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# Evidence for myosin motors on organelles in squid axoplasm

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**ABSTRACT** **Squid axoplasm has proved a rich source for the identification of motors involved in organelle transport. Recently, squid axoplasmic organelles have been shown to move on invisible tracks that are sensitive to cytochalasin, suggesting that these tracks are actin filaments. Here, an assay is described that permits observation of organelles moving on unipolar actin bundles. This assay is used to demonstrate that axoplasmic organelles move on actin filaments in the barbed-end direction, suggesting the presence of a myosin motor on axoplasmic organelles. Indeed, axoplasm contains actin-dependent ATPase activity, and a pan-myosin antibody recognized at least four bands in Western blots of axoplasm. An ≈235-kDa band copurified in sucrose gradients with KI-extracted axoplasmic organelles, and the myosin antibody stained the organelle surfaces by immunogold electron microscopy. The myosin is present on the surface of at least some axoplasmic organelles and thus may be involved in their transport through the axoplasm, their movement through the cortical actin in the synapse, or some other aspect of axonal function.**

It is now well established that organelles are transported along microtubules in the cytoplasm of a wide variety of cells (1–3). Isolated squid axoplasmic vesicles have also been reported to move on invisible tracks that are sensitive to cytochalasin but not to nocodazole, suggesting that they are moving along actin filaments (4). Indeed, myosin-mediated movements of organelles have also been implicated in intracellular targeting (5–13). By using an *in vitro* video assay of actin-based organelle motility, we show that axoplasmic organelles move toward the barbed ends of actin filaments and that one of the several myosins present in axoplasm is a prime candidate for mediating this barbed-end directed motility.

## MATERIALS AND METHODS

**Materials.** Squid (*Loligo pealii*) and horseshoe crabs (*Limulus polyphemus*) were obtained from the Marine Biological Laboratory, Woods Hole, MA. Antibodies raised against purified muscle myosin from scallop (aequipecten) and immunoabsorbed to remove antibodies to light chains were a generous gift from A. Szent-Györgyi (14). Rabbit skeletal muscle actin, muscle myosin II, and S1 were purified (15–17). Other reagents were rhodamine phalloidin (Molecular Probes), calmodulin (Boehringer Mannheim), and cytochalasin B and nocodazole (Sigma).

**Actin-Filament Elongation Off Acrosomal Processes.** Acrosomal processes of *Limulus* sperm (200 μl) were isolated (18) and stored in 50 μl of HNM (30 mM Hepes, pH 7.4/20 mM NaCl/2 mM Mg $Cl_2$) for up to a week at 4°C. For electron microscopy, globular actin (0.5 mg/ml) was polymerized off the acrosomal process for 15 min (19). Actin filaments were labeled with freshly prepared S1 (0.11 mg/ml in HNM). For video microscopy, the same protocol was followed except that polymerization proceeded in solution up to 12 h, S1 was omitted, and some preparations were labeled with rhodamine-phalloidin (0.6 μM) for 1–4 h.

**Video Microscopy of Axoplasmic Organelles on Actin Bundles.** Squid axoplasm was extruded into 3 vol of HNM containing calmodulin (10 μg/ml), preloaded with a 2 times molar excess of calcium, 10 mM Mg-ATP, and 0.5 mM dithiothreitol. In some experiments, axoplasm was treated with cytochalasin B (50 μM) and nocodazole (100 mg/ml). A typical axon yielded from 20 to 30 μl of diluted axoplasm that could be stored on ice for up to 6 h. Actin-extended acrosomal processes (2 μl) in HNM were perfused into a microscope perfusion chamber (20) and allowed to adhere for 3–5 min. After blocking with 0.04% instant milk in 1× polymerization buffer (PB) (19), 5 μl of diluted axoplasm was perfused into the chamber. Video microscopy was performed with a Nikon FXA (1.4 N.A.).

