Myosin-I Moves Actin Filaments on a Phospholipid Substrate: Implications for Membrane Targeting

Henry G. Zot, Stephen K. Doberstein, and Thomas D. Pollard

Department of Cell Biology and Anatomy, The Johns Hopkins Medical School, Baltimore, Maryland 21205

Abstract. *Acanthamoeba* myosin-I bound to substrates of nitrocellulose or planar lipid membranes on glass moved actin filaments at an average velocity of 0.2 μm/s. This movement required ATP and phosphorylation of the myosin-I heavy chain. We prepared planar lipid membranes on a glass support by passive fusion of lipid vesicles (Brian, A. A., and H. M. McConnell. 1984. *Proc. Natl. Acad. Sci. USA.* 81:6159–6163) composed of phosphatidylcholine and containing 0–40% phosphatidylserine. The mass of lipid that bound to the glass was the same for membranes of 2 and 20% phosphatidylserine in phosphatidylcholine and was sufficient to form a single bilayer. Myosin-I moved actin filaments on planar membranes of 5–40% but not 0–2% phosphatidylserine. At the low concentrations of phosphatidylserine, actin filaments tended to detach suggesting that less myosin-I was bound. We used the cooperative activation of *Acanthamoeba* myosin-I ATPase by low concentrations of actin to assess the association of phospholipids with myosin-I. Under conditions where activity depends on the binding of actin to the tail of myosin-I (Albanesi, J. P., H. Fujisaki, and E. D. Korn. 1985. *J. Biol. Chem.* 260:11174–11179), phospholipid vesicles with 5–40% phosphatidylserine inhibited ATPase activity. The motility and ATPase results demonstrate a specific interaction of the tail of myosin-I with physiological concentrations of phosphatidylserine. This interaction is sufficient to support motility and may provide a mechanism to target myosin-I to biological membranes.

MOTOR molecules that associate with membranes are required to produce many commonly observed cellular movements. For a portion of these movements such as the transport of membrane to the expanding cell surface (Fath et al., 1990; Smith, 1988), the displacement of plasma membrane at the edge of a migrating cell (Fukui et al., 1989) and the transport of proteins in the plane of the plasma membrane (Sheetz et al., 1990; Hudspeth, 1989), the motor molecule has been postulated to be a myosin. Yet there exists only indirect evidence of motility mediated by myosin associated with the surface of membranes (Kachar, 1985; Adams and Pollard, 1986; Kohno and Shimmen, 1988; Kachar and Reese, 1988; Grolig et al., 1988; Mooseker et al., 1989).

Myosin-I is a class of monomeric myosins that probably function at the membrane to mediate motility (reviewed by Korn and Hammer III, 1990; Pollard et al., 1991). This class of myosins has a single head domain that, like muscle myosin, contains binding sites for ATP and actin and transduces the energy from the hydrolysis of ATP into mechanical work. Actin stimulates ATP hydrolysis by myosin-I (Pollard and Korn, 1973), and particles containing myosin-I move along actin (Albanesi et al., 1985*a*; Adams and Pollard, 1986).

Phosphorylation of the head domain by myosin-I heavy chain kinase is required to activate myosin-I (Pollard and Korn, 1973; Hammer III et al., 1983). It has been natural to assume that myosin-I is responsible for many of the membrane-based movements that persist in the absence of myosin-II (DeLozanne and Spudich, 1987; Knecht and Loomis, 1987).

For myosin-I from *Acanthamoeba castellanii*, the activation of ATPase activity by actin is quite complex owing to a second actin binding site located in the tail, a nonfilamentous distinct structure C-terminal to the head (Albanesi et al., 1985*b*; Lynch et al., 1986). The actin binding sites on the head and tail of *Acanthamoeba* myosin-I are distinguished by the action of ATP which greatly weakens the association of actin with the head (ATP-sensitive actin binding site), but has no effect on the association of actin with the tail (ATP-insensitive actin binding site). These two sites allow myosin-I to crosslink actin filaments (Pollard and Korn, 1973; Lynch et al., 1986). This effect of crosslinking is reflected in the ATPase activity, which has three distinct phases: high ATPase activity at low actin concentration, low ATPase activity at intermediate actin concentration, and high ATPase activity at high actin concentration (Pollard and Korn, 1973). The activation at low actin concentration depends on the crosslinking of actin filaments (Albanesi et al., 1985*b*) so it is abolished by proteolytic removal of the ATP-insensitive actin binding site (Lynch et al., 1986). The crosslinking activity also allows myosin-I to contract a gel of actin filaments in vitro (Fujisaki et al., 1985).

The tails of myosin-Is also contain a domain that binds to membranes stripped of peripheral proteins (Adams and Pollard, 1989; Miyata et al., 1989) and to acidic phospholipids (Adams and Pollard, 1989; Hayden et al., 1990). Binding of myosin-I to acidic phospholipids, i.e., phosphatidylserine, phosphatidylinositol 4,5 bisphosphate, and phosphatidylglycerol and dissociation by salt (Adams and Pollard, 1989; Hayden et al., 1990) suggest that the interaction between myosin-I and phospholipids is electrostatic. This fits with the high net positive charge of the region of the tail thought to bind to stripped membranes (reviewed by Pollard et al., 1991). The binding sites proposed for phospholipids and actin on the tail of *Acanthamoeba* myosin-I are separated by linear sequence, but the finding that myosin-I bound to stripped membranes does not bind actin (Miyata et al., 1989) suggests steric hindrance between these ligands. Since an interaction between the isolated ATP-insensitive actin binding site (Lynch et al., 1986) and phospholipids has not yet been investigated, it is formally possible that this binding site also associates with phospholipids.

The binding of myosin-I to lipids provides a mechanism for the observed intracellular localization of myosin-I. Myosin-I has been found in association with plasma membranes and the surface of organelles by immunofluorescence of *Acanthamoeba* (Gadasi and Korn, 1980; Hagen et al., 1986; Baines et al., 1990; Yonemura, S., and Pollard, T. D., manuscript submitted for publication), *Dictyostelium* (Fukui et al., 1989), and vertebrate epithelium (Hoshimaru et al., 1989), by immunoelectron microscopy of cells from *Acanthamoeba* (Baines and Korn, 1990) and vertebrate epithelium (Drenkhahn and Dermietzel, 1988), and by membrane fractionation of cells from *Acanthamoeba* (Adams and Pollard, 1986; Miyata et al., 1989) and vertebrate epithelium (Mooseker et al., 1989). Myosin-I is not localized uniformly on the inner surface of the plasma membrane, but rather is concentrated at the leading edge of migrating cells (Fukui et al., 1989) and at the brush border of intestinal epithelial cells (Glenney et al., 1982). These results suggest that myosin-I is targeted to specific membrane binding sites, perhaps controlled by specific sequences in the tails of the various myosin-I isozymes.

