Methods), the immunoblot shows the relative amounts of kinesin present in the assay samples. The kinesin-depleted S2 supernatant (*B* and *C*) supported neither organelle nor microtubule and anterograde bead movement, whereas the high salt eluate (*D*) supported the movement of organelles but not microtubules or beads.

Table II. Organelle Motor Activity of Different Supernatants

Test supernatant	Frequency of movement (in movements per minute)	Organelle motor activity (as percent of control)	*n*
Control (mock-adsorbed S2)	12.3 ± 2.4	100.0	6
Kinesin-depleted S2	3.5 ± 1.5	28.5	6
Purified kinesin	0.3 ± 0.1	2.4	2
Rinse	3.6 ± 2.5	29.3	3
High salt eluate	7.4 ± 2.6	60.2	4
High salt eluate + kinesin	9.0 ± 2.7	73.1	2

The mean frequency of organelle movement (± SD) driven by different supernatants was determined as described in Materials and Methods. This value is also given as the percent of the mean organelle motor activity of the control (mock-adsorbed S2). *n* is the number of separate experiments performed; multiple video fields were analyzed for each experiment (see Materials and Methods). Kinesin was removed from S2 supernatant by immunoadsorption, and kinesin purified by antibody affinity (see Materials and Methods) was used.

In contrast, the kinesin-depleted S2 no longer supported microtubule movement on the glass coverslip and promoted predominantly retrograde movement of plastic beads on centrosome microtubules. Since at least half of the organelle movements driven by S2 were retrograde, we expected that kinesin-depleted S2 would contain ~50% of the organelle motor activity of the mock-adsorbed S2. Surprisingly, kinesin-depleted S2 had only 28% (on average) of the activity of the control (Table II). Organelle movement in both directions was inhibited to the extent that we could not measure the direction preference accurately.

These results led us to ask whether kinesin alone could induce organelle movement. Kinesin was purified from squid optic lobe by microtubule affinity (Vale et al., 1985*a*) or by affinity purification with the antikinesin resin (see Materials and Methods). Neither kinesin preparation promoted organelle movement although microtubule gliding on the glass coverslip was observed. This suggested that kinesin might require additional factors present in kinesin-depleted S2 to cause organelle movement. However, the ability of the kinesin-depleted S2 to cause organelle movement was not increased by the addition of kinesin (purified by antibody affinity; data not shown). We now had to consider the possibility that the antibody resin had removed from the S2 supernatant not only kinesin, but other factors required for organelle movement as well. It was possible that these factors had complexed with the kinesin bound to the antikinesin resin and were thus removed from the S2 supernatant.

To determine whether factors required for organelle movement could be recovered from the antikinesin resin, resin that had been preincubated with S2 supernatant was either rinsed with motility buffer alone or incubated with high salt elution buffer (motility buffer containing 0.5 M potassium chloride and 10 mM ATP). The rinse and high salt eluate were desalted, concentrated, and assayed for microtubule gliding as well as bead and organelle movement. The rinse did not promote microtubule gliding on the glass coverslip

or bead movement on microtubules and had approximately the same organelle motor activity as the kinesin-depleted S2 (29% of the control; Table II). The high salt eluate did not promote microtubule gliding or bead movement. However, this fraction contained ~60% of the organelle motor activity of the control (Table II), significantly more than the kinesin-depleted S2 or rinse ($P < 0.05$ as determined by t test).

The recovery of organelle motor activity in the high salt eluate could not be explained simply by the elution of kinesin from the resin, since the high salt eluate contained less kinesin than the kinesin-depleted S2 (Fig. 4, *C* and *D*). The low amount of kinesin in the high salt eluate did not appear to be limiting because the addition of antibody-affinity purified kinesin did not significantly stimulate organelle motor activity (Table II). In preliminary studies the high salt eluate promoted both anterograde and retrograde organelle movements.