**ATPase Assay.** Two microliters of axoplasm diluted 1:3 was added to parallel tubes containing 250 μl of HNM with 4 mM EGTA. Filamentous actin (10 μl of a stock at 7 mg/ml in HNM) was added to one tube and all tubes were brought to 0.5 ml with HNM. After 15 min, 10 μl of 0.1 M ATP was added to the tube containing actin and one of the two other tubes containing axoplasm alone. After 1 h at 4°C, protein was precipitated with trichloroacetic acid and the soluble phosphate was measured by colorimetric assay (21).

**Isolation of Organelles.** Approximately 75 μl of squid axoplasm was extruded into 75 μl of motility buffer (MB; refs. 22–24), containing protease inhibitors [leupeptin (10 μg/ml)/pepstatin A (0.7 μg/ml)/*p*-tosyl-L-arginine methyl ester (10 μg/ml)/0.5 mM dithiothreitol]. The diluted axoplasm was brought to 0.6 M KI, triturated, incubated for 10 min on ice, diluted 1:1 with MB, and centrifuged (12,000 × *g* for 5 min). The resulting supernatant (≈250 μl) was layered onto a sucrose step gradient (200 μl of 12% sucrose, 150 μl of 15% sucrose, and 60 μl of 45% sucrose in MB), and centrifuged for 1.5 h at 35,000 rpm in a Beckman SW 55.1 rotor. Supernatant and three fractions were used for up to 2 days. By electron microscopy, organelles were highly enriched in the 15% fraction.

**Western Blot Analysis of Myosin.** Squid optic lobe, axon sheath, and mantle muscle were homogenized directly in gel sample buffer (GSB; ref. 25). Axoplasm was extruded directly in 3 times its volume of GSB. Sucrose step fractions were mixed 1:1 with 4× GSB. After separation of protein by SDS/PAGE, gels were either stained with Coomassie blue or transferred to nitrocellulose (26). Blots, blocked in 5% (wt/vol) instant milk, were stained with a 1:200 dilution of anti-myosin antibody for 1.5 h. After washing, blots were incubated in 1:3000 dilution alkaline phosphatase-conjugated secondary antibody (GIBCO/BRL), washed, and developed in nitroblue tetrazolium.

**Immunogold Labeling of Purified Axoplasmic Organelles.** Glow-discharged carbon-Formvar-coated nickel grids were incubated on droplets of the 15% sucrose fraction, blocked in bovine serum albumin (10 mg/ml), incubated in 1:50 or 1:100 dilution of anti-myosin antibody, washed, and stained with

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protein A gold. Control preparations with anti-neurofilament antibody (gift of H. Gainer, National Institutes of Health) as primary antibody, or with no primary antibody were prepared in parallel. Grids were negative-stained with 1% uranyl acetate and photographed in a JEOL CX 200.

## RESULTS

**Plumes of Oriented Actin Filaments Formed on Acrosomal Processes Can Be Seen by Light Microscopy.** Upon incubation in globular actin, a plume of actin filaments formed off one end of each acrosomal process (Fig. 1*A*), whereas only a tuft formed at the other end. Decoration with the S1 fragment of myosin II revealed that the actin filaments in the plume were uniformly oriented with their barbed ends directed away from the process (Fig. 1 *A* and *B*). S1 does not decorate the actin filaments within the acrosomal process (18, 27). The acrosomal processes and the plumes of actin filaments grown off their barbed ends were visible by video-enhanced differential-interference microscopy (Fig. 1*C*) and by fluorescence after rhodamine-phalloidin staining of the actin (Fig. 1*D*).