To test further the postulated role of myosin-I at membrane surfaces, we studied the ability of a pure lipid surface to support myosin-I-mediated motility. We demonstrate here that myosin-I bound to pure lipid bilayers moves actin filaments. In addition, we show a preferential binding of myosin-I to membranes containing 5–40% phosphatidylserine, a composition consistent with that of cellular membranes. This is the first report of any molecular motor capable of supporting motility over a pure phospholipid substrate.

Materials and Methods

Proteins

Myosin-I and myosin-I heavy chain kinase were prepared from *Acanthamoeba castellanii* as described previously (Lynch et al., 1991) with minor changes for myosin-I: the lysis buffer contained 150 mM KCl; and UTP-agarose (Cat. #U9626; Sigma Chemical Co. St Louis, MO) was substituted for ADP-agarose. The isoforms were identified based on the salt concentrations required to elute each from phosphocellulose (Lynch et al., 1991). Actin was prepared from an acetone powder of rabbit fast skeletal muscle (Spudich and Watt, 1971) and gel filtered on Sephacryl S-300 (Pharmacia, Uppsala, Sweden). Fluorescent actin filaments, Rh-Ph-actin, were prepared

by incubating rhodamine phalloidin (Molecular Probes, Eugene, OR) with polymerized actin (Kron et al., 1991).

Preparation of Stripped Membranes

Stripped membranes were prepared by a modification of the method of Adams and Pollard (1989). All steps were performed at 0–4°C. *Acanthamoeba* cells were suspended in 2 vol of MEA buffer (60 mM potassium glutamate, 10 mM imidazole-HCl, pH 6.4, 2 mM EGTA, 4 mM ATP, 2 mM MgCl₂), broken with 20 strokes of a Dounce homogenizer, and centrifuged for 3 min at 3,000 rpm in a Beckman JA-20 rotor. The supernatant was then centrifuged at 38,000 rpm in a Beckman Ti45 rotor for 90 min. The membranous portion of the pellet was resuspended in 50 ml of MEA with the ATP reduced to 1 mM and centrifuged for 60 min in a Ti45 rotor as above. The pellet was resuspended in 50 ml 0.1 M NaOH with 1 mM dithiothreitol, homogenized with 10 strokes of a Dounce homogenizer, sonicated with a microtip-equipped Branson 450 Sonifier for 30 s at power level 6, and centrifuged for 60 min in the Ti45 as above. The pellet was resuspended in MEA with no ATP, homogenized, and centrifugated for 60 min in the Ti45 as above. The pellet was then resuspended in 0.5 ml MEA per gram of cells, homogenized, aliquoted, and stored at −70°C until use.

Motility Assay

The fabrication of a "Kron-style" flow cell, the formation of a film of nitrocellulose on cover slips, and the preparation of basic solutions were as described (Kron et al., 1991). We used the following solutions, preserving the original nomenclature (Kron et al., 1991). AB is 25 mM imidazole, 25 mM KCl, 4 mM MgCl₂, 1 mM EGTA. AB/BSA is AB with 0.5 mg/ml BSA (Sigma Chemical Co.).

Solutions were added sequentially to the flow cell. First, a sample containing myosin was incubated 60 s. Then nonspecific sites were blocked by incubation with AB/BSA for 60 s. Next, 0.5 mg/ml Rh-Ph-actin in AB/BSA was incubated 10 s followed by a solution change with AB/BSA. Before viewing, the solution was changed with a solution to reduce photobleaching, i.e., 3 mg/ml glucose, 0.1 mg/ml glucose oxidase (Worthington, Freehold, NJ), 0.018 mg/ml catalase (Worthington), and 50 mM DTT contained in AB/BSA. This same solution but containing 2 mM ATP was exchanged into the flow cell to start the reaction at 22°C.

Video Microscopy

Individual actin filaments were imaged with a silicon intensified video camera (MTI 65, DAGE-MTI Inc., Michigan City, IN) on an Olympus IMT-2 microscope in the epifluorescence mode using standard hardware for rhodamine fluorescence. The contrast was controlled with a video processor (Image Σ-0; Olympus Corp., White Plains, NY) without averaging or summing video frames. The output was recorded onto 1/2 in VHS format tape and single fields were photographed (FreezeFrame, Polaroid, Cambridge, MA) from taped sequences, usually averaging 10–30 frames to enhance contrast and reduce noise. During replay of video sequences, the displacement of actin filaments was measured as a pair of voltage coordinates with the aid of a video analyzer (model 321; Colorado Video Inc., Boulder, CO). The displacement in voltage was calibrated at the same magnification with a stage micrometer.

Preparation of Lipid Vesicles

Synthetically prepared dioleoylphosphatidylcholine, synthetically prepared lissamine rhodamine B sulfonyldioleoylphosphatidylethanolamine (rhodamine-labeled phosphatidylethanolamine), and brain diacylphosphatidylserine (molecular weight based on the most abundant, dioleoyl, form) were purchased from Avanti Polar Lipids (Pelham, AL). The phospholipids were dissolved in CHCl₃, combined in a molar fraction of phosphatidylcholine, and dried with a stream of N₂. Traces of solvent were removed in vacuo. The lipids were resuspended (0.5 ml) in a vortex of H₂O, sonicated until clear, and centrifuged at full speed with a microfuge for 5 min in the cold. A pellet was not detected and the supernatant was recovered.

Preparation of Planar Lipid Surfaces

The glass used to induce the fusion of lipid vesicles was prepared essentially as described (Poglitsch and Thompson, 1990). Glass cover slips were boiled in Linbro detergent (Flow Laboratories; diluted 1:4), rinsed at least 12 h with ~10 L H₂O and dried 1 h at 120°C. Before use, the glass surface was

cleansed of adsorbed organics by exposure to an isotropic argon ion plasma for 60 s. The Ar$^+$ plasma was generated using a microwave oven. First, a lyophilization flask containing the cover glasses held on edge (preferably in a glass container) was purged with argon gas for ~1 min. When a ceramic holder for the cover glasses was used it was necessary to allow the holder to out-gas in vacuo before purging with argon. Second, the contents of the flask were evacuated using a simple vacuum pump. Finally, the evacuated flask was transferred to the chamber of the microwave (500 W). The progress of the reaction was monitored visually; argon was replenished when the glow subsided.

A planar lipid surface attached to glass was prepared by passive fusion of lipid vesicles (Brian and McConnell, 1984; McConnell et al., 1986). To the inner surface of the flow cell, a sequence of degassed solutions were introduced. The buffer, AB, was added first to wet the surfaces followed by the lipid vesicle solution. After incubation for 20 min, the unbound lipid was removed and nonspecific binding sites were blocked by exchanging the solution in the flow cell twice with AB/BSA. This surface was used immediately thereafter.

