

Two Activators of Microtubule-based Vesicle Transport

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Abstract. Cytoplasmic dynein purified by nucleotide dependent microtubule affinity has significant minus end-directed vesicle motor activity that decreases with each further purification step. Highly purified dynein causes membrane vesicles to bind but not move on microtubules. We exploited these observations to develop an assay for factors that, in combination with dynein, would permit minus end-directed vesicle motility. At each step of the purification, non-dynein fractions were recombined with dynein and assayed for vesicle motility. Two activating fractions were identified

by this method. One, called Activator I, copurified with 20S dynein by velocity sedimentation but could be separated from it by ion exchange chromatography. Activator I increased only the frequency of dynein-driven vesicle movements. Activator II, sedimenting at 9S, increased both the frequency and velocity of vesicle transport and also supported plus end movements. Our results suggest that dynein-based motility is controlled at multiple levels and provide a preliminary characterization of two regulatory factors.

CYTOPLASMIC dynein is a microtubule-based, mechanochemical ATPase found in virtually all animal cells (Amos, 1989; Collins and Vallee, 1989; Euteneuer et al., 1988; Gilbert and Sloboda, 1989; Koonce and McIntosh, 1990; Lye et al., 1987; Neely and Boekelheide, 1988; Paschal et al., 1987; Schnapp and Reese, 1989; Schroer et al., 1989; Verde et al., 1991). Ultrastructurally and biochemically similar to axonemal dynein, cytoplasmic dynein is a microtubule-activated ATPase that powers movement toward the minus ends of tubulin filaments (Paschal et al., 1987; Paschal and Vallee, 1987; Sale and Satir, 1977). While axonemal dyneins function solely in ciliary and flagellar motility, serving to drive sliding of adjacent microtubule doublets, cytoplasmic dyneins are believed to power a variety of microtubule-based motile processes. As in vitro, cytoplasmic dynein within cells is thought to cause minus end-directed movement of membrane vesicles (Schnapp and Reese, 1989; Schroer et al., 1989). Examples of minus end motility include retrograde axonal transport, movement of endocytic cargo from the endosome to the lysosome (Gruenberg et al., 1989), and transport from the basolateral to apical surface of epithelia (Bomsel et al., 1990; reviewed in Schroer and Sheetz, 1991). Cytoplasmic dynein is associated with kinetochores and with microtubules of the mitotic spindle (Pfarr et al., 1990; Steuer et al., 1990; Wordeman et al., 1991), suggesting a role in chromosome-to-pole movements during prometaphase and anaphase as well as overall spindle motility. Dynein may also function as a force producer for neurite

extension and other microtubule-dependent cytoplasmic rearrangements. Given the range of possible transport functions, cytoplasmic dynein activity is likely to be subject to a host of control mechanisms.

Purified cytoplasmic dynein produces movement of microtubules on glass coverslips and plastic beads on microtubules by virtue of its well-characterized microtubule-stimulated ATPase activity (Euteneuer et al., 1988; Lye et al., 1987; Paschal et al., 1987). Such assays are useful for determining kinetic parameters of enzyme activity and for following dynein through its purification but do not necessarily provide insight into intracellular function. With this in mind, we have developed an in vitro assay that allows us to study the vesicle motor activity of cytoplasmic dynein (Schroer et al., 1988; Schroer et al., 1989). Assay samples are comprised of purified microtubules and membrane vesicles extracted with high salt to prevent their movement unless a soluble fraction is added. Activity of the soluble fraction is measured as the frequency of vesicle movements observed by video-enhanced differential interference contrast (DIC)¹ microscopy.

Using the vesicle motility assay, cytoplasmic dynein was shown to be necessary for minus end-directed movement (Schroer et al., 1989). A dynein-containing cytosol from chick embryo fibroblasts moved vesicles predominantly toward the minus ends of microtubules, and vanadate-mediated UV photocleavage (Gibbons et al., 1987), a treatment that inactivates dynein selectively (Porter, M. E., P. M. Grissom, C. M. Pfarr, and J. R. McIntosh. 1987. *J. Cell Biol.* 105:33a), resulted in profound inhibition of minus end transport. Addition of purified dynein to inactivated cytosol

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1. *Abbreviation used in this paper:* DIC, differential interference contrast.

completely restored vesicle motility although dynein by itself had little vesicle motor activity. We concluded that dynein was necessary but not sufficient for minus end vesicle transport and that other factors in the cytosol played an important role.

We have now identified and isolated from embryonic chicken brain two soluble factors that allow purified cytoplasmic dynein to move vesicles. These factors are present in partially purified dynein preparations but are successively removed during later steps in the purification. Dynein prepared by ATP-dependent microtubule affinity contains both activators and consequently has a high level of minus end vesicle motor activity. Vesicle motility is significantly reduced after velocity sedimentation and is undetectable after the final purification step, ion-exchange chromatography, although the highly purified dynein is still active in a microtubule gliding assay. This stepwise loss of vesicle motor activity allowed us to identify two activators, Activator I, which is removed by ion exchange chromatography and Activator II, which separates from dynein during velocity sedimentation. Activators I and II may represent merely a subset of the regulatory factors governing dynein-based intracellular motility.

Materials and Methods

Materials

Chick embryo fibroblasts were generously provided by Drs. Milton and Sondra Schlesinger at Washington University School of Medicine (St. Louis, MO). Taxol was provided by Dr. Matthew Suffness at the National Cancer Institute (Frederick, MD). Reagents for polyacrylamide gel electrophoresis and the Bradford protein assay were purchased from Bio-Rad Co. (Richmond, CA). 5-Bromo-4-chloro-3-indolyl phosphate for the alkaline phosphatase reaction was from Calbiochem-Behring Corp. (San Diego, CA). Ultrapure sucrose was obtained from ICN Biomedicals (Cleveland, OH). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Fertilized chicken eggs were obtained from Spafas, Inc. (Roanoke, IL). Monoclonal anti-kinesin antibody SUK4 was a kind gift of Dr. Jon Scholey (University of California, Davis, CA). Monoclonal anti-actin antibody C4 was generously provided by Dr. J. Lessard (University of Cincinnati, Cincinnati, OH). The MonoQ column was from Pharmacia LKB (Piscataway, NJ). Amicon 30 microconcentrators were purchased from Amicon Corp. (Danvers, MA).

Cytoplasmic Dynein Purification

Brains from 11-12-d old chick embryos (≈ 0.25 g/brain) were dissected, rinsed twice in PBS, then rinsed in homogenization buffer (35 mM K-Pipes, pH 7.2, 5 mM MgSO₄, 1 mM EGTA, and 0.5 mM EDTA). The wet brain pellets were divided into 5-, 10-, or 20-g aliquots, flash frozen in liquid N₂ and stored at -80°C .

