Plasma Membrane Association of *Acanthamoeba* Myosin I

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Abstract. Myosin I accounted for ~2% of the protein of highly purified plasma membranes, which represents about a tenfold enrichment over its concentration in the total cell homogenate. This localization is consistent with immunofluorescence analysis of cells that shows myosin I at or near the plasma membrane as well as diffusely distributed in the cytoplasm with no apparent association with cytoplasmic organelles or vesicles identifiable at the level of light microscopy. Myosin II was not detected in the purified plasma membrane fraction. Although actin was present in about a tenfold molar excess relative to myosin I, several lines of evidence suggest that the principal linkage of myosin I with the plasma membrane is not through F-actin: (*a*) KI extracted much more actin than myosin I from the plasma membrane fraction; (*b*) higher ionic strength was required to solubilize the membrane-bound myosin I than to dissociate a complex of purified myosin I and F-actin; and (*c*) added purified myosin I bound to KI-extracted plasma membranes in a saturable manner with maximum binding four- to fivefold greater than the actin content and with much greater affinity than for pure F-actin (apparent K_D of 30–50 nM vs. 10–40 μM in 0.1 M KCl plus 2 mM MgATP). Thus, neither the MgATP-sensitive actin-binding site in the NH_2-terminal end of the myosin I heavy chain nor the MgATP-insensitive actin-binding site in the COOH-terminal end of the heavy chain appeared to be the principal mechanism of binding of myosin I to plasma membranes through F-actin. Furthermore, the MgATP-sensitive actin-binding site of membrane-bound myosin I was still available to bind added F-actin. However, the MgATP-insensitive actin-binding site appeared to be unable to bind added F-actin, suggesting that the membrane-binding site is near enough to this site to block sterically its interaction with actin.

ACANTHAMOEBA castellanii contains approximately equal concentrations of two classes of myosins: myosin II (31) and myosin I (33, 36, for review see 26). Myosin II is a conventional myosin comprising two heavy chains and two pairs of light chains and forming typical bipolar filaments with actin-activated Mg^{2+}-ATPase activity (when the three phosphorylation sites at the tip of the tail of each heavy chain are unphosphorylated). *Acanthamoeba* myosins IA and IB are the first, and best characterized, examples of a group of monomolecular, nonfilamentous myosins that is known to include a third isozyme from *Acanthamoeba*, a myosin I from *Dictyostelium discoideum* (15), and the 110-kD protein–calmodulin complex from intestinal brush border (10, 12–14).

The comparatively short heavy chains of myosins IA and IB (140 and 127 kD, respectively [33]) each comprise a single globular head (with striking similarities of amino acid sequence [23] and functional sites [4, 9, 29, 30] to the corresponding domain of myosins II) and a short, nonhelical COOH-terminal domain (that is rich in glycine, proline, and alanine [23]). Phosphorylation of a single amino acid (21) situated between the actin-binding and ATPase sites in the globular head (30) is required for expression of the actin-activated Mg^{2+}-ATPase activity of myosin I (32, 37). Myosins IA and IB are apparently incapable of forming oligomers of any size (6). However, the heavy chains of myosins IA and IB contain a second actin-binding site on the nonhelical, COOH-terminal domain (29). The presence of two actin-binding sites in the heavy chain allows myosins IA and IB to cross-link actin filaments (5, 16) and, when phosphorylated, support superprecipitation in vitro (16). Phosphorylated myosins I can also support the translocation of latex beads along *Nitella* actin cables (6).

Several years ago, Gadasi and Korn (19), using polyclonal antibodies, provided immunofluorescence and biochemical evidence that myosins IA and IB are localized near the cell surface of fixed amoebae and are enriched in isolated plasma membranes, in contrast to myosin II which showed no evidence for membrane association. Hagen et al. (20) have reported similar observations with monoclonal antibodies. More recently, Adams and Pollard (1) reported that myosin I was apparently responsible for the translocation of phagosomes and unidentified organelles partially purified from *Acanthamoeba* along *Nitella* actin cables in vitro and presented evidence for the association of myosin I with several different membrane fractions separated by density gradient centrifugation. The present study was undertaken in an attempt to define more precisely the nature of the association

of myosin I with the plasma membrane and to determine whether the plasma membrane might be the major source of myosin I-containing vesicles in an homogenate of *Acanthamoeba*.

