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Identification of a kinesin-like microtubule-based motor protein in *Dictyostelium discoideum*

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***Dictyostelium discoideum*, a unicellular eukaryote amenable to both biochemical and genetic dissection, provides an attractive system for studying microtubule-based transport. In this work, we have identified microtubule-based motor activities in *Dictyostelium* cell extracts and have partially purified a protein that induces microtubule translocation along glass surfaces. This protein, which sediments at ~9S in sucrose density gradients and is composed of a 105 kd polypeptide, generates anterograde movement along microtubules that is insensitive to 5 mM NEM (*N*-methyl-maleimide) but sensitive to 200 μM vanadate, and has similar nucleotide-dependent microtubule binding properties to those of kinesins purified from mammals, sea urchin and *Drosophila*. This kinesin-like molecule from *Dictyostelium*, however, is immunologically distinct from bovine and squid neuronal kinesins and supports microtubule movement on glass at four-fold greater velocities (2.0 versus 0.5 μm/sec). Furthermore, AMP-PNP (adenylyl imidodiphosphate), which promotes attachment of previously characterized kinesins to microtubules, decreases the affinity of the *Dictyostelium* kinesin homolog for microtubules. Thus, an AMP-PNP-induced rigor binding may not be a characteristic of kinesins from lower eukaryotes.**

Key words: Dictyostelium/kinesin/microtubules/organelle transport

Introduction

Microtubule-based motility plays an important role in a variety of eukaryotic cell functions, including fast axonal transport of organelles (Vale, 1987) and the movement of chromosomes during mitosis (Inoue, 1981). Two force-generating proteins, kinesin and cytoplasmic dynein, that are likely to be involved in these microtubule-based movements have been identified by reconstituting microtubule movement *in vitro* (Vale *et al.*, 1985b; Paschal *et al.*, 1987). Both proteins generate unidirectional movement along microtubule polymers; kinesin supports movement towards the plus ends of microtubules (corresponding to anterograde transport of organelles in neurons) (Vale *et al.*, 1985a; Porter *et al.*, 1987), while cytoplasmic dynein supports movement towards the microtubule minus end (retrograde transport) (Paschal and Vallee, 1987).

Kinesin is a ubiquitous protein that has been isolated from various tissues and organisms including squid axons (Vale *et al.*, 1985a), bovine brain (Vale *et al.*, 1985a; Bloom *et al.*, 1988), bovine adrenal medulla (Murofushi *et al.*, 1988), sea urchin eggs (Scholey *et al.*, 1985), *Drosophila* (Saxton *et al.*, 1988) and the lower eukaryote, *Acanthamoeba* (Kachar *et al.*, 1987). Kinesin isolated from bovine brain is a complex of two heavy chain polypeptides (120 kd) and two light chain polypeptides (62 kd) (Bloom *et al.*, 1988; Kuznetsov *et al.*, 1988; Wagner *et al.*, 1989). *Acanthamoeba* and sea urchin kinesins contain a heavy chain of somewhat larger molecular weight (130 kd) and it is still undetermined whether light chains are complexed with these heavy chains (Kachar *et al.*, 1987; Porter *et al.*, 1987). Kinesin is a microtubule-activated MgATPase (Cohn *et al.*, 1987; Kachar *et al.*, 1987; Kuznetsov and Gelfand, 1986) whose microtubule and ATP binding domains are both located in the N-terminal domain of the heavy chain (Yang *et al.*, 1989; Scholey *et al.*, 1989). The C-terminus of the protein may bind to organelles or other cellular structures (Yang *et al.*, 1989; Hirokawa *et al.*, 1989).

Kinesin is primarily a soluble, cytoplasmic protein and hence could be recruited as a force-generating protein for a variety of microtubule-based movements such as organelle transport, changes in cell shape, and chromosome movement during mitosis. Kinesin's biological roles, however, have not been definitively established, although immuno-localization of kinesin on organelles supports the idea that kinesin is an organelle transport motor (Pfister *et al.*, 1989). One approach to determine the biological functions of kinesin is to examine the phenotype of cells containing a deletion of the kinesin gene. The haploid eukaryote *Dictyostelium discoideum* is a suitable organism for such studies since it contains an extensive cytoplasmic microtubule network that supports organelle movements (Roos *et al.*, 1984; Roos *et al.*, 1987) and it can be genetically manipulated by homologous recombination (De Lozanne and Spudich, 1987; Spudich, 1987; Witke *et al.*, 1987; Katz and Ratner, 1988; Manstein *et al.*, 1989). To understand the biological roles of kinesin, we have examined microtubule-based motility in *Dictyostelium*, and have partially purified and characterized a kinesin-like molecule in *Dictyostelium*.