Preparation of ATP Release. 20 g of brains were rapidly thawed at 37°C and transferred to a Dounce homogenizer (on ice). 20 ml of homogenization buffer containing a protease inhibitor cocktail (Dabora and Sheetz, 1988) and 1 mM DTT was added and the brains were homogenized (10 strokes with a type A pestle), allowed to stand for 5 min on ice, then rehomogenized (10 strokes with a type A pestle). The homogenate was centrifuged at 4°C for 15 min, 16.5 k rpm (SA-600 rotor; Sorvall Instruments, DuPont Co., Newton, CT). The supernatant was immediately removed and subjected to ultracentrifugation at 4°C for 30 min at 182,000 g. The supernatant was removed and 0.5 mM ATP, 1 mM GTP, and 20 μM taxol were added to induce microtubule polymerization. The mixture was incubated at 37°C for 30 min with occasional gentle mixing. The volume (*V*) of the sample was noted, then the sample was subjected to ultracentrifugation at 25°C for 30 min at 182,000 g to pellet microtubules. Meanwhile, phosphocellulose-purified bovine tubulin (Williams and Lee, 1982; enough to give a tubulin concentration of 0.25 mg/ml in volume *V*) was thawed, 1 mM GTP, and 20 μM taxol were added, and the microtubules polymerized by incubating at 37°C for 15 min. The polymerized microtubules were stored at room

temperature for less than 30 min until subsequent use. Following ultracentrifugation the brain supernatant was removed, made 4 mM in AMP-PNP and MgSO₄, the bovine microtubules were added, and the sample was incubated at room temperature for 20 min with occasional gentle mixing. The sample was then layered onto a two-step sucrose gradient composed of 12.5 and 25% sucrose in homogenization buffer containing 1 mM GTP and 20 μM taxol. (The volume of the gradient was usually 5 to 10 ml, such that a 39 ml SW 28 tube would be completely filled when the gradient was overlaid with the microtubule/supernatant mixture.) The gradient was then subjected to ultracentrifugation at 25°C for 45 min at 105,000 g. The final supernatant and the gradient steps were removed by aspiration and the pearlescent microtubule pellet was resuspended in homogenization buffer (total volume used was 0.1 to 0.15 \times V) containing 10 mM ATP, 5 mM (additional) MgSO₄, 1 mM GTP, and 20 μM taxol (ATP release buffer). The microtubule pellet was resuspended as follows. Using a P-1,000 pipetman, 0.5-ml aliquots of ATP release buffer were gently pipetted at the edge of the pellet. The release buffer was repeatedly triturated to break up the pellet, taking great care to avoid bubble formation. The resuspended microtubules were transferred into a thickwall SW50.1 tube. Multiple fresh aliquots of release buffer were added to the pellet until it was completely resuspended. The microtubules were incubated at room temperature for 15 min, then subjected to ultracentrifugation at 25°C for 30 min at 188,000 g. The final supernatant (ATP release) was removed and immediately placed on ice. Approximately 20% of total dynein was recovered in the ATP release (Gill et al., in press).

Velocity Sedimentation. The ATP release (2.5 to 3 ml total) was then layered onto two 11.5 ml, 5-20% linear gradients of sucrose in homogenization buffer containing 0.5 mM ATP and 1 mM DTT. The gradients were centrifuged at 4°C for 13.5 h at 145,000 g in a SW 40 rotor. Sedimentation standards were catalase (11.3S) and thyroglobulin (19S). The bottom of the tube was punctured with a 19 gauge needle, taking care to avoid the microtubule pellet, then 1.3 ml fractions were collected. The fractions were immediately analyzed by SDS-PAGE.

To analyze motor activity of the gradient fractions it was necessary to remove sucrose. A 0.5 ml aliquot of a fraction (or a pool of fractions) was diluted with 1.5 ml homogenization buffer and concentrated by ultrafiltration in an Amicon 30 microconcentrator. This was repeated to arrive at a final volume of 100-200 μl , having a sucrose concentration of $\leq 1.25\%$ and protein concentration of 50-100 $\mu\text{g/ml}$.

MonoQ Chromatography. The 20S (or 9S) sucrose gradient fractions were pooled, passed through a 0.2- μm filter, and loaded onto a HR5/5 MonoQ column. The column was run at a flow rate of 1 ml/min and monitored by absorbance at 280 nm. After loading, the column was washed with homogenization buffer until the absorbance returned to baseline (10-15 ml). Material was eluted from the column with a 15-ml linear gradient of 0 to 250 mM KCl followed by a 25-ml linear gradient of 250 to 400 mM KCl. Fractions of 1 ml were collected or the fraction collector was manually advanced after each peak of absorbance. At the end of the run the column was washed with 1 M KCl.

The fractions comprising each peak were pooled, then desalted and concentrated in an Amicon 30 microconcentrator. Only 0.5 ml of MonoQ dynein (peak 2) was used because of its high concentration (≈ 0.14 mg/ml) after chromatography. To ensure that the final KCl concentration was ≤ 50 mM each pool was subjected to multiple cycles of dilution (with homogenization buffer) and reconcentration. The final volume of each pool was 50 to 150 μl having protein concentrations of ≈ 200 $\mu\text{g/ml}$. The concentrated MonoQ pools were stored on ice for further use.

UV Photocleavage

Vanadate-mediated UV photocleavage was performed as described previously (Schroer et al., 1989).

Vesicle Preparation

KI-washed chick embryo fibroblast membranes were isolated and treated with 0.6 M KI to extract weakly associated proteins (including kinesin and dynein) as described (Schroer et al., 1989). 40 μl of crude membranes were KI extracted, then resuspended in 30 μl homogenization buffer. The protein concentration was 3 mg/ml. Membranes were used at a dilution of $\sim 1:5$ to give a final concentration of 0.65 mg/ml in the motility assay.

Motility Assays

Vesicle Motor Activity. In the assays described below, the microtubule substrate and vesicles are prepared in a standardized manner so the activity of

any soluble sample is reproducible from experiment to experiment (Schroer et al., 1988; Schroer et al., 1989). Each video field contained $\approx 200 \mu\text{m}$ of microtubule polymer; vesicles were used at 0.65 mg/ml. In the absence of soluble motors vesicle movements are not observed. Samples were examined using video-enhanced contrast, DIC microscopy as described (Schnapp, 1986). Data were recorded on 3/4 in U-matic or S-VHS videocassettes. Images from the video monitor were recorded on Plus-X film (ASA125) and printed through a 50 line per inch Ronchi ruling to minimize video scan lines.

Preparation of Randomly Oriented Microtubule Substrate. Vesicles stuck to gliding microtubules are difficult to distinguish from vesicles being translocated along stationary microtubules, producing artifactually high measurements of organelle motor activity. To avoid this problem we developed a procedure for the preparation of an immobilized microtubule substrate. Microtubule gliding was not observed in samples prepared in this manner.

A 20- μl aliquot of freshly thawed bovine brain tubulin (5 mg/ml) was polymerized by the addition of 5 μl of homogenization buffer containing 5 mM GTP and 100 μM taxol. The mixture was incubated for 15 min at 37°C and then added to 175 μl homogenization buffer containing 1 mM GTP and 20 μM taxol, mixing by gentle trituration. The diluted microtubule stock (160 μl) was then added to 12 ml of homogenization buffer plus 1 mM GTP and 20 μM taxol that was prewarmed to 37°C.

Polymerized microtubules were sedimented onto eight coverslips as follows. 50 ml round-bottom centrifuge tubes were plugged with ≈ 8 ml RTV-silicon rubber (Dow-Corning Co., Midland MI) as a level coverslip support for centrifugation. Round coverslips (7/8-in diam) were prewashed with two cycles of 25% HNO_3 and 2 M NaOH, rinsed extensively with distilled water, and baked dry. A circle of ≈ 8 mm (inner diameter) made from silicon high vacuum grease (Dow Corning Co., Midland MI) was drawn on each coverslip to serve as a sample well and reduce lateral shearing. Coverslips were then placed into the eight plugged centrifuge tubes and each was overlaid with 1.5 ml of the polymerized microtubule mixture. The tubes were centrifuged at 25°C for 10 min at 7,000 rpm (HS-4 rotor).