By SDS-PAGE analysis, the polypeptide compositions of the rinse and the high salt eluate were the same as the kinesin-depleted S2, indicating that a significant amount of kinesin-depleted S2 was present in the antikinesin resin before elution. To reduce the background level of kinesin-depleted S2 in the high salt eluate, the resin was washed with motility buffer before preparation of the high salt eluate or rinse. This high salt eluate had similar organelle motor activity to the original high salt eluate (67% of the control; 8.2 ± 2.0 movements per minute). The high salt eluate was enriched in a subset of polypeptides when compared to the rinse, however these polypeptides represented only a small fraction of the total protein (data not shown). Therefore, we could not be confident which, if any, of the polypeptides enriched in the high salt eluate were involved in organelle movement.

Discussion

Microtubule gliding and bead movement assays have recently led to the purification of several microtubule-based motors (Vale et al., 1985*a*; Kuznetsov and Gelfand, 1986; Kachar et al., 1987; Lye et al., 1987; Porter et al., 1987; Paschal et al., 1987; Bloom et al., 1988; Euteneuer et al., 1988). However, it is not clear whether any of these proteins are directly involved in microtubule-based organelle movement. Microtubule gliding and bead movement might well have requirements different from those for organelle movement. Motors bind nonspecifically to glass and plastic to produce microtubule and bead movement; purified preparations of motors are sufficient for movement in these assays and relatively high concentrations of motors are required for microtubule gliding. In contrast, it is likely that organelles bind motors specifically via protease-sensitive (Gilbert and Sloboda, 1984; Schroer et al., 1985; Vale et al., 1985*d*) membrane receptors. Our present results indicate that while purified kinesin (from optic lobe) by itself is not capable of driving organelle movement, kinesin can drive organelle movement in the presence of other, as yet unidentified soluble factors.

It is obvious that microtubule and bead assays do not provide adequate criteria for identification of organelle motors. In this report we have measured organelle movement directly using a quantitative reconstitution assay. Our original assay for organelle movement (Vale et al., 1985*d*) had the disadvantage that the frequency of organelle movement in the absence of soluble supernatant (the assay background) was high and variable. Although a high speed axoplasmic supernatant or purified kinesin seemed to increase the frequency of organelle movement, the magnitude of the enhancement was also variable (1–30-fold; Vale et al., 1985*a*). To develop a more reproducible assay we purified the organelles further by KI washing, concentrated the organelles by centrifugation, and standardized the concentrations of microtubules and organelles used in the assay. KI-washed organelles are free of neurofilaments and do not move unless soluble factors are added. Because of the improved signal/noise ratio (the difference between the frequency of organelle movement in the presence and absence of supernatant), we were able to use this assay to measure reliably the intermediate levels of activity obtained in subfractions of S2 supernatant.

Role of Kinesin and Other Factors in Organelle Movement

It was previously reported that purified kinesin could stimulate the movement of partially purified organelles (Vale et al., 1985*a*). We show here that kinesin does not promote the movement of more extensively purified (KI washed) organelles, and that additional factors appear to be required for organelle movement. Similar results were obtained with highly purified elasmobranch synaptic vesicles which moved in much the same way as the endogenous organelles when introduced into squid axoplasm, suggesting that the foreign vesicles bound to and were moved by squid organelle motors (Schroer et al., 1985). In a reconstituted assay with purified kinesin, the synaptic vesicles did not move on microtubules (Schroer and Sheetz, 1988). These results are consistent with our current idea that both kinesin and additional axoplasmic components are necessary for organelle movement.

Purified optic lobe kinesin will not substitute for the factors that are removed from axoplasmic supernatants by the immunoadsorbent. We have considered alternative explanations for this key result. It is possible that optic lobe kinesin is not competent to drive organelle movement (perhaps this activity is lost during purification) while the axoplasmic kinesin in the high salt eluate is by itself sufficient for the organelle movement we observe. However, this does not readily explain the fact that the kinesin-depleted S2 contains more kinesin but significantly less organelle motor activity than the high salt eluate. A simpler explanation of our results is that the high salt eluate contains other factors which allow organelle movement to occur in the presence of a low amount of kinesin.