Results

Microtubule-based motility in cells extracts of Dictyostelium

Transport of organelles along microtubules and movement of microtubules along glass surfaces can be observed in cytoplasmic extracts of squid giant axons (Vale *et al.*, 1985c; Allen *et al.*, 1985; Gilbert *et al.*, 1985). To determine whether a similar transport system exists in *Dictyostelium*, we examined *Dictyostelium* extracts for microtubule-based movements. When taxol-stabilized bovine microtubules and ATP were added to a low-speed supernatant of *Dictyostelium* (S1), bidirectional transport of organelles along microtubules was observed by video-enhanced microscopy. Furthermore,

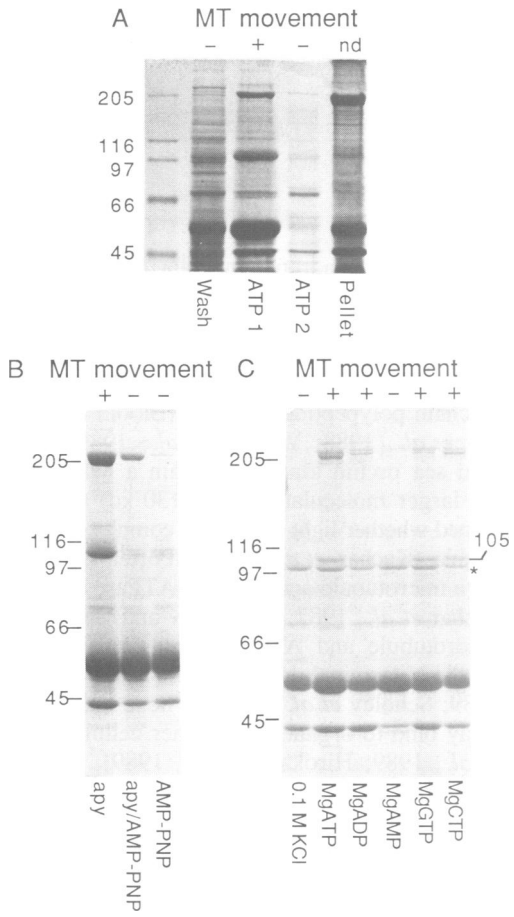

Fig. 1. Purification of *Dictyostelium* motility protein by microtubule binding. High speed supernatants (S2) were incubated with 1 mg/ml apyrase (apy) (except for the samples shown in B, lanes 2 and 3), 20 μM taxol and 0.5 mg/ml taxol-stabilized bovine microtubules for 1 h on ice. Microtubules were sedimented and the pellets were incubated with the appropriate nucleotide (10 mM), usually MgATP. The microtubules were resedimented and the resulting supernatant (release) was assayed for the ability to move microtubules on glass in the presence of 1 mM added ATP (indicated above each lane; nd = not done).; (**A**) Standard preparation. Lane 1, 50 mM KCl wash; lane 2, first MgATP release; lane 3, second MgATP release; lane 4, microtubule pellet following both releases. (**B**) Microtubule binding conditions. Proteins bound to microtubules were released with MgATP. Lane 1, microtubule binding in presence of 1 mg/ml apyrase; lane 2, microtubule binding in presence of 1 mg/ml apyrase and 2 mM AMP-PNP; lane 3, microtubule binding in presence of 2 mM AMP-PNP. (**C**) Nucleotide dependent release from microtubules. Lane 1, 0.1 M KCl; lane 2, MgATP; lane 3, MgADP; lane 4, MgAMP; lane 5, MgGTP; lane 6, MgCTP. The 100 kd polypeptide (marked by *) present in all lanes in C was observed only when proteins were prepared from strain AX3, and was not seen in preparations from strain AX4.

a high speed supernatant (S2) adsorbed onto a glass slide supported translocation of microtubules along the glass surface. In some S2 preparations, bidirectional gliding of microtubules was observed; a microtubule would move in a linear path for several microns, stop and then undergo movement in the opposite direction. These observations suggest that *Dictyostelium*, like squid axons (Vale *et al.*, 1985b), contains two motile activities that produce movement in opposite directions along microtubules.