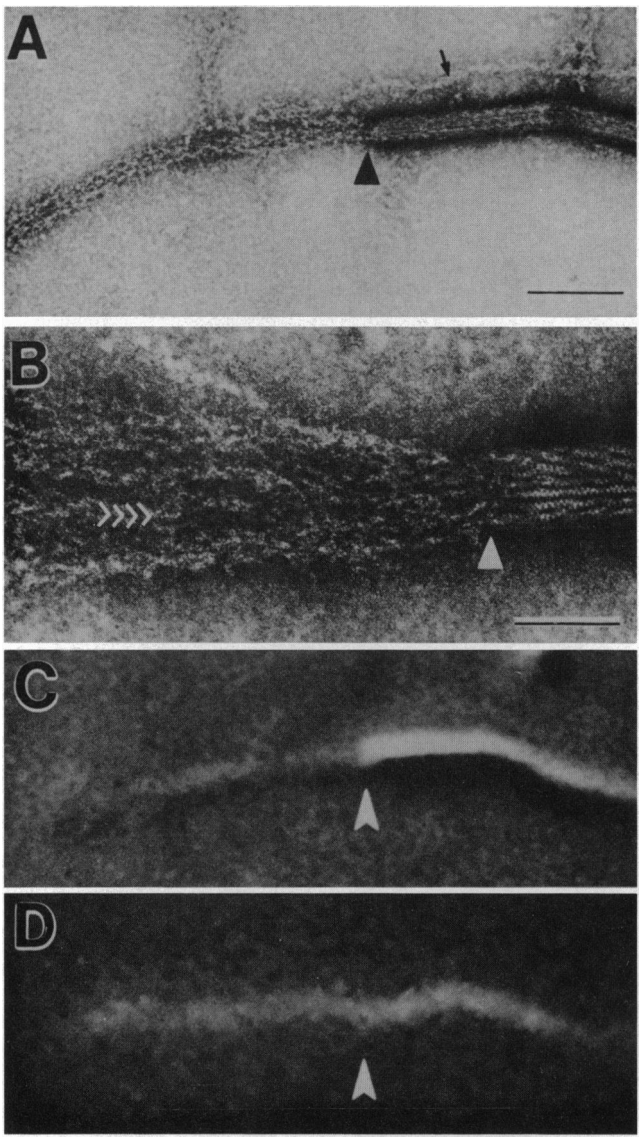


FIG. 1. Oriented actin filaments polymerized off acrosomal processes can be detected by light microscopy. (*A*) At arrowhead, an isolated fragment of acrosome gives rise to plume of actin filaments seen by negative staining. (Bar = 0.25 μm.) (*B*) S1 decoration of the acrosomal actin filaments, showing that they are oriented barbed end out (as indicated by white arrowheads) when they originate from the end of the acrosome (indicated by white arrowhead). Filaments occasionally escaping from the bundle may reverse their course (small arrow in *A*). Only short tufts of filaments form from the opposite ends of these acrosomes. (Bar = 0.1 μm.) (*C*) Direct visualization of actin plume in organelle movement assay by video-enhanced differential contrast microscopy. White arrowhead indicates the end of the acrosomal process. (*D*) Rhodamine-phalloidin staining confirms that the plume is composed of actin filaments. White arrowhead is at end of acrosomal process.

**Axoplasmic Organelles Move Toward the Barbed End of Actin Filaments.** Oriented actin filaments on acrosomal processes served as a substrate for the directed motility of vesicles in extruded squid axoplasm, but no movements were apparent on the acrosomal processes themselves. Vesicles frequently attached near the origin of the plume of actin filaments and moved distally, where they escaped (Fig. 2*A*) or became stuck, leaving many vesicles attached to the outermost region of the plume. Vesicles moved inward toward the acrosomal process as well, but these movements could be along individual filaments that had bent backward (Fig. 1*A*). Only the largest vesicles were unable to move along actin plumes. In four preparations, >30 moves of 1 μm or more were observed; >80% of these were in the barbed-end direction. Some vesicles moving in either direction made jerky discontinuous movements, as if entangled in invisible filaments. It was difficult to measure an average velocity of vesicle movement because the visible part of the plume is short (1–1.5 μm), but short bursts up to 0.1 μm/s were recorded (Fig. 2*A*).