Trials were conducted to determine optimal conditions for planar lipid formation (Table I). A substrate suitable for motility was produced at 5.0 mM lipid with 10 and 20% phosphatidylserine but not with 40% phosphatidylserine. A poor substrate was also found with all lipid vesicles tested at 0.5 mM when H_2O was the solvent. However, the formation of a planar lipid surface was apparently achieved for all lipid vesicles at 0.5 mM when suspended in a buffered salt solution (AB). Hence, for subsequent experiments we used lipid vesicles that were suspended in AB to form planar lipid surfaces.

Measurement of Lipid Binding to Glass

The binding of lipids to glass was measured using a "Berg-style" flow cell of construction previously described (Berg and Block, 1984) with minor alterations to the lower surface to allow for disassembly following each experiment. The glass on the upper side of the flow cell was prepared for the fusion of lipid vesicles. A round cover glass (12 mm, #1 thickness) covered the precisely machined aperture of the flow cell and was held in place on the edge with grease; this provided a region of 56.1 mm^2 for the formation of a planar lipid surface. The planar lipid surface was formed as described above using lipid vesicles containing ^{14}C-phosphatidylcholine. Gliding filaments on this surface were recorded with the video microscope.

After recording filament movements, the flow cell was disassembled while submerged in a water bath. The surface, containing the planar lipids, was covered by a piece of filter paper before transferring the lipids to the counting solution to prevent contact of the lipid surface with air. The lipids and associated glass and filter paper were counted together. In all cases, a separate piece of filter paper from the water bath detected negligible counts. This control eliminated the possibility that a significant number of counts associated with the glass were picked up by the protective filter paper from the medium. The specific activity was calculated by counting a known concentration of lipid stock in parallel.

Miscellaneous Procedures

The rate of ATP hydrolysis was determined as described (Pollard, 1982). Protein was determined by the Bradford (1976) assay using BSA as the standard.

Results

Myosin-I on Nitrocellulose Supports Gliding Motility of Actin

The gliding movement of actin filaments over *Acanthamoeba* myosin-I immobilized on nitrocellulose-coated glass required ATP and phosphorylation of the myosin-I heavy chain (Fig. 1 *A*). Actin filaments bound stably to nitrocellulose coated glass pretreated with myosin-I followed by a blocking solution containing BSA. With the subsequent addition of ATP and myosin-I heavy chain kinase, virtually all of the actin filaments glided over the surface after a prolonged delay (Fig. 2). This lag before movement probably represented the time required for the phosphorylation of the full population

of myosin-I, a reaction that is inhibited by binding of actin filaments to the head of myosin-I (Brzeska et al., 1988, 1989). Preincubation of myosin-I with heavy chain kinase and ATP before coating the nitrocellulose reduced the lag before actin filament gliding to ~30 s, so we used this prephosphorylation step routinely before addition of myosin-I to the flow cell.

The gliding of actin filaments over phosphorylated myosin-I–coated nitrocellulose (Fig. 1*A*) closely resembled that produced by other myosins (Kron and Spudich, 1986; Toyoshima et al., 1987; Collins et al., 1990; Sellers and Kachar, 1990; Yamada et al., 1990; Warshaw et al., 1990). We observed gliding filaments on nitrocellulose with both myosin-IB and myosin-IA isoforms, although we report on only the results with myosin-IB here. The movements, rarely along straight paths, were marked by both gradual and acute turns. During the first 5–10 min of movements, long filaments (30–40 μm) were fragmented continuously into a larger population of short filaments <5 μm long. These breaks frequently occurred at sharp bends. Most of the short filaments continued to move beyond the duration of all experiments, so that ultimately all parts of every field had moving filaments. Few filaments detached completely from the substrate or attached to the substrate during the period of observation.

The gliding velocity of actin over myosin-I-coated nitrocellulose varied about a mean of 0.2 μm/s (Fig. 3) reminiscent of muscle myosin, which has a mean velocity of 3–4 μm/s (Kron and Spudich, 1986). The instantaneous velocity of individual filaments varied with time and included some brief stops. These fluctuations most likely reflected the activity of a constantly changing population of underlying myosins (possibly including those that had become denatured or disoriented) encountered by a moving filament. Therefore, we feel that the velocity distribution of a large number of individual filaments provided an unbiased estimate of the average activity of myosin bound in all parts of the flow cell. We measured the velocity as the linear displacement of a filament during a fixed segment of time. Since the actual curved path of the filament was often longer than this linear displacement, the values in Fig. 3 are an underestimate of the actual velocities.

Three other test surfaces were unable to support gliding filaments. After the standard incubation with phosphorylated myosin-IB and the addition of ATP, actin filaments did not move and most detached from surfaces of both untreated glass and glass coated with BSA. Siliconized glass incubated with phosphorylated myosin-I bound actin filaments, which remained immobile in the presence of ATP.