Coverslips were removed from the centrifuge tubes by vacuum using a pasteur pipet tipped with silicon rubber tubing. Care was taken to ensure that a pool of liquid remained in the central well on each coverslip since drying of the microtubule substrate caused a marked loss of activity. The coverslips were placed on stands in a humidified chamber. Each sample well was blocked with heat-inactivated FCS that had been dialyzed against homogenization buffer, made 1 mM in GTP and 20 μM in taxol, and clarified by centrifugation for 10 min at 27 psi in an airfuge (Beckman Instruments Inc., Palo Alto, CA). 10 μl of the buffer in each coverslip well was removed and immediately replaced with 10 μl of serum, then incubated for 10 min at room temperature. At the end of the incubation 10 μl of the liquid in the well was removed and immediately replaced with 20 μl of homogenization buffer plus 1 mM GTP and 20 μl taxol. The coverslips were left in the humidified chamber until further use. Microscopic analysis revealed the microtubules to be evenly dispersed across the central well.

Sample Preparation. The standard sample recipe was 1.5 μl KI-washed membranes, 1 μl carrier protein (FCS prepared as above), 0.7 μl of 20 mM ATP and 4 μl of a motor sample. Motor samples were mixtures of 20S or MonoQ-purified dynein and a sucrose gradient fraction (or pool), a MonoQ pool, or buffer alone (homogenization buffer plus 1 mM GTP and 20 μM taxol). The sample was premixed and drawn up into a pipet (P20 pipetman), ready to dispense. The buffer in the central well of one coverslip (prepared as above) was removed completely and then immediately replaced with the sample. The coverslip was then inverted onto a second coverslip, sealed with VALAP (equal parts Vaseline, lanolin, and paraffin; see Brady et al., 1985), swabbed with ethanol, and then distilled water to remove buffer salts, viewed in the video microscope, and scored for vesicle motor activity as described previously (Schroer et al., 1988). Video fields were selected with a microtubule density of $\approx 200\text{-}\mu\text{m}$ -polymer length per 400 μm^2 area (one video field).

Directionality Assay. Assays were performed on microtubules regrown from isolated centrosomes as described previously (Schroer et al., 1989), except that a motor sample (see above) was used instead of chick embryo fibroblast cytosol.

Vesicle Motor Activity. This was determined by counting the number of vesicle movements that occurred in one video field in a 2–4-min interval. The data presented are the mean (\pm SEM) of at least six video fields from two or more experiments performed on separate occasions (see Schroer et al., 1988); in most cases the total number of video fields scored was 10 or more. A movement was considered to be any unidirectional vesicle translocation over a distance greater than 1 μm . Only movements faster than 0.3 $\mu\text{m/s}$ were considered in the activity measurements. Velocity measurements

on randomly selected vesicles from at least two independent experiments were made using the Measure program of Dr. Steven Block (Sheetz et al., 1986). The velocity analysis included vesicle movements slower than 0.3 $\mu\text{m/s}$.

Microtubule Gliding. These activities of 20S and MonoQ dynein were assayed as described (Vale et al., 1985).

SDS-PAGE, Immunoblot, and Protein Assay

Samples were analyzed by SDS-PAGE as described (Laemmli, 1970). Immunoblotting was performed as described (Towbin et al., 1979) using anti-kinesin mAb SUK4 (Ingold et al., 1988), anti-actin mAb C4 (Lessard, 1988), anti-dynein mAbs 440.1 and 70.1, or mAb 150.1 against the M_r 150,000 component of 20S dynein and MonoQ peak 3 (Steuer et al., 1990). Goat anti-mouse second antibody was conjugated to alkaline phosphatase. Soluble samples were assayed for protein according to (Bradford, 1976) and membrane samples according to (Schaffner and Weissman, 1973) using BSA as a standard.

Results

Loss of Vesicle Motor Activity during Purification of Dynein

Since we determined previously that chick embryo fibroblast cytosol, but not highly purified chick embryo brain dynein, could promote vesicle movement, we examined when in the dynein purification the ability to move vesicles was lost. To do this, the vesicle motor activities of different fractions from the purification were measured quantitatively. For the vesicle motility assay, membrane vesicles and a soluble fraction were combined and examined by video-enhanced contrast light microscopy using an immobilized microtubule substrate prepared by centrifuging taxol-stabilized microtubules onto glass coverslips. Vesicle movements were counted and activity was scored as the number of movements per minute; the direction of transport was determined on centrosome microtubules (Schroer et al., 1988; Schroer et al., 1989; see Materials and Methods).

The first steps in the dynein purification are cosedimentation with microtubules followed by ATP-dependent release. No vesicle motor activity was detected in the initial brain extract. In contrast, the ATP release had a high level of vesicle motor activity (Fig. 1 B and Table I; this represents both plus end- and minus end-directed motility). Although the ATP release contains kinesin and dynein which cause bidirectional movement, as shown in Table II, vesicle movements were predominantly (89%) toward microtubule minus ends. The finding of a high level of minus end-directed vesicle motor activity suggested that the ATP release, like cytosol, contained accessory factors that activate dynein-driven vesicle transport.

Vesicle Motor Activity of 20S Dynein

Use of velocity sedimentation to further fractionate the ATP release resolved two peaks of protein, one that sedimented at 20S and the second that sedimented broadly near $\approx 9\text{S}$. The polypeptide composition of the sucrose gradient fractions was analyzed by SDS-PAGE (Fig. 1 A, lanes 1–9), and, as expected, the 20S peak contained the characteristic M_r 440,000, 70,000, and 55,000 polypeptide subunits of cytoplasmic dynein (Paschal et al., 1987), as well as various minor components. The 9S component contained kinesin and several other polypeptides, including high molecular weight polypeptides that co-migrated with the heavy chain of dy-