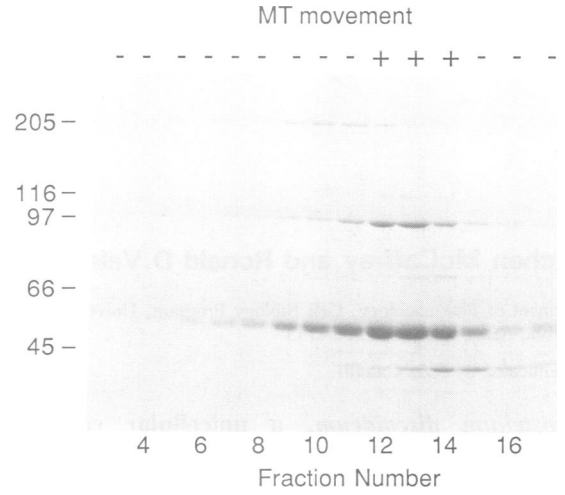

Fig. 2. Sucrose gradient purification of *Dictyostelium* motility protein. One ml of a MgATP release (Figure 1A, lane 2) was loaded on a 5–25% sucrose gradient. Samples were removed from the bottom of the gradient (bottom = fraction 1), analyzed by electrophoresis and assayed for the ability to support microtubule movement on glass.

Partial purification of a motility protein by microtubule affinity

The motile activities in the high speed supernatant samples were ATP-dependent, suggesting that they were driven by a soluble ATPase(s). Since many microtubule motility proteins bind to microtubules with a high affinity in the absence of ATP (Paschal *et al.*, 1987; Lye *et al.*, 1987; Scholey *et al.*, 1985; Euteneuer *et al.*, 1988), we prepared a high speed *Dictyostelium* supernatant (S2), depleted it of ATP with apyrase, and then added taxol-stabilized bovine microtubules. The microtubules wih bound proteins were sedimented and then resuspended in buffer supplemented with 10 mM MgATP to release potential motility proteins. This ATP-release supernatant supported unidirectional movement of microtubules on glass; no bidirectional movement of microtubules was observed (n = 21 preparations). The most prominent polypeptide in this motile fraction migrated at 105 kd in SDS polyacrylamide gels. This polypeptide was released from microtubules with 10 mM MgATP but not with 50 mM KCl (Figure 1A). The other prominent proteins in this sample include tubulin (due to some depolymerization of the bovine microtubules during the ATP release step), a 220 kd polypeptide that was identified as myosin by immunoblotting using an anti-*Dictyostelium* myosin antibody (not shown; antibody provided by Dr J.Spudich), the 42 kd actin polypeptide, and an unknown 75 kd polypeptide that was also released from microtubules by the KCl wash.

Binding of the *Dictyostelium* motor activity to microtubules and its subsequent release were both nucleotide-dependent. Without added apyrase, the motor activity did not cosediment with microtubules, probably because of the presence of endogenous nucleotide in the extract (not shown). Unlike neuronal and sea urchin kinesins which sediment with microtubules in the presence of the nonhydrolyzable ATP analog, adenylyl imidodiphosphate (AMP-PNP) (Vale *et al.*, 1985a; Scholey *et al.*, 1985; Brady, 1985), the *Dictyostelium* motor activity did not cosediment with microtubules in the

Table I. ATPase activity of partially purified *Dictyostelium* motor protein

	nmoles PO_4/min/mg		microtubule concentration	
	−MT	+MT	mg/ml	fold stimulation
Prep 1	147	387	0.8	2.6
Prep 2	80	440	1.0	5.5
Prep 3	67	667	2.0	10.0
Prep 4	200	650	2.0	3.3

Four *Dictyostelium* motility protein preparations were assayed for ATPase activity in the presence and absence of bovine microtubules (MTs) at the indicated concentrations. The procedure for the ATPase assay is described in Materials and methods.

presence of AMP-PNP or even in the presence of AMP-PNP and apyrase (Figure 1b). These results indicate that AMP-PNP may interfere with binding of the motility protein to bovine microtubules. Once bound to microtubules after apyrase treatment, the protein(s) responsible for motor activity was released from microtubules in the presence of MgATP, MgADP, MgGTP and MgCTP (10 mM each) (Figure 1c). In contrast, proteins released from microtubules with buffer containing 0.1 M KCl or 10 mM MgAMP did not support microtubule motility (Figure 1c).