Gliding Motility of Actin Filaments over Myosin-I on Pure Lipid Substrates

Myosin-I bound to a pure phospholipid bilayer immobilized on a glass surface supported the gliding motility of actin filaments that was almost indistinguishable from gliding over nitrocellulose (Fig. 1 *B*). For this reconstitution of actin movements relative to a lipid membrane, we used a simplified, defined system of purified components–purified myosin-I and actin filaments attached to a planar lipid membrane supported on a glass surface cleaned previously of organic compounds by etching in an argon plasma. When this lipid surface was pretreated with phosphorylated myosin-I, actin filaments bound and began moving with roughly the
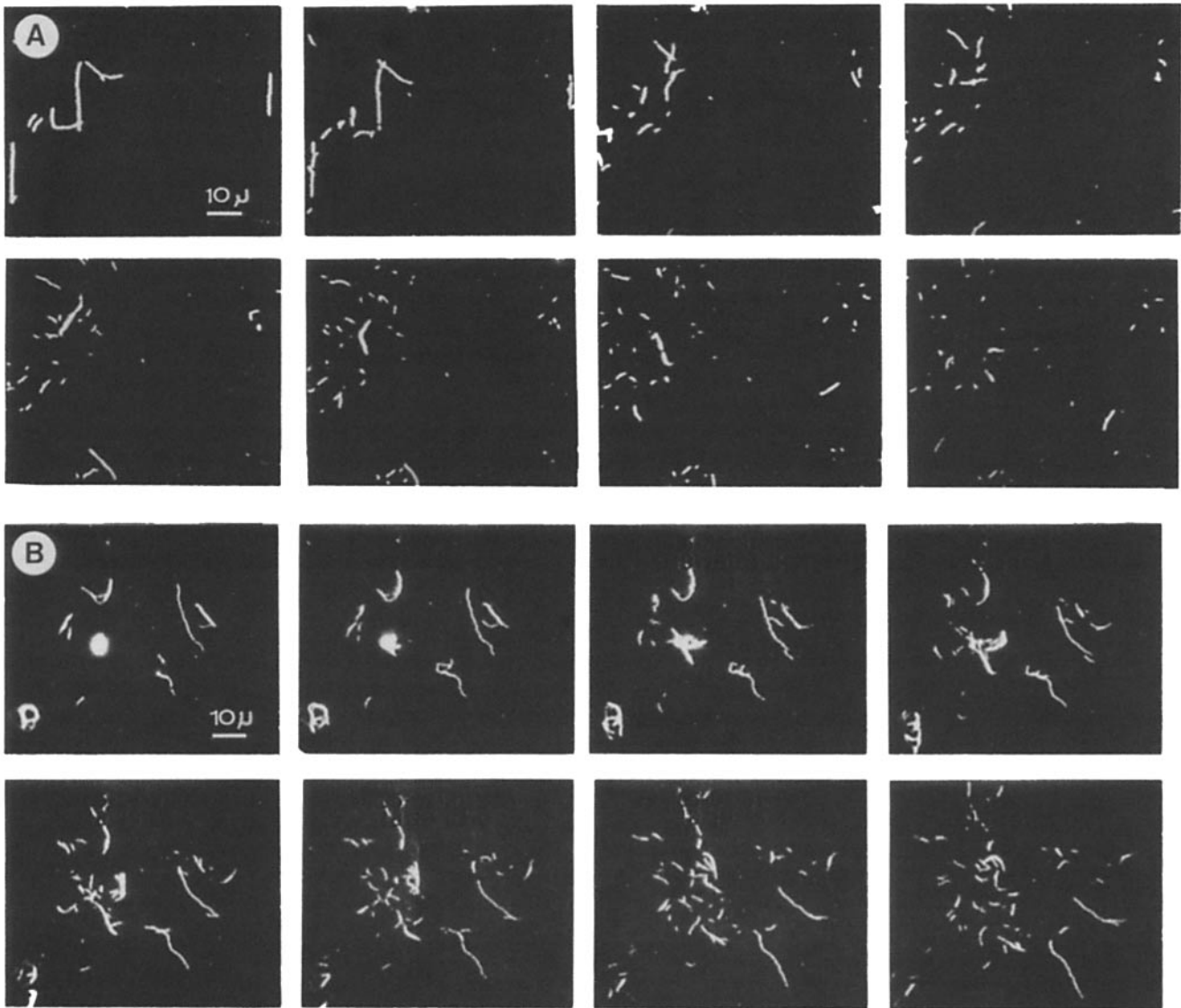
Figure 1. Fluorescence micrographs demonstrate gliding actin filaments on nitrocellulose and phospholipid surfaces. (*A*) Nitrocellulose binds myosin-I; actin filaments (*white*) are immobilized close to the surface in the absence of ATP. The sequence shows the same field at 0, 30, 60, 90, 120, 150, 210, and 270 s. At 10 s, ATP was added to the flow cell and actin filaments are displaced along their long axis; this is apparent in the figure from the change in the positions of virtually all of the filaments. The changes in the field show that the filaments initially sever and eventually move in all parts of the field. In both *A* and *B*, filaments moved continuously and new filaments attached to the surface rarely. (*B*) A planar membrane substrate binds myosin-I; actin filaments (*white*) are more loosely bound near the surface in the absence of ATP. The sequence shows the same field at 0, 10, 20, 30, 40, 50, 60, and 70 s. At 2 s, ATP was added to the flow cell. As time increases, the filaments become more closely associated with the surface and are displaced along their long axis, thereby demonstrating motility. Single filaments emerge continually from the side of apparent bundles of filaments. Movements eventually occur in all parts of the field. Conditions: 277 nM myosin-IB in AB was incubated with 2.7 nM myosin-I heavy chain kinase and 1 mM ATP for 20 min and then added to both surfaces in Kron-style flow cells. The reaction was started with the buffer containing ATP (Materials and Methods) and 5.4 nM myosin-I heavy chain kinase. The planar membranes were prepared from vesicles that contained 20% phosphatidylserine (2,500 nmol phosphatidylcholine and 500 nmol phosphatidylserine).

same 30-s lag after addition of ATP as on nitrocellulose (Fig. 1 *B*).

A continuing effort to quantitate subtle differences in the gliding of actin filaments on nitrocellulose and lipid substrates shows that they are almost identical. The planar lipid substrates supported all of the actin filament movements exhibited by myosin-I on nitrocellulose. The similarity extended to the velocity distributions (Fig. 3) and the breaking of long filaments at acute bends. At early times after the addition of ATP, filaments that were loosely bound to the sub-

strate quickly attached along their entire length and moved, a behavior that may be different than myosin-I on nitrocellulose. Another possible difference was several instances of clumps of filaments that resolved into many individual filaments by peeling away from the core (Fig. 1 *B*). We conclude that the lipid substrate does not affect the basic mechanicochemical properties of myosin-I.

The number of attached and moving actin filaments depended on the composition of the lipid substrate for the myosin-I (Fig. 4 and Table I and II). Here we consider the
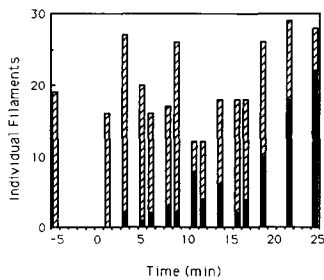
Figure 2. Myosin-I heavy chain kinase is required to initiate gliding filaments. The total number of filaments (*open-striped bars*) and the filaments that moved at least 1 μm (*closed bars*) were counted. Counts were made for 1 min at the times indicated in the central 1,100 μm² of the field of view. At times 11–12 and 16–17, one field was analyzed; otherwise different fields were analyzed. Conditions: 277 nM myosin-IB in buffer AB was introduced into a Kron-style flow cell and allowed to bind passively the surface of nitrocellulose. The buffer initiating the reaction contained ATP (Materials and Methods) and was present for the duration of the experiment. 11.2 nM myosin-I heavy chain kinase was introduced at time 0.

effect of the phosphatidylserine content of bilayers composed primarily of phosphatidylcholine. In a subsequent paper, we will report on other lipids. On a phosphatidylcholine substrate with 0–2% phosphatidylserine few actin filaments bound after myosin-I pretreatment. After addition of ATP, the vast majority of these filaments remained immobile or detached from the surface (Fig. 4). Planar lipid membranes of 5–10% phosphatidylserine pretreated with myosin-I bound actin filaments, but their ATP-dependent movement was erratic (Fig. 4). In one experiment, a high fraction of filaments was immobile. In another trial, a high fraction of filaments made short sporadic movements separated by rests. Short filaments often moved and detached from the surface or reattached to the surface and moved. In contrast, on a surface of 20–40% phosphatidylserine, large numbers of actin filaments bound and virtually all of them were motile with relatively few interruptions after addition of ATP (Fig. 1 *B* and 4).