Electron microscopy of organelle–acrosome complexes showed that the attached particles are membrane-bound organelles and that they are in direct contact with the actin filaments extending from the acrosomal process (Fig. 2*B*). Microtubules were apparent in some preparations, even after nocadazole treatment, and electron microscopy also revealed the presence of neurofilaments. While microtubules did not participate in the organelle–actin plume complexes, occasionally, an organelle that had moved on actin filaments subsequently attached to and moved along a separate microtubule.

**High Molecular Mass Myosins Are Present in Squid Optic Lobe and in Axoplasm.** Myosins, the only known barbed-end-directed actin-based motors, have a well-characterized enzymatic activity—hydrolysis of ATP in $K_2$EDTA that is enhanced by actin filaments (28–31). While other molecules may also hydrolyze ATP in EDTA, only the myosins increase ATPase activity in actin. Actin-dependent ATPase activity was measured to test for the presence of active myosin. When 2 μl of axoplasm was incubated alone in ATPase assay buffer, an average of <5 nmol of phosphate was released per hour (Table 1). In contrast, when exogenous ATP was added, axoplasm hydrolyzed 15 nmol of phosphate per hour. This hydrolysis was further enhanced 2-fold with the addition of filamentous actin. To remove endogenous actin, axoplasm was treated with cytochalasin prior to addition of ATP, which decreased baseline ATP hydrolysis by a factor of 10.

A polyclonal anti-myosin antibody that recognizes myosins from a wide range of invertebrates (14) was used to detect myosins in squid optic lobe, axoplasm, and muscle. The antiserum recognized purified aequipecten myosin and a 220-kDa band in squid muscle (data not shown), and two bands in optic lobe: a faint band at 220 kDa and a strong band at 230 kDa (Fig. 3, lanes 1 and 4). In the axonal sheath (Fig. 3, lanes 2 and 5), only a 230-kDa band was detected. In contrast, the strong 220-kDa band in axoplasm probably corresponds to the faint lower band in optic lobe (Fig. 3, lanes 3 and 6). At least three other bands are detected in axoplasm, at ≈235, 240, and 150 kDa. Since the cortical cytoskeleton is left behind during extrusion, it is likely that the myosins detected in axoplasm are present in the internal cytoplasm