In all instances, the presence of motile activity in these microtubule binding and release experiments correlated with the presence of the 105 kd polypeptide, implicating it as the motility protein. A 75 kd and a 125 kd protein were present in minor amounts in all release samples, but the binding and release of these proteins from microtubules was not nucleotide-dependent. A 100 kd polypeptide, found only in preparations of *Dictyostelium* strain AX3 (Figure 1c), also did not exhibit nucleotide-dependent binding to microtubules. In addition to the 105 kd polypeptide, a 260 kd, a >300 kd polypetide, and the 220 kd myosin bound to microtubules in the presence of apyrase and were released with ATP. The relative amounts of these proteins obtained after different microtubule binding and release conditions did not correlate with microtubule motility, as did the 105 kd polypeptide.

Sucrose density gradient fractionation

To purify the *Dictyostelium* motility protein(s) further, the supernatant released from microtubules with MgATP was loaded onto a sucrose density gradient (Figure 2). Microtubule motility cofractionated with the 105 kd polypeptide at ~9S, again implicating it as the force-producing protein. The major contaminant in the motile fractions was depolymerized bovine tubulin. The 125 kd polypeptide which cofractionated with the 105 kd protein in Figure 2 was not found in all preparations. The high molecular weight polypeptide (>300 kd) migrated at a sedimentation value of ~20S, similar to that of cytoplasmic dyneins. No motility was observed in these fractions, however.

Properties of the Dictyostelium motility protein

Since microtubule-activated ATPase activity is a characteristic property of microtubule-based motility proteins, the ATPase activities of four separate preparations of partially purified *Dictyostelium* motor protein were examined (Table I). All of the samples contained ATPase activity that increased between 2.6- and 10-fold in the presence of

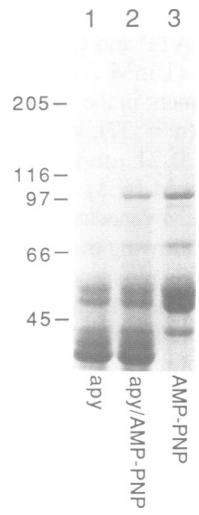

Fig. 3. Rebinding of partially purified kinesin to microtubules. Sucrose gradient fractions containing the peak of the 105 kd protein were incubated with 5 μM taxol and 0.4 mg/ml taxol stabilized bovine microtubules in the presence of apyrase (apy) (1 mg/ml) lane 1, apyrase (1 mg/ml) and AMP-PNP (2 mM) lane 2, or AMP-PNP (2 mM) lane 3. The microtubules were sedimented and the supernatants, depleted of proteins that had bound to the microtubules, were analyzed by electrophoresis. The polypeptides smaller than 40 kd observed in lanes 1 and 2 are seen in samples incubated with apyrase. Control experiments indicate that AMP-PNP does not significantly inhibit ATP hydrolysis by apyrase.

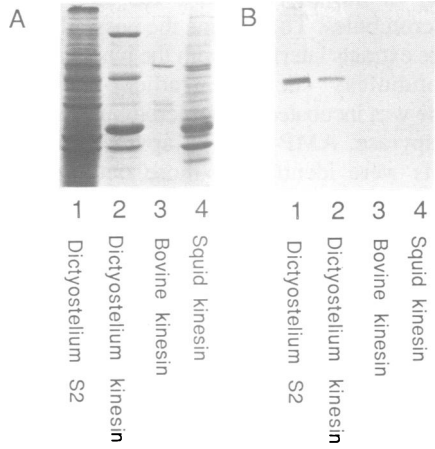

Fig. 4. Cross-reactivity of anti-*Dictyostelium* kinesin antibodies. Two identical gels were run with the following samples: Lane 1, *Dictyostelium* high speed supernatant (S2); lane 2, *Dictyostelium* kinesin (MgATP release); lane 3, bovine kinesin; lane 4, squid kinesin. One gel was stained with Coomassie blue (**A**), while the other gel was blotted to nitrocellulose and probed with 1/500 dilution of anti-*Dictyostelium* kinesin serum (**B**).

microtubules to a level of 670 nmol PO_4/min/mg. The differences in the degree of microtubule stimulation were mostly due to variations in the basal level of activity, which appeared to correlate with the amount of tubulin contamination present in the preparations (data not shown).