Measurement of Lipids Bound to Glass Substrates

Direct chemical measurement established that within the 10% error of the method, the cleaned glass surface bound the same amount of lipid in planar membranes composed of phosphatidylcholine with either 20% or 2% phosphatidylserine (Table II). We used a flow cell with a precisely known surface area to support the lipid membrane and to view actin gliding. Vesicles containing trace amounts of radioactive
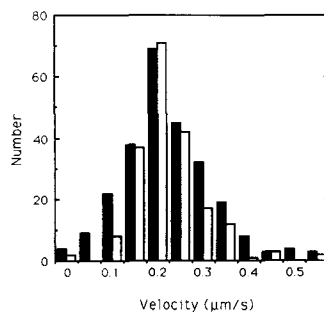

Figure 3. The velocity of actin filaments is the same on nitrocellulose and lipid surfaces. The velocity of individual actin filaments were calculated for myosin-IB bound to surfaces of nitrocellulose (*closed bars*) and planar lipids (*open bars*). The velocities from two separate experiments for each condition were calculated at 15-s intervals from the displacement of actin filaments and grouped into ranges of 0.05 μm/s. We typically analyzed 30–50% of the filaments in a given field; a total of five and five fields were analyzed for nitrocellulose and lipid surfaces. The experimental conditions were the same as Fig. 1.
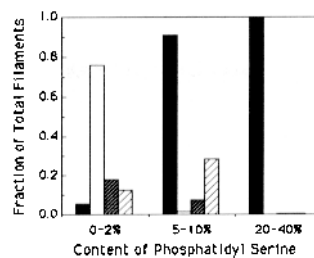

Figure 4. The gliding of actin filaments is dependent on the concentration of phosphatidylserine in planar lipids. The number of filaments were counted before the addition of ATP and the fate of each filament was determined after the addition of ATP. Additional fields were monitored, but to overcome a natural bias toward selecting fields with many attached and moving filaments, the analysis was restricted to the first field; filaments in this field were fixed and stationary initially and events unfolded unpredictably after addition of ATP. To quantitate these events, the fates of all filaments in the first field were determined and grouped into categories that reflected the probable activity of myosin-I. These categories included filaments that moved at any time during the video sequence in the plane of the substrate for a clearly discernable distance of 1 μm (*closed bars*), filaments that remained bound but did not move (*closed-striped bars*), filaments that did not move and detached completely from the surface (*open bars*), and filaments that moved subsequent to attachment to the surface or detached from the surface subsequent to moving (*open-striped bars*). We analyzed 88, 186, and 219 filaments from 9, 6, and 12 experiments, respectively, for 0–2, 5–10, and 20–40% phosphatidylserine. Conditions: To form planar lipid surfaces, lipid vesicles of 0–40% phosphatidylserine were prepared from 2,500 nmol phosphatidylcholine and 0–1,000 nmol phosphatidylserine, diluted to 0.5 mM in AB, and incubated 20 min in Kron-style or Berg-style flow cells. 277 nM myosin-IB and 2.7 nM myosin-I heavy chain kinase in AB and containing 1 mM ATP were preincubated 20 min and added to the flow cell. The reaction was started with the buffer containing ATP (Materials and Methods) and 5.4 nM myosin-I heavy chain kinase.

phosphatidylcholine enabled us to determine the mass of lipid bound to the glass. Although the total bound lipid was similar, planar membranes composed of 20% phosphatidylserine supported a high proportion of moving actin filaments (94%) while planar membranes composed of 2% phosphatidylserine did not (Table II). Hence, the ability of the planar lipid membrane to support motility depended solely on the content of phosphatidylserine rather than a measurable effect of phosphatidylserine on the binding of phosphatidylcholine vesicles to the glass.

From the amount of lipid bound to the glass, we calculated that the packing density of the lipid molecules on the glass is most consistent with a bilayer as expected from previous work (Brian and McConnell, 1984; Poglitsch and Thompson, 1990). For this calculation we assumed that the lipids spread uniformly on the glass. This is reasonable since regions without lipid would be expected not to support motility and gliding actin filaments were observed uniformly in every field in all parts of the cover slip. The ratio of the surface area and the number of lipid molecules bound gives 68 and 75 Å² per lipid molecule as the calculated surface areas for a single bilayer (Table II). These values are near the surface area of 72 Å² per lipid molecule that was measured for a single bilayer of phosphatidylcholine (Small, 1967; Johnson, 1973) and inclusion of phosphatidylserine is not expected to change this value dramatically under our experimental conditions (Israelachvili et al., 1980). The packing area for a monolayer, which would be about 35 Å² per molecule, is lower than expected and the energetics for

Table I. Summary of Conditions for the Formation of a Planar Lipid Surface

Phosphatidyl-serine*	Total Lipid‡	Solvent or buffer§	Number experiments	Fraction filaments moved‖	Fraction filaments detached¶
%	mM				
10	5.0	H₂O	1	1.00	0
20	5.0	H₂O	1	0.79	0
40	5.0	H₂O	2	0	0.60
10	0.5	H₂O	1	0	0.82
20	0.5	H₂O	3	0	0.78
40	0.5	H₂O	3	0	0.53
10	0.5	AB	2	0.87	0
20	0.5	AB	8	1.00	0
40	0.5	AB	4	0.99	0

* Lipid vesicles were prepared with 2,500 nmol phosphatidylcholine and 250, 500, and 1,000 nmol phosphatidylserine corresponding to 10, 20, and 40%.
‡ Concentration of lipid vesicles incubated 20 min in a Kron-style flow cell outfitted with a clean glass surface for planar lipid formation.
§ Lipid vesicles were diluted into H₂O or buffer, AB.
‖ The total number of filaments in the first field of each experiment were counted before and after the addition of ATP. The filaments that were displaced at least 1 μm were counted as "moved." Conditions: 277 nM myosin-IB and 2.8 nM myosin-I heavy chain kinase were preincubated 20 min in AB containing 1 mM ATP before adding to the flow cell. The reaction was started with the addition of the buffer containing ATP (Materials and Methods) and 5.4 nM myosin-I heavy chain kinase.
¶ Filaments that did not move and were released completely from the surface were counted as "detached."

the formation of a monolayer film are unfavorable owing to polar surfaces provided by the glass and solvent. While small, localized regions of defects in the membrane are not excluded by our data, it is unlikely that large regions of multilayers are formed since the packing area of just two bilayers layers would exceed 100 Å² per molecule.