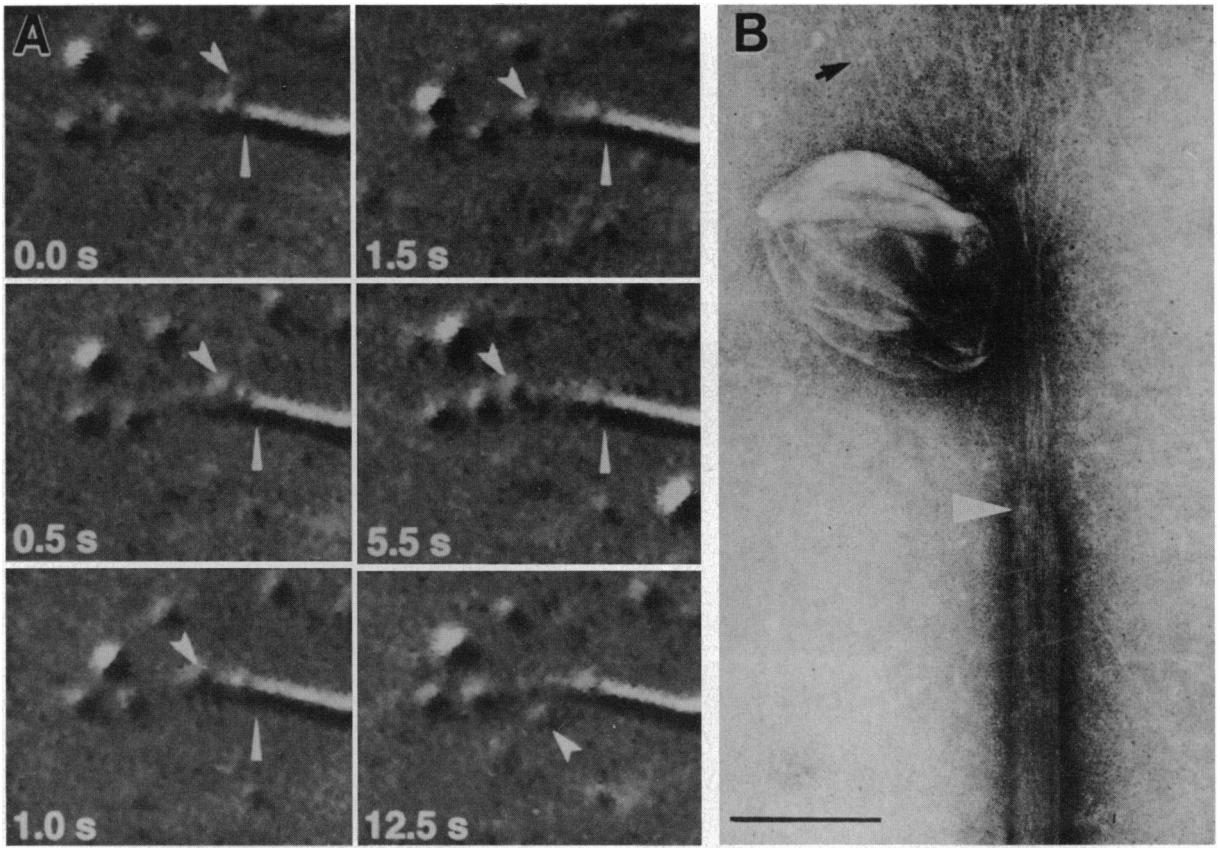


FIG. 2. Axoplasmic organelles attach to and move toward the barbed ends of actin plumes. (*A*) Organelle at curved arrowhead attaches to tuft between 0.0 and 0.5 s, moves continuously toward the end of the tuft between 0.5 and 1.5 s, stops, and returns to Brownian movement at 12.5 s. The highest continuous velocity is 6 μm/min. Plain arrowhead marks origin of actin tuft. (*B*) Negative stain of organelle on plume of actin filaments at the barbed end of an acrosomal process (white arrowhead). The organelle appears to contact multiple actin filaments (individual actin filament at small black arrow). (Bar = 0.25 μm.)

where transport takes place. The differences in molecular mass between the three larger myosins are minor and could represent phosphorylation or different isoforms. These myosins migrate with the prominent band composed of neurofilament protein, and thus, their relative proportion cannot be calculated by comparison of Coomassie-stained bands.

**A High Molecular Mass Myosin Copurifies with Organelles and Is Present on Isolated Organelles.** To determine whether any of the myosins in axoplasm are potential candidates for a role in actin-based motility of organelles, it was necessary to know whether they are associated with organelles. Axoplasm in MB was KI-extracted and fractionated in a four-step sucrose density gradient to separate organelles from other axoplasmic components (22–24). By SDS/PAGE, the banding pattern of axoplasm, sucrose gradient supernatant, and the other sucrose gradient fractions appeared essentially the same with the bulk of the protein remaining in the supernatant (Fig. 4*A*, lanes 1–4). By Western blot analysis, all of the myosins found in axoplasm were present in the supernatant (Fig. 4*B*, lane 4). In contrast, only one of these bands, ≈235 kDa, was also present in the organelle fraction (Fig. 4*B*, lane 2), suggesting that this myosin is a good candidate to be involved in organelle transport.

Decoration of these isolated organelles with anti-myosin antibodies suggested that this myosin was associated with

Table 1. Actin-activated ATPase activity in squid axoplasm

| Assay | Phosphate released | |
|---|---|---|
| | Mean, nmol/h | Range |
| Axoplasm alone | 5 | 0.5–9 |
| Axoplasm + ATP | 15 | 0.6–19* |
| Axoplasm + ATP + actin | 30 | 22–38 |

Five experiments were performed for each assay condition.
*Low value occurred after addition of cytochalasin.