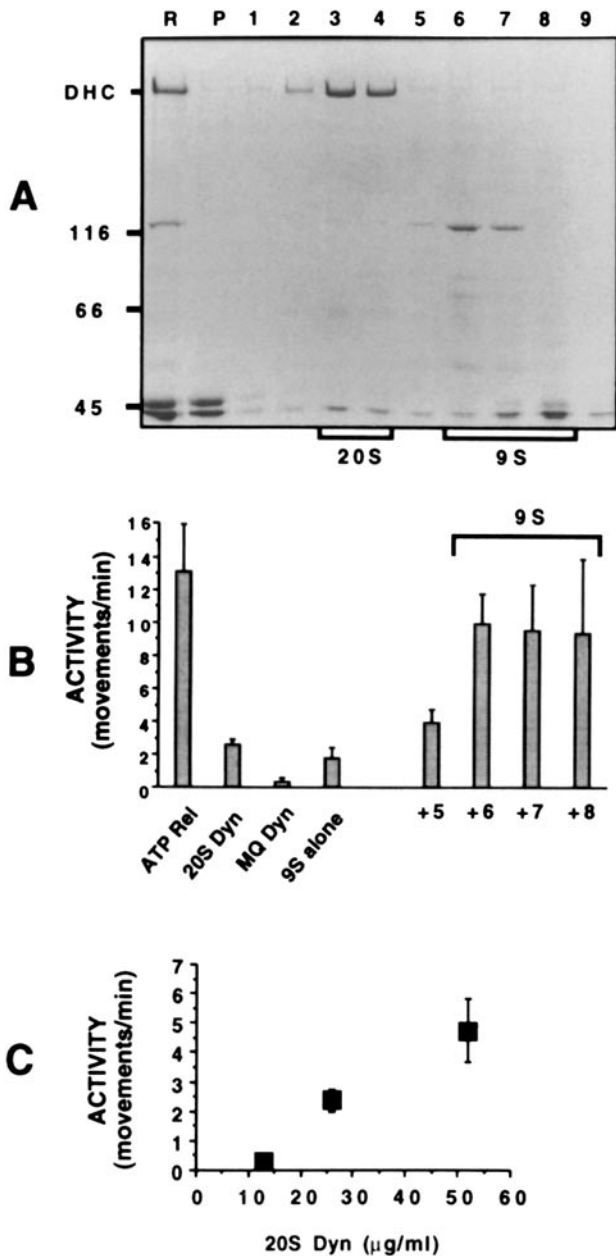


Figure 1. Loss of vesicle motor activity during purification of dynein. (A) Polypeptide compositions of the ATP release and sucrose gradient fractions. ATP-sensitive microtubule-binding proteins (ATP release) were subjected to velocity sedimentation into a 5–20% linear sucrose gradient. The starting material and sucrose gradient fractions were run on a 6% SDS–polyacrylamide minigel that was stained with Coomassie blue. Lane R, ATP release; lane P, an aliquot ($\approx 1\%$) of the gradient pellet; lanes 1–9, fractions 1–9. The 9S and 20S fractions are indicated in brackets. Molecular weight markers (in kD) are on the left. DHC, Dynein heavy chain; $M_r \approx 440,000$. (B) The vesicle motor activities of the ATP release (ATP Rel), 20S dynein (20S Dyn), MonoQ-purified dynein (MQ Dyn), and the 9S fraction (9S alone; a pool of fractions 6, 7, and 8; bracketed) were measured. In samples ATP Rel, 20S Dyn, and 9S alone, the “motor mixture” (see Materials and Methods) was composed of equal parts motor and buffer. To detect any activity of MonoQ dynein (MQ Dyn) the sample was assayed undiluted. Bars +5, +6, +7, and +8 represent the activities of mixtures of 20S dynein with fraction 5, 6, 7, or 8. The data are the average (mean \pm SEM) of activity measurements from at least three independent experiments. (C) The concentration dependence of 20S

Table I. Cytoplasmic Dynein Purification: Vesicle Motor Activity

Sample	Minus end-directed vesicle motor activity (movements/min)	Dynein ($\mu\text{g/ml}$)	Specific vesicle motor activity (activity/mg/ml dynein)
ATP release	11.7	38	308
20S dynein	2.6	26	100
MonoQ dynein	0.3	100	3

Activity: values for total vesicle motor activity are from the data in Fig. 1 B. ATP release: activity is corrected for the fact that vesicle movements were 89% minus end directed (Table II). 20S and MonoQ dynein activities are not corrected because they were 100% minus end directed (Table II). The data are the average of at least three independent experiments. Dynein concentration: protein concentrations of 20S and MonoQ dynein were determined by protein assay (see Materials and Methods). The dynein concentration in the ATP release was estimated by comparing the Coomassie blue staining intensity of the dynein heavy chain on SDS-PAGE with a dynein standard. The concentrations given represent the final dynein concentrations in the vesicle motility assay. At lower protein concentrations MonoQ dynein had undetectable vesicle motor activity (see Fig. 3).

nein. Compared with the ATP release, 20S dynein (Fig. 1 A, lanes 2–4) showed reduced ability to move vesicles (Fig. 1 B and Table I), with all residual movements being minus end directed. However, despite overall loss in vesicle motor activity, 20S dynein retained full activity in a microtubule gliding assay, promoting movement at a velocity of $0.5 \mu\text{m/s}$ as reported previously (Lye et al., 1987; Paschal et al., 1987; Schroer et al., 1989). We reported earlier that 20S dynein alone was not sufficient for vesicle motility (Schroer et al., 1989), but in that study dynein was assayed at concentrations of $<15 \mu\text{g/ml}$, significantly lower than the concentrations used here ($50\text{--}100 \mu\text{g/ml}$; see Materials and Methods). We have now determined the dose dependence of dynein concentration on vesicle motor activity (Fig. 1 C); activity increases linearly with concentrations $\geq 15 \mu\text{g/ml}$, explaining the apparent discrepancy.

That more highly purified dynein retained full activity to bind to and move along microtubules, but was less active for vesicle transport supported the view that one or more components required to mediate vesicle movement were being lost during purification. To examine this, 20S dynein was recombined with other fractions from the sucrose gradient and assayed for vesicle motility. As seen in Fig. 1 B, mixing the enzyme with the 9S fractions resulted in restoration of full vesicle motor activity, in which most movements were minus end-directed (82%, Table II). Although the 9S component by itself had low vesicle motor activity (Fig. 1 B), the effect of mixing 20S dynein with the 9S fractions was synergistic because the activity of the mixture was greater than the sum of the separate activities.

The discovery that vesicle motor activity increases with increasing dynein concentration (Fig. 1 C) suggested that the enhancement of minus end motility might be due to dynein components in the 9S fractions. However, this is not the case.

Dynein on vesicle motor activity was determined. Motility assay samples were prepared in which the motor mixture was composed of undiluted 20S dynein ($60\text{--}110 \mu\text{g/ml}$ protein) or dynein diluted 1:2 or 1:4 with buffer. Vesicle motor activities were averaged (mean \pm SEM) from four independent experiments. No motility was observed at dynein concentrations less than $15 \mu\text{g/ml}$.

Table II. Direction of Vesicle Movements

Sample	Minus end directed	<i>n</i>
	%	
ATP release	89	62
20S dynein	100	42
MonoQ dynein	100	10
20S dynein + 9S fraction	82	95
20S dynein + Activator II	23	241
Activator II	14	159

The direction of vesicle movements was determined using microtubules polymerized from centrosomes (see Materials and Methods). Each sample was tested in at least three independent experiments.

n, The total number of vesicle movements analyzed.

9S fraction, Pool of sucrose gradient fractions 6, 7, and 8 (see Fig. 1 A).

Activator II, Pool E isolated by MonoQ chromatography of the 9S fraction (see Fig. 6).

No dynein polypeptides were detected in the 9S fractions by immunoblot analysis with antibodies against the M_r 440,000 heavy chain and 70,000 intermediate chain polypeptides (mAbs 440.1 and 70.1; Steuer et al., 1990; Steuer et al., manuscript in preparation). Further, following exposure of the 9S fractions to vanadate-mediated UV photocleavage to inactivate dynein, the ability to stimulate vesicle motility was retained (data not shown) indicating that the activator(s) in the 9S fraction was a component other than dynein.

An Activator of Vesicle Motility in 20S Dynein

The finding of vesicle motor activity in the 20S fractions indicated that some dynein in the preparation was active for vesicle motility. This might result from the presence of a dynein activator in the preparation or the permanent activation of a subpopulation of the enzyme, or both. To distinguish among these possibilities 20S dynein was purified further by ion exchange chromatography on a MonoQ column. As seen in Fig. 2, MonoQ chromatography separated the material into three major peaks of protein (Fig. 2 A; peaks 1, 2, and 3). SDS-PAGE analysis (Fig. 2 B) showed peak 2 to contain exclusively the M_r 440,000 dynein heavy chain plus the 70,000 and 55,000 dynein polypeptides (Fig. 2 B, lanes 24–28). We will refer to peak 2 as MonoQ dynein. Trace amounts of dynein heavy chain also trailed into later column fractions. Peak 3 (Fig. 2 B, lanes 33–35) contained major polypeptides of M_r 50,000 and a doublet of M_r 150,000 plus other components including a polypeptide of M_r 45,000 that reacted with an anti-actin antibody on immunoblots (data not shown). Peak 1 contained a M_r 45,000 protein (Fig. 2 B, lane 21) that also reacted with the anti-actin antibody on immunoblots (data not shown).