Microtubule motility induced by the *Dictyostelium* motility protein was further characterized with regard to nucleotide dependence, velocity, polarity of movement, and pharmacological sensitivity. A minimum protein concentration of ~40 μg/ml of the 105 kd polypeptide was required to

observe movement. Motility was nucleotide-dependent and could be induced by ATP and GTP but not by AMP- PNP, ADP, TTP nor ITP (1 mM concentrations). The velocity of microtubule movement in the presence of 1 mM ATP was 2.0 ± 0.17 μm/sec (n = 37), and in the presence of 2 mM GTP was 1.0 ± 0.21 μm/sec (n = 9). Movement was insensitive to 5 mM NEM and to 20 μM vanadate, but was blocked by 200 μM vanadate. The polarity of movement was determined by following the movement of microtubules polymerized from the plus ends of axonemes (Vale and Toyoshima, 1988a). These motility assays were performed in the presence of 1 mg/ml NEM-modified tubulin to prevent any minus end microtubule growth that might occur during the assay. In all instances, microtubules moved with the minus end of the microtubule leading (n = 53), which indicates relative movement of the motor towards the plus end of the microtubule. This direction of movement is the same as the kinesin class of motility proteins (Vale *et al.*, 1985a). The nucleotide requirements for movement and the pharmacological sensitivities to NEM and vanadate are also characteristic of kinesin but not of dynein molecules (Porter *et al.*, 1987; Vale *et al.*, 1985a; Vallee *et al.*, 1988).

Microtubule binding studies with partially purified kinesin

The results of the microtubule binding experiments with high speed supernatants indicated that AMP-PNP may decrease the affinity of the *Dictyostelium* 105 kd polypeptide for bovine microtubules. To examine the possibility that factors in the crude extracts interfered with the binding of this protein to microtubules, sucrose gradient-purified 105 kd polypeptide was incubated with microtubules in the presence of either apyrase, AMP-PNP, or apyrase and AMP-PNP. The results were identical to those obtained with crude extracts; the 105 kd polypeptide bound microtubules in the presence of apyrase and was completely removed from the supernatant, while in the presence of AMP-PNP or AMP-PNP and apyrase, the 105 kd polypeptide did not bind to microtubules and remained in the supernatant (Figure 3). Control experiments verified that bovine kinesin cosedimented with microtubules in the presence of AMP-PNP under the same conditions (data not shown). AMP-PNP also released the 105 kd polypeptide from microtubules once it was bound in the presence of apyrase (data not shown). These results indicate that, unlike kinesin from other organisms, the affinity of the *Dictyostelium* 105 kd polypeptide for microtubules is decreased by AMP-PNP.

Immunoreactivity of anti-Dictyostelium kinesin antibodies

An antiserum against electrophoretically purified 105 kd protein was raised in rabbits. The immune serum reacts strongly with the 105 kd protein in lysates of *Dictyostelium* and in supernatants released from microtubules with MgATP (Figure 4). The serum did not cross-react with bovine or squid kinesin, nor did anti-squid kinesin antiserum cross-react with *Dictyostelium* kinesin, although it recognizes mammalian (Neighbors *et al.*, 1988), sea urchin (Scholey *et al.*, 1985) and *Drosophila* (Saxton *et al.*, 1988) kinesins. This result suggests that the *Dictyostelium* 105 kd polypeptide is immunologically distinct from kinesins isolated from higher eukaryotes.