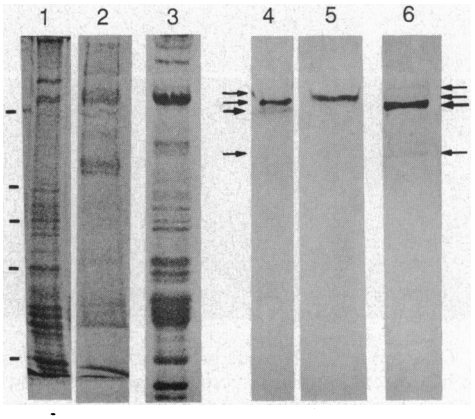


FIG. 3. Anti-myosin antibodies recognize at least four bands in axoplasm. Coomassie-stained SDS/PAGE 8% gel (lanes 1–3) and corresponding Western blots (lanes 4–6) probed with anti-myosin of optic lobe (lanes 1 and 4), axon sheath (lanes 2 and 5), and extruded axoplasm (lanes 3 and 6). The antibodies detect four bands in axoplasm with apparent molecular masses of 150, 220, 235, and 240 kDa (arrows). The 230-kDa band is enriched in optic lobe and sheath, and the 220-kDa band is enriched in axoplasm. The 150-, 235-, and 240-kDa bands are only detected in axoplasm. Molecular mass standards indicated at left are myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *a* (98 kDa), bovine serum albumin (68 kDa), and ovalbumin (45 kDa).

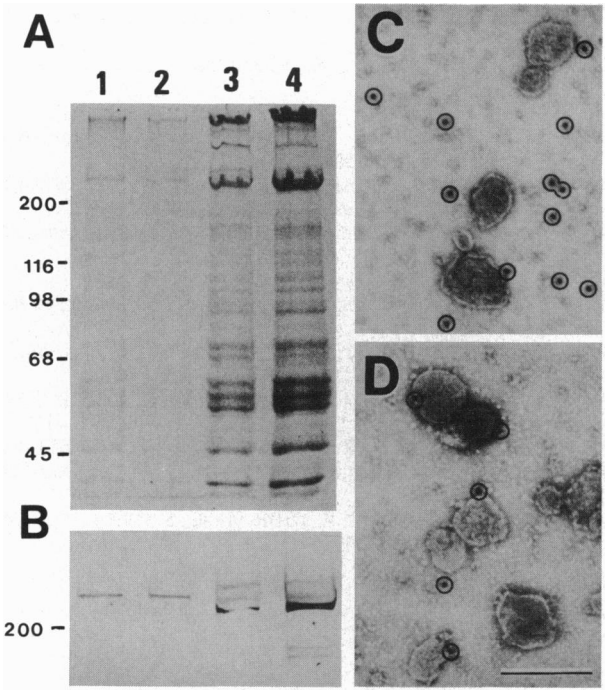


FIG. 4. Axoplasmic myosin copurifies with axoplasmic organelles and is localized by immunogold to the surface of isolated organelles. Coomassie-stained SDS/PAGE 8% gels (*A*) and corresponding anti-myosin Western blot (*B*) of fractions from a sucrose density step gradient of axoplasm. Lanes: 1, 45% sucrose; 2, 15% sucrose; 3, 12% sucrose; 4, cytosol. By electron microscopy, organelles are enriched in the 15% fraction. All four myosin bands detected in axoplasm are present in the cytosol, while only one of these, migrating at 235 kDa, is detected in the organelle (15%) fraction (lane 2). Positions of molecular mass standards are at the left. Negative-stained organelles are labeled with antibody to neurofilament (*C*) or myosin (*D*). Gold label is indicated by circles. Analysis of distributions of 15-nm gold particles showed that organelles are selectively stained with myosin (see text). (Bar = 0.25 μm.)

some organelles (Fig. 4*D*). Counts from 10 randomly selected electron micrographs revealed that gold particles were 12 ± 6.5 (mean ± SD) times more concentrated in the vicinity of an organelle than in the background in anti-myosin-stained preparations, whereas gold particles were only 2 ± 0.4 times more concentrated in the vicinity of an organelle than in the background in preparations stained with anti-neurofilament antibodies (Fig. 4*C*).