In control experiments, we evaluated the integrity of the planar membrane from the pattern of emission of fluorescently labeled phospholipids as described previously (McConnell et al., 1986). Planar membranes were formed in buffer AB

Table II. The Ability of Planar Lipids to Support Gliding Filaments Depends on the Content of Phosphatidylserine and Not on the Mass of Lipid Bound to Glass

	Content of Phosphatidylserine*		
	2%	20%	20%/H₂O
Total experiments‡	3	4	1
Total fields§	23	21	8
Total filaments bound‖	61	471	6
Total filaments moved¶	0	442	0
Lipid bound** (mean pmol ± SD)	274 ± 31	249 ± 26	83
Mean Å²/lipid molecule‡‡ (bilayer)	68	75	226

* Lipid vesicles were prepared with phosphatidylcholine (250 nmol), phosphatidylserine (5 or 50 nmol), ¹⁴C-phosphatidylcholine (9.4 nmol, 1 μCi) and incubated 20 min in a Berg-style flow cell.
‡ 87 nM myosin-IB and 2.8 nM myosin-I heavy chain kinase were preincubated 20 min in AB/BSA containing 1 mM ATP.
§ A cruciform pattern of individual fields were selected reaching to the edges of the flow cell.
‖ Filaments were counted throughout the duration of the sequence for each field.
¶ The filaments that at any time moved at least 1 μm.
** The specific activities were 3.4 and 3.2 cpm/pmol total phospholipid for vesicles containing 2 and 20% phosphatidylserine.
‡‡ Based on 56.1 mm², the area of glass in contact with the solution in the flow cell.

with vesicles of phosphatidylcholine containing 1% rhodamine-labeled phosphatidylethanolamine. The rhodamine fluorescence was found in a narrow focal plane near the surface of the glass support. Occasionally, small regions of intense fluorescence, perhaps resulting from aggregates of phospholipids, were observed but these were rare if the glass support was treated with an argon plasma, which is part of our standard procedure. With the standard procedure for preparing planar membranes, a uniform intensity of fluorescence was observed in all fields such that no areas devoid of fluorescence were detected. The uniformity of the fluorescence indicated that the supported lipid membrane contained no discontinuities that could be detected by fluorescence microscopy.

Support of myosin-I–based actin filament binding and motility appears to require a complete lipid bilayer on the glass surface. For example, when our usual concentration of vesicles containing adequate quantities of phosphatidylserine were reacted with glass in pure water rather than our usual buffer, only a small mass of lipid bound to the glass and the surface did not support myosin-I based motility of actin filaments (Table II). The small amount of bound lipid suggested that the lipid did not cover the complete surface with water as a solvent. In preliminary experiments, lipid vesicles suspended in water and containing a fluorescent phospholipid were seen to associate with the glass as discrete particles rather than a continuous sheet. This may indicate that, in certain solvents, lipid vesicles bind to the glass without fusing. The reduced mass of lipid bound in water is an important negative control for nonspecific association of a constant amount of lipid to the substrate. The water itself was not the problem, since a high concentration of the same lipid vesicles did support motility (Table I).

Acanthamoeba Membranes Inhibit the Activation of Myosin-I ATPase by Actin

Since the tight binding of actin filaments to a substrate anchor can prevent gliding (Prochniewicz and Yanagida, 1990; Warshaw et al., 1990), we were curious why the ATP-insensitive actin binding site on the tail of myosin-I did not prevent the motility of actin filaments in our assays. One possibility was that association of the myosin-I tail with phospholipids blocked the ATP-insensitive actin binding site on the tail of myosin-I as suggested by the work of Miyata et al. (1989) on myosin-I bound to isolated Acanthamoeba plasma membranes.

We tested this hypothesis with an ATPase assay that depends on the association of the ATP-insensitive site of Acanthamoeba myosin-I with actin filaments (Fig. 5). Acanthamoeba myosin-I crosslinks low concentrations of actin filaments into bundles, because it has actin binding sites on both the head and the tail (Albanesi et al., 1985b; Lynch et al., 1986). The high local concentration of actin in these bundles strongly activates ATP hydrolysis by the ATP-sensitive site in the head (Albanesi et al., 1985b). This activation is manifested only at low actin concentrations (<2 μM) (Fig. 5 A, solid symbols). Higher actin concentrations do not favor these bundles, so the ATPase activity becomes dependent solely on the binding isotherm for actin to the low affinity, ATP-sensitive site on the head (Fig. 5 A and inset, solid symbols). Consequently, the ATPase activity at low actin
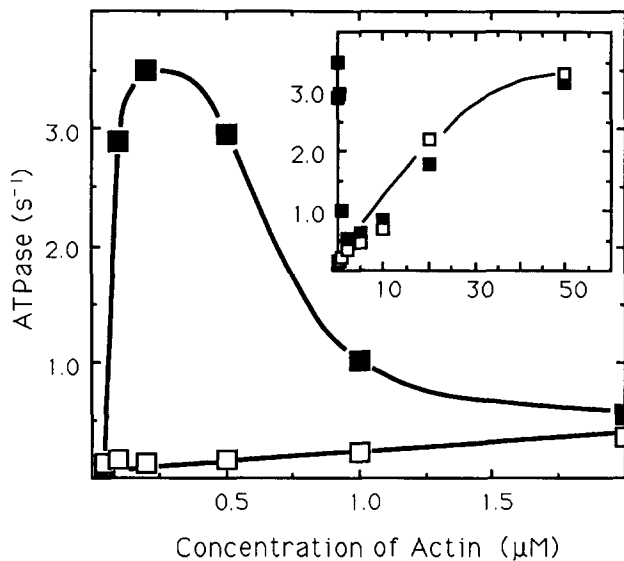
Figure 5. Stripped Acanthamoeba membranes block the activation of myosin-I ATPase by low concentrations of actin. Myosin-IB was preincubated 22°C for 10 min alone (■) or with stripped Acanthamoeba membranes (□) and the activation of ATP hydrolysis by actin was measured. The inset shows the activity over an expanded range of actin concentrations and the axes of the graph have the same units as the surrounding graph. Conditions: Each reaction (1 ml) contained 33 pmol myosin-IB, 2.7 pmol myosin-I heavy chain kinase, 15 mM imidazole, pH 7.0, 2 mM MgCl₂, 1 mM EGTA, and 2 mM ATP. When present, Acanthamoeba stripped membranes were at 5 μg/ml (protein content). The reaction was started with the addition of actin and proceeded for 10 min at 22°C. The inorganic phosphate that was released in the absence of actin was subtracted as basal activity.

concentrations depends on the accessibility of actin to both of its binding sites on myosin-I.