Discussion

We have identified and partially purified a *Dictyostelium* microtubule-based motility protein that translocates microtubules when adsorbed to a glass slide. This motile activity consistently cofractionates with a 105 kd polypeptide using a variety of microtubule binding and release conditions and on sucrose density gradients. As is expected for a microtubule-based motor protein, fractions containing the 105 kd polypeptide also demonstrate microtubule-activated ATPase activity. Although less abundant polypeptides than the 105 kd polypetpide are present in partially purified *Dictyostelium* motor preparations, their relative concentrations in different preparations do not correlate with microtubule movement, as does the amount of the 105 kd polypeptide. Furthermore, since movement of microtubules on glass requires a rather high protein threshold concentration of kinesin or dynein (>15 μg/ml; Vale and Toyoshima, 1988b), it is unlikely that a minor contaminant, rather than the abundant 105 kd polypeptide, is responsible for motility. Whether the 105 kd polypeptide has associated light chains remains to be determined.

Many properties of the 105 kd *Dictyostelium* motor protein are similar to kinesin molecules isolated from other organisms. The size of this polypeptide is similar to that of the kinesin heavy chain isolated from other sources (110−135 kd), and the sedimentation coefficient (~9S) of the native *Dictyostelium* molecule is the same as that of kinesins from sea urchin (Scholey *et al.*, 1985) and bovine brain (Bloom *et al.*, 1988; Kuznetsov *et al.*, 1988). The *Dictyostelium* motor protein utilizes ATP or GTP to move microtubules on glass, and a variety of nucleotides, including ATP, GTP and ADP, release the 105 kd *Dictyostelium* motor from a rigor binding interaction with microtubules. Such nucleotide-dependent motility and microtubule binding properties are characteristic of kinesin (Vale, 1987; Porter *et al.*, 1987) but not of dynein molecules (Shpetner *et al.*, 1988; Vale and Toyoshima, 1989; Mitchell and Warner, 1980; Paschal *et al.*, 1987). The insensitivity of movement to NEM and low concentrations of vanadate are characteristic pharmacological features of kinesins (Vale, 1987). Most importantly, the *Dictyostelium* motility protein supports anterograde movement along microtubules, the same direction as that produced by kinesin, and opposite to that of dynein. Taken together, these pieces of evidence strongly suggest that the 105 kd polypeptide is the kinesin homolog in *Dictyostelium*.

Certain properties of the *Dictyostelium* kinesin homolog reveal its divergence from its higher eukaryotic counterparts. First, polyclonal antibodies raised against *Dictyostelium* kinesin do not cross-react with squid or bovine kinesins and vice versa. In contrast, anti-squid kinesin antibodies react strongly with kinesins from *Drosophila*, sea urchin and mammals. The velocity of microtubule movement on glass induced by the *Dictyostelium* motor protein (2.0 μm/sec) is also 4-fold faster than the movement generated by most other kinesins in a similar assay system (Scholey *et al.*,1985; Vale *et al.*, 1985a; Saxton *et al.*, 1988). Interestingly, a partially purified kinesin-like molecule from the unicellular organism *Acanthamoeba castellanii* also promotes comparatively fast velocities of movement of 3−4 μm/sec (Kachar *et al.*, 1987).

The most intriguing difference between the kinesin-like molecule from *Dictyostelium* and kinesins isolated from

higher eukaryotes is the effect of the non-hydrolyzable ATP analog, AMP-PNP, on microtubule binding. Kinesin was first isolated on the basis of its ability to form a rigor-like association with microtubules in the presence of AMP-PNP (Vale *et al.*, 1985a; Scholey *et al.*, 1985; Brady, 1985). In contrast, AMP-PNP decreases the affinity of dynein for microtubules (Mitchell and Warner, 1980) and of myosin for actin (Greene and Eisenberg, 1980). Thus, it has been thought that the AMP-PNP-induced rigor binding state is an unique feature of the kinesin class of molecules (Vale, 1987), and it has been speculated that AMP-PNP rigor binding may reflect certain underlying differences in the mechanochemical cycle of kinesin compared with other motor proteins (e.g. Lasek and Brady, 1985). In this study, we find that the *Dictyostelium* motor protein, which is kinesin-like in its other properties, has a lower affinity for microtubules in the presence of AMP-PNP. Thus, an AMP-PNP-induced rigor binding state appears not to be a defining characteristic of kinesin molecules as was previously thought, a point that should be considered in developing strategies to purify kinesin from other lower eukaryotes or plants.