## DISCUSSION

We used an actin assay consisting of plumes of actin filaments polymerized off the ends of acrosomal processes to characterize the actin-based transport system in squid axoplasm. This assay has the advantages that the plume of actin filaments can be observed directly by video microscopy and that these actin filaments have a uniform polarity. Native organelles are readily observed moving distally along the plume of the actin filaments. Since long actin filaments form only at the barbed ends of acrosomal processes, these organelle movements must be toward barbed ends.

The only actin-based motors known are the myosins, which are barbed-end-directed motors (29–31). The extruded axoplasm contains several myosins, and one of these, an ≈235-kDa protein, copurifies with KI-stripped organelles. The same myosin antibody that recognized this single band in immunoblots of isolated organelles also labeled the surfaces of organelles by immunogold. Although this myosin is larger than other myosins known to be involved in vesicle transport (30), it appears to be a prime candidate for a transport motor. The myosin enriched in optic lobe and axon sheath may well be the 200-kDa myosin previously purified from optic lobe that is thought to be a contractile rather than a motor protein (32).

While the present results establish that axoplasmic organelles move toward the barbed ends on actin filaments and that at least one myosin is associated with these organelles, it remains unclear whether these motors operate in intact axoplasm and what role they might have if they do. They might form a system redundant with and possibly assisting in anterograde transport of organelles (4). However, it is not clear that the membrane vesicles that move on actin filaments in the acrosome assay are identical with the vesicular organelles that move on microtubules in the intact axon.

There are, however, several reasons to believe that the myosin on organelles might not function as a motor in the axon, where there are actin filaments in the vicinity of the microtubules. These filaments, however, are very short, <1 μm long, and their polarity is unknown (33, 34). In fact, a component of short filaments in the axonal growth cone are known to have mixed polarities (35). Furthermore, anti-kinesin antibodies block anterograde movements of organelles (36). Since isolated organelles driven by kinesin can, in the absence of any actin filaments, consistently make concerted movements at the fast anterograde transport velocity (24), which is 10 times greater than that of organelles on actin filaments, there is no obvious need for a myosin motor in axoplasm.

Vesicular myosins in axoplasm could be inactive while being carried down the axon, where activation would permit transport of organelles along cortical actin filaments to sites of interaction with the plasma membrane. For example, vesicles in the microvilli of the intestinal brush border are apparently transported along actin filaments to the apical membrane (7, 10). Thus, there are several axonal functions other than fast axonal transport that might be served by the organelle-associated myosin motor demonstrated here.

**Note Added in Proof.** G. Langford *et al.*[§] also reported barbed-end-directed movements of axoplasmic organelles on actin filaments.

[§]Langford, G., Kuznetsov, S. & Weiss, D., The General Scientific Meeting at the Marine Biological Laboratory, Aug. 17, 1993, Woods Hole, MA.