Acanthamoeba membranes stripped of peripheral proteins by mild exposure to alkali not only bound myosin-I (Adams and Pollard, 1989), but also inhibited the cooperative activation of the myosin-I ATPase by low concentrations of actin filaments (Fig. 5). This inhibition was reproducible for three preparations of myosin-I and both of two preparations of stripped membranes. Since the inhibition of the ATPase was restricted to the low concentrations of actin (Fig. 5, inset), the membranes are unlikely to affect the ATPase site in the head directly. Rather, these results are consistent with a specific effect of membranes on the binding of actin filaments to the ATP-insensitive site on the tail of myosin-I. This established the ATPase assay as a simple test for the association of lipids with the tail of myosin-I.

Pure Lipids Inhibit the Activation of Myosin-I ATPase by Actin

The ATPase assay provided evidence that the ability of pure phospholipids to block the association of actin with the tail of myosin-I depends on both the concentration and the content of phosphatidylserine in the membranes (Fig. 6). We used a fixed concentration of actin in the cooperative activation range (0.26 μM) and solutions that were identical to those used in the motility experiments to test vesicles prepared from phosphatidylcholine and phosphatidylserine. Pure phosphatidylcholine vesicles did not inhibit ATPase ac-
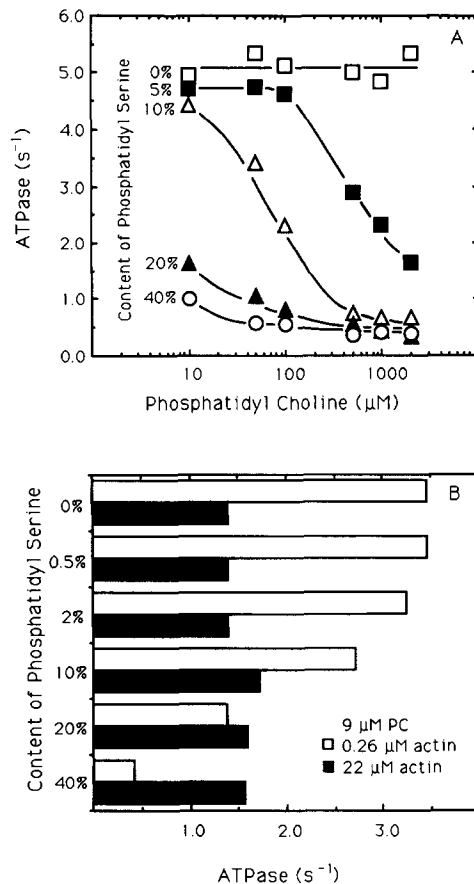

Figure 6. The actin activation of the myosin-I ATPase is blocked by pure phospholipid vesicles containing phosphatidylserine. (A) With the actin concentration fixed, ATPase activity is dependent on the concentration of phosphatidylserine. ATP hydrolysis was measured at a low concentration of actin (0.26 μM) in the presence of lipid vesicles containing 0% (□), 5% (■), 10% (△), 20% (▲), and 40% (○) phosphatidylserine in a background of phosphatidylcholine. Trials were carried out at increasing concentrations of total lipid and normalized to the content of phosphatidylcholine. (B) At a higher concentration of actin, the ATPase activity is constant despite a large change in the concentration of phosphatidylserine. ATP hydrolysis was measured in the presence of vesicles containing 9 μM phosphatidylcholine and various molar fractions of phosphatidylserine. The concentrations of actin were 0.26 μM (open bars) and 22 μM (closed bars). The absence of a significant change in activity at 22 μM actin indicates that phosphatidylserine inhibits the cooperative activation phase (Fig. 5) and not the mass action phase (Fig. 5, inset) of ATPase activity. Conditions: The vesicles, composed of 0-40% phosphatidylserine, were prepared with 2,500 nmol of phosphatidylcholine and 0-1,000 nmol phosphatidylserine. Reaction tubes (0.5 ml) contained 11.5 pmol myosin-IB and 0.6 pmol myosin-I heavy chain kinase in AB, preincubated 20 min in the presence of 2 mM ATP and, subsequently, 5 min with the lipids. Reactions were started with the addition of actin. Hydrolysis of ATP in the absence of actin was subtracted as basal activity.

tivity even at the highest lipid concentration tested, 2 mM (Fig. 6 A). We obtained similar results with buffers of lower ionic strength and vesicles of larger diameter prepared by extrusion (Hope et al., 1985) containing phosphatidylcholine alone or with 10% phosphatidylserine. For vesicles that contained both phosphatidylcholine and phosphatidylserine, the inhibition of the ATPase increased with total lipid concentra-

tion and the fraction of phosphatidylserine (Fig. 6 *A*). In addition to the results with myosin-IB (Fig. 6), myosin-IA likewise was inhibited by vesicles containing phosphatidylserine and *Acanthamoeba* stripped membranes (data not shown). As in the case of stripped membranes, the pure lipids did not inhibit the ATPase at high (22 μM) actin concentrations (Fig. 6 *B*), so the effect is more likely on the tail than on the head.

These results suggest that phosphatidylserine in a background of phosphatidylcholine associates specifically with the tail of myosin-I and prevents the association of the ATP-insensitive site with actin filaments. This binding to phosphatidylserine likely accounts for the phosphatidylserine dependence of the in vitro motility of actin filaments.

Discussion

We used the gliding filament assay (Kron and Spudich, 1986; Toyoshima et al., 1987; Kishino and Yanagida, 1988) to show that *Acanthamoeba* myosin-I supports the gliding movement of actin filaments equally well on surfaces of nitrocellulose or planar phospholipid bilayers containing 5–40% phosphatidylserine. With planar membranes as a substrate, actin filaments tend to detach with 0–2% phosphatidylserine, move and detach with 5–10% phosphatidylserine, and move continuously with 20–40% phosphatidylserine. Taking into account that the density of myosin-I is likely reflected by the tendency of the filaments to remain bound, the ability of a planar lipid bilayer to promote motility appears to be mediated by an association of myosin-I with phosphatidylserine. We confirmed this specific association with a simple assay based on the ATPase of *Acanthamoeba* myosin-I and showed that vesicles prepared from 5–40% phosphatidylserine in a background of phosphatidylcholine block the binding of actin to the ATP-insensitive site of myosin-I. Thus, pure phospholipids are utilized as a substrate to move actin filaments by myosin-I, the only motor molecule of any kind known to have this capability.