The bidirectional movement of organelles along microtubules in *Dictyostelium* suggests that cells contain motor proteins other than the 105 kd molecule, which produces only anterograde movement. In other systems where bidirectional organelle movements have been characterized, a retrograde motor with dynein-like characteristics has been identified (Paschal and Vallee, 1987; Schnapp and Reese, 1989). We also observe a high molecular weight polypeptide that exhibits certain dynein-like properties such as ATP-dependent binding to microtubules and sedimentation at 20S in sucrose density gradients. This protein has been more extensively characterized by M.P.Koonce and J.R.McIntosh (manuscript submitted), who have obtained additional evidence that this protein is indeed a cytoplasmic form of dynein.

The biological functions of *Dictyostelium* kinesin are undetermined, although the similar velocities of kinesin-induced microtubule movement *in vitro* (2.0 μm/sec) and organelle transport *in vivo* (2−3 μm/sec; Roos *et al.*, 1987) suggest that it may drive the anterograde movement of organelles along microtubules. The ability to replace a wild type gene in *Dictyostelium* with a specific mutation (De Lozanne and Spudich, 1987; Witke *et al.*, 1987; Manstein *et al.*, 1989) will allow us to examine the biological roles of kinesin using genetic as well as biochemical approaches. We are in the process of cloning the kinesin gene in *Dictyostelium* to achieve these goals.

Materials and methods

Materials

Taxol was generously supplied by Dr Matthew Suffness at the National Cancer Institute. All nucleotides, *N*-ethyl-maleimide (NEM), orthovanadate, protease inhibitors and apyrase (Grade V:Potato) were obtained from Sigma Chemical Co. Ultra-pure sucrose was obtained from Bethesda Research Laboratories. Freunds adjuvant was from Gibco, Inc. Goat anti-rabbit IgG alkaline phosphatase-conjugated antibodies was obtained from Bio-Rad Laboratories. Other reagents were from Fischer Scientific, Inc.

Cell extracts and protein isolation

Dictyostelium discoideum (strain AX4 for most experiments, AX3 for the experiment shown in Figure 1c) was grown axenically in HL5 medium to mid or late log phase (5×10^6 cells/ml). Cells were harvested by centrifugation at 1500 *g*, washed twice with Tris-buffered saline (TBS; 150 mM NaCl, 10 mM Tris, pH 7.7), and resuspended in an equal volume of 30% sucrose in M75 (75 mM PIPES, 4 mM $MgCl_2$, 5 mM EGTA, 0.1 mM EDTA, pH 6.85) containing 1 mM dithiothreitol (DTT), 1 μg/ml leupeptin, 1 μg/ml pepstatin, 10 μg/ml soybean trypsin inhibitor, 5 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone, 1 μg/ml chymostatin, and 1 mM PMSF. Cells were sonicated on ice with 4−5 × 15 s bursts, and examined by microscopy for lysis (generally >95%). All of the following procedures were carried out at 4°C in the presence of DTT and protease inhibitors. Cell lysates were centrifigued at 15 000 *g* for 20 min to remove whole cells, nuclei and large membranes. The supernatant (S1) was then centrifuged at 175 000 *g* for 90 min. The high speed supernatant (S2) was incubated with apyrase (1 mg/ml) for 10 min on ice, unless otherwise noted for binding studies. Taxol (5 μM) and phosphocellulose purified, taxol-stabilized bovine microtubules (0.5 mg/ml) were then added and incubated for 60 min on ice, prior to centrifugation at 35 000 *g* for 30 min. All microtubule incubations were carried out on ice in the presence of taxol since incubation at a higher temperature resulted in some protein degradation. To remove weakly associated proteins, the microtubule pellets were resuspended in 1/5 of the original S2 volume with M75 + 50 mM KCl and resedimented through a cushion of 10% sucrose in M75 + 5 μM taxol. These microtubule pellets were resuspended in 1/20 volume with M75 supplemented with $MgCl_2$ to 10 mM, taxol to 5 μM, and 10 mM nucleotide (ATP unless indicated). The microtubules were again sedimented and the supernatant (containing the ATP-released motor activity) was removed and stored on ice.