It is not necessarily surprising that we were able to detect organelle motor activity in a fraction that contained little kinesin and promoted neither bead nor microtubule movement. Organelles may require only a few or even a single motor for movement to occur. We have estimated the minimum amount of kinesin necessary to move an organelle as follows. The M_r of kinesin is 3×10^5 (Cohn et al., 1987; Bloom et al., 1988) and the M_r of a 160-nm-diam organelle is estimated to be 7.2×10^8 (derived from Wagner et al., 1978). The ratio of kinesin mass to organelle mass in a 1:1 complex is therefore 1:2,400. According to this threshold value calculation, a sample containing organelles mixed with the high salt eluate contains >50 kinesin molecules per organelle (see Materials and Methods for details). Our results are consistent with the hypothesis that kinesin is the anterograde or-

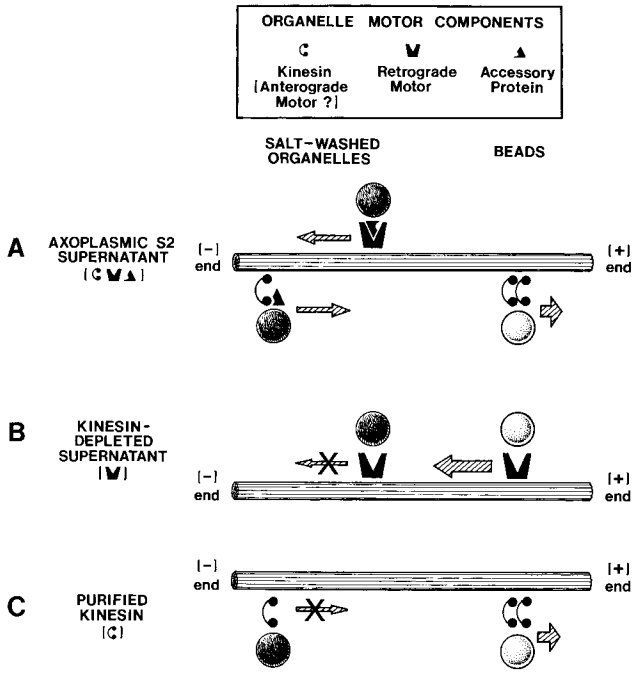

Figure 5. Hypothetical model of organelle and bead movement. The movements of organelles and plastic beads observed in the in vitro assay are compared here. Arrows indicate the directions of movement. The length of each arrow indicates the relative velocity (see text and Vale et al., 1985*a*, *b*), and the breadth of each arrow indicates the relative amount of movement that occurs in the direction specified. (*A*) Axoplasmic S2 supernatant contains an anterograde motor (kinesin), a retrograde motor (cytoplasmic dynein?), and other soluble factors. We propose that organelles require both kinesin and other factors for anterograde movement. (The same may apply for retrograde organelle movement.) Because all the necessary components are present, both anterograde and retrograde organelle movements are observed in axoplasmic S2 supernatant. Beads move primarily anterogradely (probably because they nonspecifically bind kinesin better than the retrograde motor; see also Vale et al., 1985*d*). (*B*) Immunoadsorption of S2 supernatant with the antikinesin resin removes both kinesin and the other factors necessary for organelle movement. Since the kinesin-depleted supernatant still contains the retrograde motor, retrograde bead movement is observed (see also Vale et al., 1985*b*), but retrograde organelle movement does not occur. (*C*) Purified kinesin promotes anterograde bead movement (Vale et al., 1985*b*), but anterograde organelle movement does not occur in the absence of the other necessary factors.

ganelle motor, although it remains possible that the high salt eluate contains an unidentified anterograde motor that is driving the movements we observe.

A hypothetical model that explains our results is depicted in Fig. 5. Organelle movement is more complicated than bead movement, since bead movement can be driven by either purified kinesin or the kinesin-depleted supernatant, although neither of these preparations support organelle movement (Fig. 5, *B* and *C*; Vale et al., 1985*a*, *b*). According to the model, organelles do not move unless a mechanochemical motor (kinesin or cytoplasmic dynein) and other soluble factors (accessory proteins, depicted as a single species for simplicity) are present. S2 supernatant (*A*) and the high salt eluate of the antikinesin resin appear to contain all the components necessary for organelle movement. The motors and other factors may act by forming an organelle motor complex. We are currently working to purify these other factors to reconstitute organelle movement entirely from purified components.

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