MonoQ dynein was assayed for motor activity in the microtubule gliding and vesicle motility assays. Like 20S dynein, MonoQ dynein promoted robust microtubule gliding at a velocity of 0.5 $\mu\text{m/s}$, but in contrast with 20S dynein had nearly undetectable vesicle motor activity (Fig. 1 B; Fig. 3 and Table I). The few vesicle movements observed were

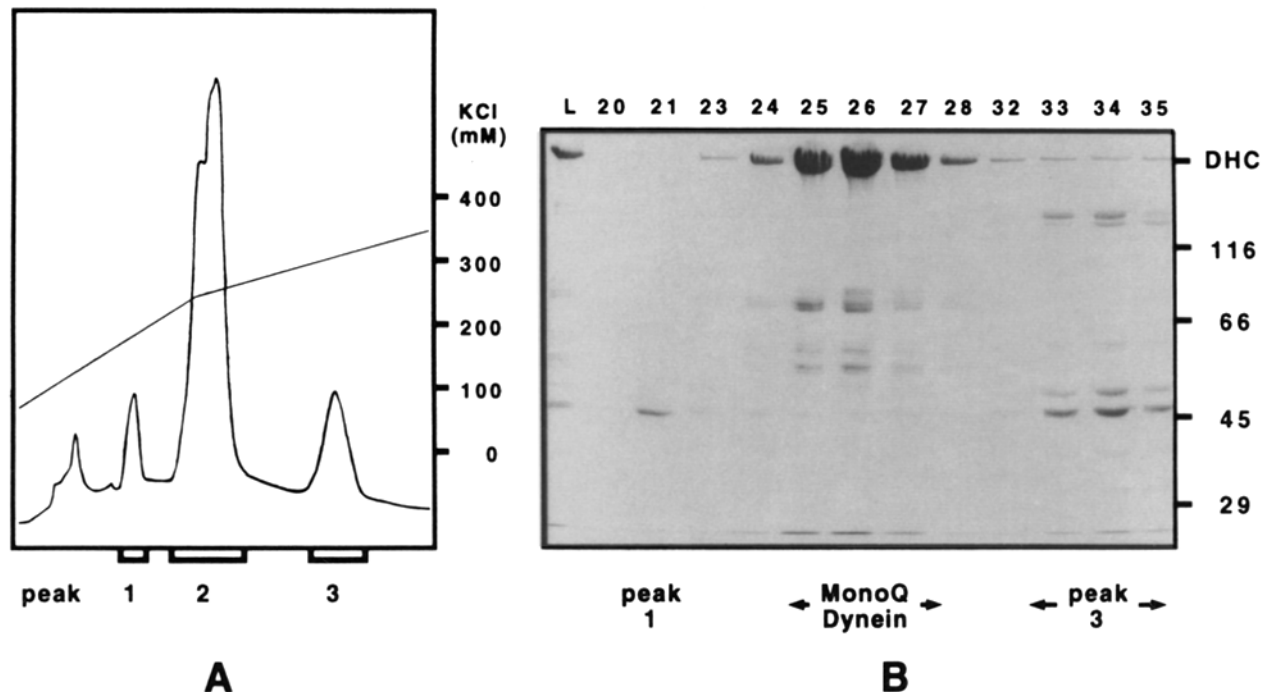


Figure 2. MonoQ chromatography of 20S dynein. (A) A representative column profile depicting absorbance at 280 nm; only the portion of the chromatographic run representing the KCl elution is shown. The salt gradient (80 to 400 mM KCl) is superimposed on the absorbance tracing. The eluate from 0 to 150 mM KCl did not contain protein. Peaks 1, 2 (MonoQ Dynein), and 3 are indicated, containing 10, 70, and 30 $\mu\text{g/ml}$ protein, respectively. (B) SDS-polyacrylamide gel analysis of the column load and elution profile. Fractions comprising peaks 1, 2, and 3 are indicated in brackets. Lane L, the 20S dynein loaded on the column. Lanes containing column fractions 20–28 and 32–35 are labeled accordingly. All lanes contained equal amounts of sample. The 7.5% gel was stained with Coomassie blue. Molecular weight markers (in kD) are indicated on the right. DHC, dynein heavy chain; $M_r \approx 440,000$.

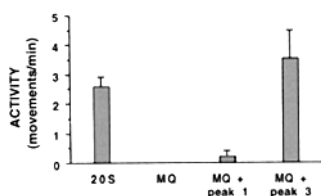


Figure 3. Identification of Activator I. The vesicle motor activities of mixtures of MonoQ dynein with MonoQ peaks 1 and 3 (bars MQ + peak 1 and MQ + peak 3; equal parts dynein and either peak) were determined. The activities

of 20S dynein (20S; $\approx 40 \mu\text{g/ml}$), MonoQ dynein (MQ; $\approx 50 \mu\text{g/ml}$) are shown for comparison (this concentration of MonoQ dynein was insufficient for vesicle movement). The data represent the average values (mean \pm SEM) obtained from experiments performed on at least three separate occasions.

minus end directed (Table II). MonoQ dynein retained the capacity to drive vesicle movement since activity could be restored (5.8 ± 0.7 movements/min) by addition of the 9S fraction. In an attempt to identify the activator responsible for the residual motility of 20S dynein, peaks 1 and 3 were recombined with MonoQ dynein using a concentration of enzyme insufficient for vesicle motility (Fig. 3). Peak 1 neither stimulated motility when mixed with MonoQ dynein nor did it have activity by itself. In contrast, the mixture of peak 3 and MonoQ dynein promoted minus end-directed vesicle movements although peak 3 had little activity by itself. We will refer to the activity in peak 3 as Activator I.

Effects of Dynein Activators on Velocity of Vesicle Movements

Although the vesicle motor activity of dynein is increased both by factors that sediment at 9S and by Activator I, the two activators have different effects on the velocity of vesicle movement. As seen in Fig. 4 and Table III, vesicle movements driven by 20S dynein showed a mean velocity of $0.6 \mu\text{m/s}$. On rare occasion, MonoQ dynein moved vesicles at $0.6 \mu\text{m/s}$ and the mixture of MonoQ dynein with Activator

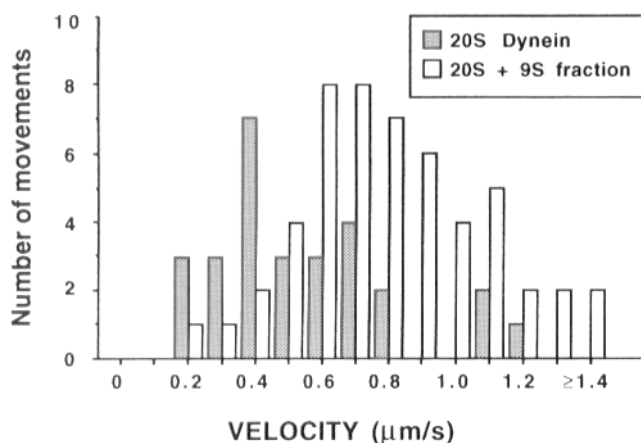


Figure 4. Velocities of vesicle movements driven by 20S dynein and 20S dynein plus the 9S fractions. The histogram shows the distribution of velocities ($\mu\text{m/s}$) of vesicle movements exhibited by the two samples. For 20S dynein (\blacksquare), the mean velocity (\pm SD) was $0.6 \pm 0.2 \mu\text{m/s}$ ($N = 28$). For 20S dynein plus 9S fractions (\square), the mean velocity (\pm SD) was 0.8 ± 0.3 ($N = 51$). The velocities of 20S dynein vs. 20S dynein plus the 9S fraction or Activator II were significantly different as determined by *t* test ($P > 0.001$). These data are also listed in Table II.