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1. Allen, R. D., Metuzals, J., Tasaki, I., Brady, S. T. & Gilbert, S. P. (1982) *Science* **218,** 1127–1128.
2. Vale, R. D., Schnapp, B. J., Reese, T. S. & Sheetz, M. P. (1985) *Cell* **40,** 559–569.
3. Vale, R. D., Reese, T. S. & Sheetz, M. P. (1985) *Cell* **42,** 39–50.
4. Kuznetsov, S. A., Langford, G. M. & Weiss, D. G. (1992) *Nature (London)* **356,** 722–725.
5. Adams, R. J. & Pollard, T. D. (1986) *Nature (London)* **322,** 754–756.
6. Espereafico, E. M., Cheney, R. E., Matteoli, M., Nacimento, A. A. C., De Camilli, P. V., Larson, R. E. & Mooseker, M. S. (1992) *J. Cell Biol.* **119,** 1541–1557.
7. Fath, K. R. & Burgess, D. R. (1993) *J. Cell Biol.* **120,** 117–127.
8. Johnston, G. C., Prendergast, J. A. & Singer, R. A. (1991) *J. Cell Biol.* **113,** 539–551.
9. Lillie, S. H. & Brown, S. S. (1992) *Nature (London)* **356,** 358–361.

10. Mooseker, M. S., Conzelman, K. A., Coleman, T. R., Heuser, J. E. & Sheetz, M. P. (1989) *J. Cell Biol.* **109,** 1153–1161.
11. Sanders, G., Lichte, B., Meyer, H. E. & Kilimann, M. W. (1992) *FEBS Lett.* **311,** 295–298.
12. Sheetz, M. P., Block, S. M. & Spudich, J. A. (1986) *Methods Enzymol.* **134,** 531–544.
13. Sheetz, M. P., Chasan, R. & Spudich, J. A. (1984) *J. Cell Biol.* **99,** 1867–1871.
14. Walliman, T. & Szent Györgyi, A. G. (1981) *Biochemistry* **20,** 1176–1187.
15. Pardee, J. D. & Spudich, J. A. (1982) *Methods Enzymol.* **85,** 164–181.
16. Kron, S. J., Toyoshima, Y. Y., Uyeda, T. Q. P. & Spudich, J. A. (1991) *Methods Enzymol.* **196,** 399–416.
17. Hynes, T. R., Block, S. M., White, B. T. & Spudich, J. A. (1987) *Cell* **48,** 953–963.
18. Tilney, L. G. (1975) *J. Cell Biol.* **64,** 289–310.
19. Pollard, T. D. (1986) *J. Cell Biol.* **103,** 2747–2754.
20. Bearer, E. L. (1991) *J. Cell Biol.* **115,** 1629–1638.
21. Taussky, H. H. & Shorr, E. (1952) *J. Biol. Chem.* **202,** 675–685.
22. Schroer, T. A., Schnapp, B. J., Reese, T. S. & Sheetz, M. P. (1988) *J. Cell Biol.* **107,** 1785–1792.
23. Schnapp, B. J. & Reese, T. S. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1548–1552.
24. Schnapp, B. J., Reese, T. S. & Bechtold, R. (1992) *J. Cell Biol.* **119,** 389–399.
25. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
26. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
27. Bullitt, E. S. A., DeRosier, D. J., Coluccio, L. M. & Tilney, L. G. (1988) *J. Cell Biol.* **107,** 597–611.
28. Pollard, T. D. & Korn, E. D. (1973) *J. Biol. Chem.* **248,** 4682–4690.
29. Titus, M. A. (1993) *Curr. Opin. Cell Biol.* **5,** 77–81.
30. Cheney, R. E. & Mooseker, M. S. (1992) *Curr. Opin. Cell Biol.* **4,** 27–35.
31. Korn, E. D. & Hammer, J. A. (1990) *Curr. Opin. Cell Biol.* **2,** 57–61.
32. See, Y. P. & Metuzals, J. (1976) *J. Biol. Chem.* **251,** 7682–7689.
33. Metuzals, J. & Tasaki, I. (1978) *J. Cell Biol.* **78,** 597–621.
34. Fath, K. R. & Lasek, R. J. (1988) *J. Cell Biol.* **107,** 613–621.
35. Lewis, A. K. & Bridgman, P. C. (1992) *J. Cell Biol.* **119,** 1219–1243.
36. Brady, S. T., Pfister, K. K. & Bloom, G. S. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1061–1065.