Additional purification of the motor activity was achieved by loading 1 ml of the MgATP release on an 11 ml 5−25% sucrose gradient in M75 which was centrifuged in a Beckman SW41 rotor for 12 h at 34 000 r.p.m. Fractions (0.5 ml) were collected from the bottom of the tube and assayed for motile activity.

Bovine brain tubulin, NEM-modified tubulin and microtubules were prepared as previously described (Vale and Toyoshima, 1988a).

Motility assays

12 μl of S2 or partially purified kinesin samples were adsorbed to a glass slide (Clay Adams, Gold Seal) in a drop for 5 minutes at room temperature. 1.4 μl of taxol-stabilized bovine microtubules (0.3 mg/ml) and 1.4 μl of 10 mM ATP were added, and a coverslip was placed on top of the mixture. When nucleotides other than ATP were tested for their ability to promote microtubule movement, they were pretreated with 50 units/ml hexokinase and 10 mM glucose to remove any contaminating ATP. Microtubule movement on the glass slide was examined by dark field microscopy as previously described (Vale and Toyoshima, 1988a). To assay for drug sensitivity, the kinesin preparation was incubated with different concentrations of orthovanadate or NEM in an Eppendorf tube for 5 min, after which 10 min DTT was added to the NEM-treated samples. The samples were then adsorbed to a glass slide for 5 minutes and assayed for microtubule movement. Velocities of microtubule movement were measured using a computer program developed by Dr Steve Block (described in Sheetz *et al.*, 1986).

The polarity of movement was determined by following the movement of *Tetrahymena* axonemes that had microtubules selectively polymerized from their plus ends (Vale and Toyoshima, 21988a). Axonemes were incubated with 1 mg/ml each of unmodified and NEM treated tubulin and 1 mM GTP for 15 min at 37°C, which results in selective growth from the axoneme plus ends (Huitorel and Kirschner, 1988). Polymerization was terminated and the microtubules were stabilized by diluting the sample 50-fold in M75 + 20 μM taxol. Motility assays for polarity were performed with the addition of 1 mg/ml NEM tubulin to prevent any minus end microtubule growth that might occur during the assay. Bovine kinesin was used as a control to confirm that the polartiy of movement generated by kinesin was with the minus end of the microtubule leading.

ATPase assay

ATP hydrolysis was measured using the colorimetric assay of Kodama (Kodama *et al.*, 1986). A 50 μl reaction mixture containing ~0.2 μg of *Dictyostelium* kinesin in M75 buffer diluted 1:1 in H_2O containing 0.1 mM ATP was prepared in the presence or absence of bovine brain microtubules. After a 10 min incubation, which is within the linear range of ATP hydrolysis by the peak fractions, the reaction was stopped by addition of 150 μl of M75 followed by 200 μl of Malachite Green reagent (Kodama *et al.*, 1986). Color development after 10 min was measured at 640 nm with a spectrophotometer and was compared to a standard curve of inorganic phosphate.

Antibody production

Two New Zealand white rabbits were immunized with 50 μg of 105 kd polypeptide that had been electroeluted from a Coomassie stained

polyacrylamide gel. Primary injections were performed using antigen mixed 1:1 with complete Freunds adjuvant. Booster injections (at 3 week intervals) were mixed with incomplete Freunds adjuvant. Sera were collected prior to immunization and after 2 and 3 booster injections.

Electrophoresis and electrophoretic transfer blots

SDS polyacrylamide gel (7.5%) electrophoresis was performed according to the method of Laemmli (1970). Proteins were visualized by Coomassie blue staining. For immunoblotting, unstained gels were electrophoretically transferred to nitrocellulose filters as described in Towbin *et al.* (1979). Filters were incubated in 5% nonfat dry milk in TBS for at least one hour. Following extensive washing in TBS, 0.1% Tween 20 (wash buffer), primary antibody was added to TBS containing 0.01% Tween 20 and 1 mg/ml bovine serum albumin (antibody buffer) and incubated for 2 h. After several washes, second antibody (goat anti-rabbit IgG alkaline phosphatase-conjugated) was added at a dilution of 1:3000 in antibody buffer, and incubated for 1 h. The alkaline phosphatase reaction was conducted according to the manufacturer's protocol (Bio-Rad Laboratories). All incubations were done at room temperature.

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