Table III. Velocity of Vesicle Movements

Sample	Velocity (mean \pm SD)	Range	<i>n</i>
	($\mu\text{m/s}$)	($\mu\text{m/s}$)	
20S dynein	0.6 ± 0.2	0.2–1.2	28
MonoQ dynein + Activator I	0.7 ± 0.3	0.3–1.2	20
20S dynein + 9S	0.8 ± 0.3	0.2–2.3	51
20S dynein + Activator II	0.8 ± 0.4	0.4–2.1	46

Velocities were measured as described in Materials and Methods; data were collected from at least four separate experiments for each sample. The distribution of velocities of 20S dynein and 20S dynein plus the 9S fraction are shown in Fig. 4.

I promoted movement at similar velocities to 20S dynein (Table III). The maximal velocity of movement was significantly increased when 20S dynein was mixed with the 9S fraction (Fig. 4), with vesicles having a mean velocity of $0.8 \mu\text{m/s}$ and some as fast as $2.3 \mu\text{m/s}$ (Table III). Thus, the dynein activator(s) present in the 9S fraction increase(s) both frequency and velocity of vesicle movements, while only the frequency of movement is enhanced by Activator I.

Purified Dynein Causes Membranes to Bind to Microtubules

The requirement for dynein activators in vesicle motility could arise from the inability of purified dynein to bind vesicles effectively. To test whether dynein alone could interact with membranes we examined mixtures with 20S or MonoQ dynein and microtubules. As seen in Fig. 5, more vesicles bound to microtubules (Fig. 5, B and C) than in the presence of 20S dynein plus the 9S fractions (Fig. 5 A). In addition to promoting typical vesicle translocations (e.g., Fig. 5 D), 20S dynein caused vesicles to undergo short, bidirectional displacements of $\leq 1 \mu\text{m}$ (Fig. 5 E). These “tethered” movements were unique to purified dyneins and were not observed in the ATP release, mixtures of 20S dynein and the 9S fractions or in whole cytosol. If soluble protein (motor and carrier protein; see Materials and Methods) was omitted from the assay, membranes also decorated microtubules but no movement occurred. The observation that MonoQ-purified dynein induced vesicles to bind to microtubules suggests that, in the absence of activators, dynein retains the capacity to bind membranes.

An Activator in the 9S Fraction Stimulates Minus End-directed Transport and Supports Plus End-directed Transport

In an attempt to identify other dynein activator(s), the 9S fraction was subjected to MonoQ chromatography, yielding several distinct peaks of protein (Fig. 6 A). The predominant polypeptide in the starting mixture (Fig. 6 B, lane L), kinesin, trailed across the column in a broad peak (Fig. 6 C). MonoQ peaks A through H were desalted and assayed for motor activity by themselves. Peaks A, B, and C all contained the plus end motor kinesin (Fig. 6 C) and supported microtubule gliding but not vesicle motility. In contrast, peak E, which we will refer to as Activator II, supported vigorous vesicle motility (Fig. 7). On centrosome microtubules, vesicle movements powered by Activator II alone were 87%