It is curious that both *Acanthamoeba* and brush border myosin-I move actin filaments about 10-fold faster in the gliding filament assay than they move plastic beads in the *Nitella* cable assay of Sheetz and Spudich (1983). Both the gliding filament assay and the *Nitella* cable assay are thought to measure the simple maximum mechanical velocity which is characteristic of the head domain of a given myosin, since myosins from striated muscle (Sheetz and Spudich, 1983; Kron and Spudich, 1986), smooth muscle (Umemoto and Sellers, 1990), and platelets (Umemoto and Sellers, 1990) have roughly the same velocity in the two systems under a wide range of conditions. In contrast, for *Acanthamoeba* myosin-I gliding is 0.2 μm/s (this report) and bead movement is 0.01–0.08 μm/s (Albanesi et al., 1985*a*). For chicken brush border myosin-I gliding is 0.1 μm/s (Collins et al., 1990) and bead movement is 0.014 μm/s (Mooseker and Coleman, 1989). The difference could be in the myosin-I preparations, but may also be related to coupling myosin-I to the beads, especially since in vitro movement of membranes on *Nitella* actin cables by *Acanthamoeba* myosin-I (Adams and Pollard, 1986) and brush border myosin-I (Mooseker et al., 1989) appears to be faster. Rapid movements by myosin-I of actin-associated particles (reported in abstract form by Zot, H. G., S. K. Doberstein, and T. D. Pollard [1990]. Transport of stripped vesicles by myosin-I from *Acanthamoeba. Biophys.*

J. 57:212a.) are also consistent with this discussion, but the interpretation of these preliminary experiments has been complicated by the presence of endogenous motors in the *Nitella* preparation (H. Zot, unpublished observations).

Some of the characteristics of natural bilayer membranes are exhibited by supported planar membranes that have been prepared by the technique of vesicle fusion (Brian and McConnell, 1984). This technique produces a supported membrane which is laterally continuous as judged by a uniform appearance of fluorescently labeled phospholipids incorporated into the planar membrane (Brian and McConnell, 1984; Poglitsch and Thompson, 1990). In addition, component phospholipids of supported membranes have a measured lateral diffusion constant in the range expected for phospholipids in a lipid bilayer (Brian and McConnell, 1984). We also find uniform fluorescence of rhodamine-labeled phosphatidylethanolamine in a supported planar membrane and independently show that the mass of supported membrane is most consistent with a bilayer structure.

Motility on phosphatidylcholine is an important test of the adequacy of the planar membrane. While a planar membrane of phosphatidylcholine does not support motility, it is possible that myosin-I is bound but is not oriented properly to move actin filaments. A lack of association with phosphatidylcholine has been shown directly with both myosin-I and a proteolytic fragment of myosin-I that retains a binding site for phosphatidylserine (Adams and Pollard, 1989). Consistent with this absence of an interaction is the inability of vesicles of phosphatidylcholine to inhibit the actin activated ATPase activity of myosin-I. Thus, for planar membranes composed of pure phosphatidylcholine, the lack of motility is more likely to be the result of poor association of myosin-I than misorientation of bound myosin-I. Taken together, the lack of association with phosphatidylcholine, the apparent continuity of the planar membrane, and the inability of plain glass and glass blocked with BSA to support motility, the evidence supports the conclusion that an interaction of myosin-I with a physiological concentration of phosphatidylserine enables planar membranes to support motility.

The phosphatidylserine content of *Acanthamoeba* plasma membranes is likely to be adequate to support some types of myosin-based motility. The phosphatidylserine content of 27% (Ulsamer et al., 1971) is in the middle of the range (5–40%) of phosphatidylserine shown here to support motility. This content of phosphatidylserine could explain the observed fractionation of myosin-I with naturally occurring membranes (Adams and Pollard, 1986, 1989; Mooseker et al., 1989; Miyata et al., 1989).

On the other hand, the phosphatidylserine content of biological membranes cannot fully explain the concentration of myosin-I at the leading edge of moving cells (Fukui et al., 1989; Yonemura, S., and T. D. Pollard, manuscript submitted for publication), the concentration of myosin-I in the brush border (Glenney et al., 1982), or the localization of myosin-IC to the contractile vacuole (Baines et al., 1990). This apparent targeting of myosin-I isozymes to specific membrane subdomains is likely to require additional interactions. One possibility is that myosin-I which is bound through a primary interaction with membrane phospholipids may be sorted by secondary interactions with receptors, perhaps proteins, that are present in particular membrane subdomains and are specific for particular sequences in the tail

domains of the various myosin-I isozymes (Pollard et al., 1991). This idea is supported by the presence of distinct sequences in the tail domains of the isozymes of myosin-I (Titus et al., 1989; Jung et al., 1989). Identification and characterization of these hypothetical receptors will be required to gain further insight into the targeting of myosin-I to specific membranes.

We have speculated (Pollard et al., 1991) that the basic domain identified for the *Acanthamoeba* isozymes myosin-IB, myosin-IC, and myosin-ID (Jung et al., 1987, 1989) is the consensus lipid binding domain of myosin-I. However, a number of possible myosin-Is do not have tail sequences obviously related to the *Acanthamoeba* basic domain (Horowitz and Hammer III, 1990; Mercer et al., 1991; Johnston et al., 1991). No information is available on the binding of these variants to lipids, so a generalization regarding the structure(s) mediating the association of myosin-Is with lipids must await further study.

Other motors that work at the membrane level could share the membrane binding mechanism of myosin-I. There is direct evidence that dynein is a motor for the movement of axonal vesicles (Schnapp and Reese, 1989; Schroer et al., 1989) and indirect evidence that dynein is a motor for vesicle movement in *Reticulomyxa* (Schliwa et al., 1991). Similarly, kinesin has been shown to support the movement on microtubules of isolated vesicles (Schroer et al., 1988) and tubular membranes of endoplasmic reticular origin (Vale and Hotani, 1988; Dabora and Scheetz, 1988). However, in the only test of a direct association of kinesin with pure phospholipids, tubulovesicular structures composed of phosphatidylcholine and phosphatidylglycerol were not transformed into a reticular network by kinesin (Vale and Hotani, 1988). Future investigation of motility with pure phospholipids may be hampered by a requirement for presently undefined cytoplasmic factors as has been described for dynein (Schnapp and Reese, 1989; Schroer et al., 1989) and kinesin (Schroer et al., 1988).

While arguing for a general role of myosin-I in movements at the interface between the actin cytoskeleton and membranes, our results also have an obvious connection to the transport of membrane vesicles and actin filaments, such as myosin-I near expanding membrane regions predicted for the developing brush border (Fath et al., 1990) and neuronal growth cones (Smith, 1988). The role of myosin-I in the delivery of vesicles and actin filaments to the developing cell surface is supported by the report of a myosin-I homologue in mice, *dilute*, which is thought to be necessary for the development of dendrites in neuronal cells (Mercer et al., 1991). Further evidence for the role of myosin-I in the transport of vesicles comes from the temperature-sensitive yeast mutant MYO2. In these cells, an absence of the myosin-I homologue, MYO2, results in the accumulation of vesicles in the main cell body (Johnston et al., 1991). The approaches we have used here provide a system in which the activities of these and other motors may be assessed.

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