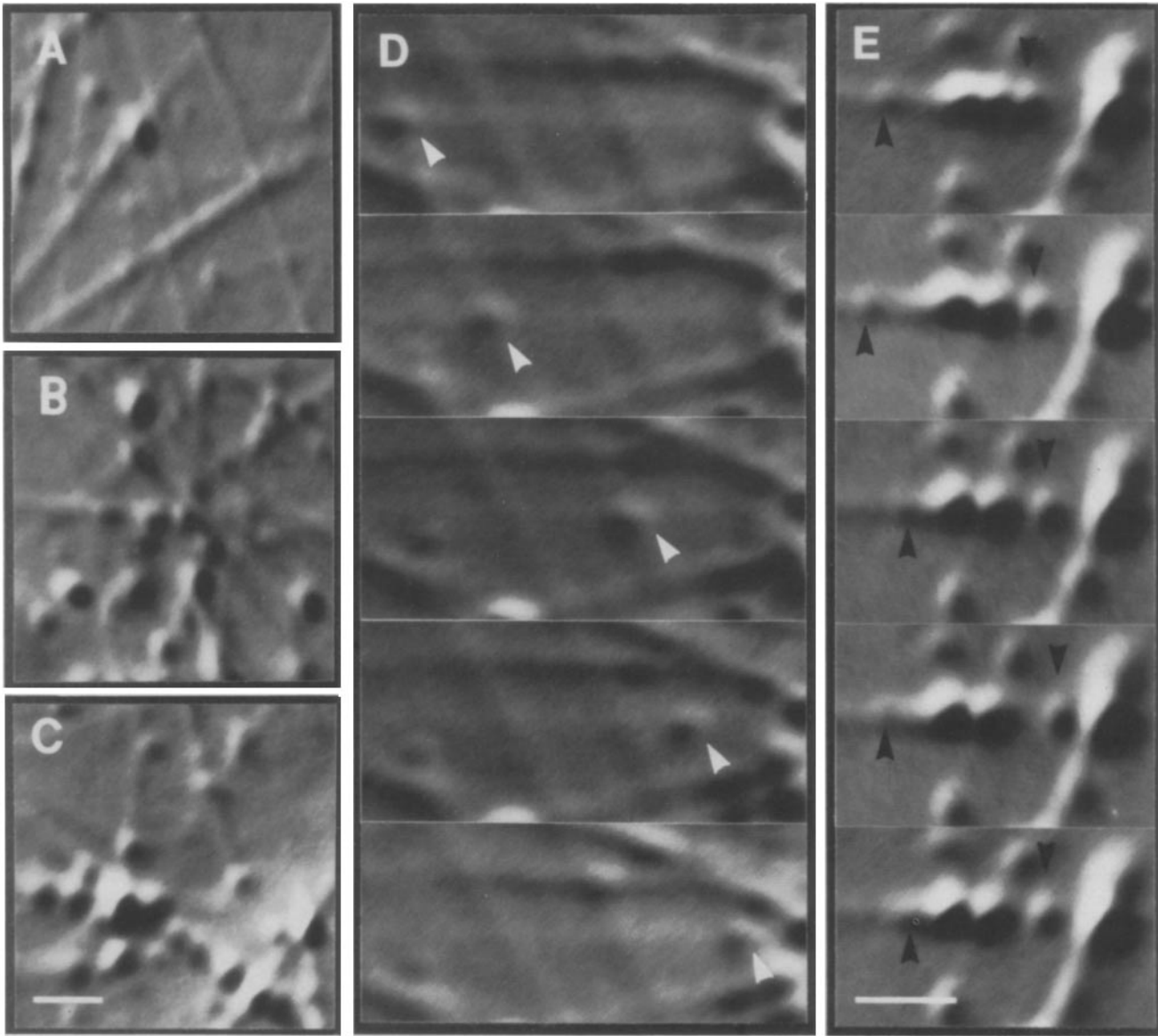


Figure 5. Purified dynein causes membrane vesicles to bind to microtubules. Representative samples from the vesicle motility assay are depicted in photographs taken from the video monitor. All samples contained microtubules, vesicles, motors, and ATP (as detailed in Materials and Methods). In the absence of added motors few vesicles bound to microtubules and the samples resembled *A*. (*A*) Assay with 20S dynein plus the 9S fraction, showing the standard mixture of vesicles with the microtubule substrate. (*B* and *C*) Assay with 20S dynein (*B*) or MonoQ dynein (*C*) alone. Many vesicles are bound to microtubules. (*D* and *E*) Sequential micrographs demonstrating vesicle movements. (*D*) 20S dynein plus 9S fraction: a moving vesicle (*arrowhead*) is shown at 1-s intervals. (*E*) 20S dynein alone: The back and forth movements of two vesicles (*arrowheads*) are shown at 8-s intervals. Bars, 1 μm .

plus end directed (Table II), suggestive of kinesin (Fig. 6 *C*, lane *E*) activity.

Activator II also stimulated the activity of 20S dynein (Fig. 7), yielding a significant enhancement of minus end motility (2.1 minus end-directed movements/min vs. 0.2 minus end-directed movements/min on centrosome microtubules). In some (but not all) experiments peak D (Fig. 6 *B*, lane *D*) drove vesicle movements when mixed with dynein. (This variability is likely to stem from the peak E activator contaminating the peak D fractions.) 20S dynein plus Activator II promoted vesicle movements at the same mean velocity ($0.8 \pm 0.4 \mu\text{m/s}$; Table III) and with a distribution similar to 20S dynein plus the 9S fractions. Addition of 20S dynein to Activator II did not affect plus end vesicle movements.

Fig. 6 shows that Activator II elutes from the MonoQ column at a salt concentration similar to that which elutes dynein, and contains polypeptide bands in the molecular weight range of cytoplasmic dynein heavy chain. However, only trace levels of dynein could be detected in Activator II by immunoblot analysis (mAbs 440.1 and 70.1; Steuer et al., 1990; data not shown). To further exclude the possibility that dynein was responsible for its stimulatory effects, Activator II was subjected to vanadate-mediated UV-photocleavage or was UV-irradiated under control conditions (Lye et al., 1987; Schroer et al., 1989). When compared by SDS-PAGE, neither sample contained the expected M_r 220,000 and 190,000 UV-cleavage fragments nor was loss of a high molecular weight polypeptide detected. In mixtures with 20S dynein

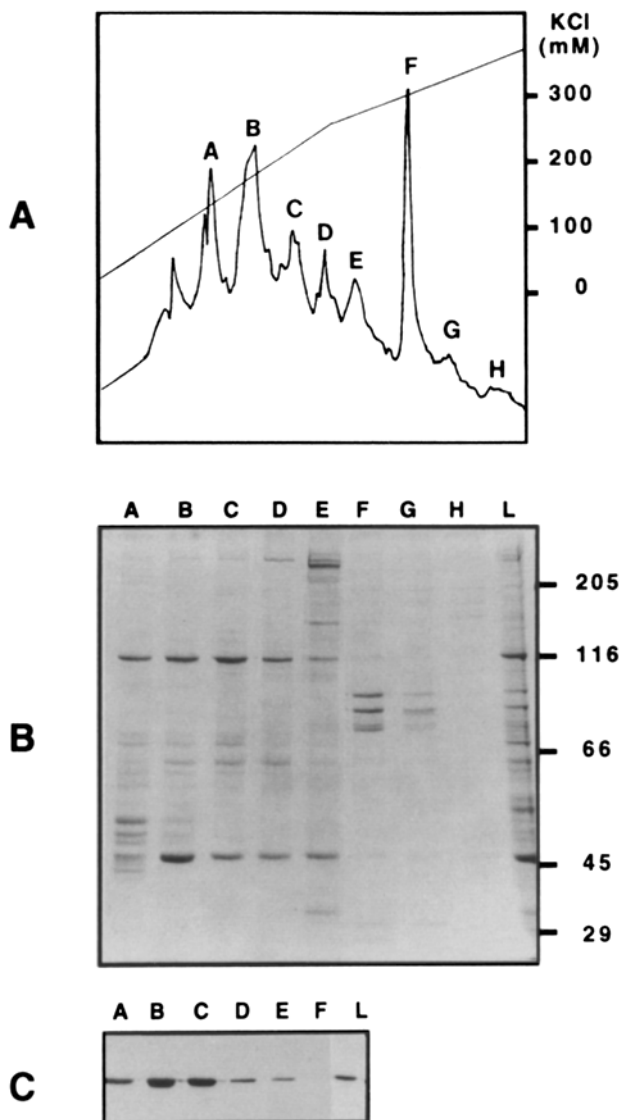


Figure 6. MonoQ chromatography of 9S fraction. (A) A representative column profile depicting absorbance at 280 nm; only the portion of the chromatographic run representing the KCl elution is shown. The salt gradient (80 to 400 mM KCl) is superimposed on the absorbance tracing. The eluate from 0 to 150 mM KCl did not contain protein. (B) SDS-PAGE analysis of the column load and KCl elution. Lanes A-H, peaks A through H, lane L, Pooled 9S fractions (fractions 6 through 8 in Fig. 1 A) loaded onto the column. The 7.5% gel was stained with Coomassie blue; 15 μ l of each sample was loaded per lane. Molecular weight markers (in kD) are indicated on the right. (C) Distribution of kinesin in the MonoQ column fractions. Peaks A-F were analyzed by anti-kinesin immunoblot (mAb SUK4; Ingold et al., 1988), the 9S fraction loaded on the column (L) is also shown. Only the relative molecular weight range of kinesin heavy chain (M_r 116,000) is shown; the antibody did not recognize any other polypeptides.

the two samples had similar vesicle motor activities (UV-cleaved: 16.5 ± 1.5 movements/min; control: 13.8 ± 1.7 movements/min), as they did when assayed by themselves (data not shown). We conclude that the stimulatory effect of Activator II on vesicle transport is not due to a low level of an "activated" cytoplasmic dynein. Activator II is also distinct from Activator I, judging from the lack of reactivity on

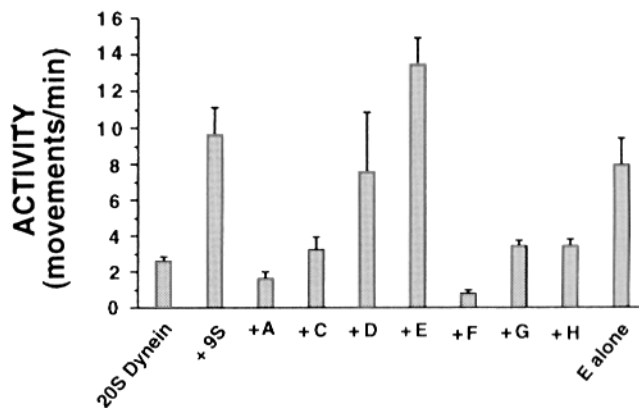


Figure 7. Identification of Activator II. Vesicle motor activity of 20S dynein alone (20S Dynein; $\approx 40 \mu\text{g/ml}$) and "motor mixtures" of 20S dynein plus the 9S fraction (+9S) or MonoQ peak A, C, D, E, F, G, or H (+A-H). Motor mixtures were composed of equal parts 20S dynein and the peak indicated. The activity of peak E by itself is also shown (E alone, diluted 1:2 with buffer). The activity of peak E plus 20S dynein was significantly greater than the sum of the activities of 20S dynein and pool E by themselves as determined by *t* test ($P > 0.05$).

immunoblots with an antibody against the M_r 150,000 doublet component of Activator I (mAb 150.1; Steuer et al., 1990; data not shown). These findings suggest that Activator II stimulates both kinesin and dynein-based vesicle motility.

Discussion

Cytoplasmic dynein drives intracellular processes such as neurite extension, mitotic movements, and vesicle transport, activities that must be carefully coordinated to ensure normal cell function. Using an assay for vesicle motility *in vitro*, we showed previously that transport requires soluble factors (activators) in addition to cytoplasmic dynein; the latter provides motile force while the activators govern the overall magnitude and velocity of movement. On the basis of their ability to stimulate purified dynein to move vesicles, we have now identified Activators I and II, the first described regulators of cytoplasmic dynein activity.

Activator Effects on Dynein

That purified dynein or kinesin immobilized on an inert anionic surface has motor activity, while dynein bound to a vesicle does not (Schroer et al., 1988; 1989) indicates that membrane binding inhibits motility unless necessary activators are available. It may be possible to glean insight into mechanisms by which dynein activity is controlled by examining details of vesicle movement in the presence and absence of the two activators. Judging from the behavior of vesicles *in vitro*, dynein can exist in multiple states when complexed with membranes and microtubules. The enzyme is either immotile (MonoQ dynein alone), active but capable of only basal motility (plus Activator I), or active at increased velocity (plus Activator II), states which may correlate with dynein ATPase activity. The vesicle oscillations promoted by highly purified dyneins may be similar to movements of microtubules mediated by flagellar dynein made incapable of ATP hydrolysis (Vale et al., 1989). Purified dynein bound to vesicles and microtubules may be unable to

utilize energy owing to lack of the necessary activator(s). A detailed comparison of the vesicle oscillations and microtubule movements has not yet been performed, so the mechanistic similarity of the two is unknown. Since motor ATPase and resulting transport velocity are commonly coupled (Cohn et al., 1987; Vale et al., 1984), the effects of Activator II on velocity suggest further enhancement of dynein ATPase activity.

Mechanisms for Activation

Regulation of highly coordinated microtubule-based movements such as bidirectional transport of pigment granules in chromatophores (Rodzialis and Haimo, 1986; Thaler and Haimo, 1990), sperm motility (Tash, 1989), and mitosis (Moreno and Nurse, 1990) is unequivocally mediated by phosphorylation, although direct involvement of microtubule-based motors is not proven. Such modification affects all subunits of axonemal dynein (reviewed by King and Witman, 1989) and the M_r 70,000 intermediate chains of the cytoplasmic isoform (A. Adamson, M. Anderson, and T. Schroer, unpublished results). Kinesin heavy and light chains are both phosphorylated *in vitro* (Buster, D., M. Lohka, and J. M. Scholey. 1990. *J. Cell Biol.* 111:418a) as predicted from primary sequence analysis (Yang et al., 1989; Cyr, J. L., G. S. Bloom, and S. T. Brady. 1990. *J. Cell Biol.* 111:146a). Since the kinesin light chain and dynein intermediate chains are located distal to the microtubule-binding heads (Hirokawa et al., 1989; Steuer et al., manuscript in preparation) their phosphorylation may profoundly affect membrane, chromosome, and/or spindle binding and consequent motility.

That even the major components of Activators I and II are recovered in substoichiometric amounts compared with dynein suggests a catalytic mechanism for activation. Alternatively, despite copurification with microtubule motors, significant amounts of either activator may be lost earlier in the dynein purification and in cells the activators may be present in high concentration. Comparative immunoblot analysis of dynein and the M_r 150,000 polypeptide doublet reveals that the two proteins are stoichiometric in brain tissue but that significant amounts of dynactin are lost upon microtubule binding and release (Gill et al., in press). Further, judging from its abundance, Activator I may function as a structural component of the vesicle motor. However, it appears that neither activator is required to bind dynein to membranes, although it is not known whether the binding we observe reflects a physiological interaction.

Identity of Activator Polypeptides

We concluded previously that kinesin plays a key role in bidirectional vesicle transport since its immunodepletion from axonal cytosol profoundly inhibits motility in both plus end and minus end directions (Schroer et al., 1988). Yet kinesin by itself is not sufficient for vesicle motility (this report and Schroer et al., 1988), nor can it serve as an activator of dynein-driven vesicle motility, just as purified dynein does not activate kinesin to move vesicles (see Fig. 7, bar C). Our earlier findings are most consistent with the existence of activator(s) shared by kinesin and cytoplasmic dynein, in keeping with a model wherein the "vesicle motor" is a protein complex composed of the two motors plus activator(s) (Sheetz et al., 1989). Its stimulatory effects on both motors make

Activator II an appropriate candidate for a shared activator, further study of which awaits purification of the active component.

Considering its simple polypeptide composition (assuming a major component is an activator), identification of the active polypeptide(s) in Activator I should be straightforward. Of potential interest is the M_r 150,000 component, common in 20S dynein preparations (Neely and Boekelheide, 1988; Schnapp and Reese, 1989; Hirokawa et al., 1990; Verde et al., 1991), which when adsorbed by mAb 150.1 prevents stimulation of dynein-based vesicle motility by Activator I (Gill et al., in press). The M_r 150,000 doublet is one component of a complex formed by the Activator I polypeptides (Fig. 2 B, peak 3), since all are removed by immunodepletion (Gill et al., in press) and in hydrodynamic studies behave as an aggregate of $M_r \geq 10^6$ (D. M. Eckley, D. Hammond and T. Schroer, unpublished results) that sediments at $\approx 20S$ (Gill et al., in press).

The primary structure of the M_r 150,000 component, named dynactin, has been determined (Gill et al., in press; Holzbaur et al., 1991). The predicted protein structure contains two highly conserved, α -helical coiled-coil domains, a motif commonly found in proteins capable of homodimerization (e.g., kinesin, myosin II, tropomyosin, spectrin and intermediate filament proteins). Of the two dynactin isoforms we observe, only the lower M_r species is brain specific (Gill et al., in press; Steuer et al., manuscript in preparation), suggesting a specialized role in neurons. Dynactin has extensive sequence homology to the *Drosophila* protein Glued, which when mutated results in defects in basic cell function and neuronal development, as expected for a required component of the vesicle transport machinery.

Further Implications

The participation of dynein in such diverse transport functions as vesicle motility and mitosis raises interesting questions about mechanisms for coordination of enzyme activity. Recent studies in *Xenopus* eggs demonstrate that dynein provides alternating roles during the cell cycle, promoting membrane movements only in interphase extracts (Allan and Vale, 1991), while in mitotic extracts prominent microtubule-microtubule motility is observed (Verde et al., 1991). Coordinate regulation of these complementary activities is thought to be mediated by cell cycle-dependent phosphorylation of components that target dynein to the appropriate organelle. Isolated as facilitators of vesicle motility *in vitro*, Activators I and II are examples of factors that may enhance the interaction of dynein with vesicles or other target(s) to control activity *in vivo*.

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