Materials and Methods

Acanthamoeba myosins IA and IB were purified as described by Lynch et al. (29), and myosin I heavy chain kinase was purified by the method described by Hammer et al. (21). Rabbit antisera were raised against purified myosins IA and IB (19). The anti-myosin IB antibody was affinity purified by adsorption to and elution from nitrocellulose strips containing bound myosin IB heavy chain (28) before using it to immunolocalize myosin IB in cells. Anti-myosin II rabbit antiserum was raised against a synthetic peptide corresponding in sequence to the first 20 amino acids of the 29-residue-long nonhelical tailpiece at the COOH terminus of the heavy chains (7). FITC-conjugated goat anti-rabbit IgG and horseradish peroxidase-conjugated goat anti-rabbit IgG were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). A monoclonal antibody IgG_1 (ATCC HB80) against *Dictyostelium* actin was obtained from American Type Culture Collection (Rockville, MD). Rhodamine-conjugated phalloidin was from Molecular Probes Inc. (Junction City, OR). Actin purified from rabbit muscle according to the procedure of Spudich and Watt (38) was gel filtered on Sephadex G-200. All other chemicals were reagent grade. All protein concentrations were determined by the method of Bradford (8) using BSA as standard. NH_4^+,EDTA-ATPase activity was measured according to Pollard and Korn (36) using an assay buffer containing 0.4 M NH_4Cl, 35 mM EDTA, 2 mM ATP, 25 mM Tris-HCl, pH 7.5. Myosin I contents of membrane fractions determined by ATPase activities were converted to protein contents by reference to the specific activity of pure myosin I. SDS-PAGE was by the system described by Laemmli (27), and immunoblot analyses were performed as described by Towbin et al. (39) and Hawkes et al. (22). Coomassie blue-stained gels were quantified by scanning at 580 nm with a scanning densitometer (GS-300; Hoefer Scientific Instruments, San Francisco, CA) and using standard curves prepared with pure actin, myosin IA, and myosin IB.

Isolation of Plasma Membranes

Acanthamoeba castellanii (Neff strain) was grown to a density of 10^6 cells/ml at 29°C in 1-liter culture flasks as described by Pollard and Korn (36). Plasma membranes were isolated according to the recently developed procedure of Clarke et al. (11). Briefly, cells at 2×10^7/ml in a solution containing 0.35 M sucrose, 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES),[1] pH 7.4, and protease inhibitors (1 μg/ml pepstatin, 1 μg/ml antipain, 0.1 μg/ml leupeptin, and 0.3 mM diisopropylfluorophosphate) were homogenized by 12–15 strokes of a homogenizer (Dounce; Kontes Glass Co., Vineland, NJ). Unbroken cells and nuclei were removed by brief centrifugation at 230 *g*, and a crude plasma membrane fraction was pelleted from the supernatant at 800 *g*, resuspended in buffered 0.25 M sucrose, and then pelleted at 620 *g*. This pellet was resuspended and fractionated by density gradient centrifugation in 0.25 M sucrose and 18% Percoll in 10 mM TES, pH 6.9, for 40 min at 48,000 *g*. The plasma membrane fraction near the top of the gradient was diluted in 10 mM TES, pH 6.9, and pelleted at 12,000 *g*. The density gradient centrifugation was repeated, and the purified plasma membranes were suspended in 10 mM TES, pH 6.9.

Extraction of Myosin I from the Plasma Membranes

Plasma membranes (30 μg) were incubated in 200 μl of 10 mM TES, pH 7, containing 0.6 mM PMSF, 2 mM DTT, and various concentrations of KCl with and without 2 mM MgATP for 15 min at room temperature. 25% of the mixture was retained for ATPase assays, and the remainder was centrifuged in an airfuge at 120,000 *g* for 20 min. The pellet was resuspended in the original buffer and all fractions were assayed for their NH_4^+,EDTA-ATPase activities, a measure of myosin I content.

Extraction of Actin from the Plasma Membranes

The stock suspension of plasma membranes was diluted about fourfold in 10 mM TES, pH 7, containing a final concentration of 0.6 M KI and 30 μM sodium thiosulfate, then incubated on ice for 2 h, and centrifuged in an airfuge at 150,000 *g* for 30 min. The pellet was suspended in 10 mM TES, pH 7, repelleted, and then resuspended in 10 mM TES, pH 7.

Binding of Exogenous Myosin IA and IB to Plasma Membranes

KI- and KCl-extracted plasma membranes (10–30 μg) and 0.25–2 μg of myosin IA or 0.5–8 μg of myosin IB were mixed in 200 μl of buffer containing 0.1 M KCl, 2 mM MgATP, 1% BSA, 2 mM DTT, 0.6 mM PMSF, and 10 mM TES, pH 7, and incubated for 15 min at room temperature. An aliquot was removed and the remainder was pelleted in an airfuge at 120,000 *g* for 20 min. The NH_4^+,EDTA-ATPase activities of the mixture before centrifugation and of the pellet and supernatant after centrifugation were determined.

Binding of F-Actin to Plasma Membranes

KI-extracted plasma membranes with bound exogenous myosin IA were suspended with F-actin in 200 μl of 15 mM imidazole, pH 7.5, containing 2 mM $MgCl_2$, 1 mM EGTA, 2 mM MgATP, and myosin I heavy chain kinase (20 ng/ml) to phosphorylate the myosin heavy chain. After incubation at room temperature for 10 min, the mixture was centrifuged at 15,000 *g* for 15 min, the supernatant was removed, and the pellet was suspended in the original buffer. The actin contents of supernatants and pellets were quantified by SDS-PAGE.

Immunolocalization of Myosin IB

Cells were allowed to attach to poly-L-lysine-coated glass slides for 5 min at room temperature, while being flattened under an agar slip according to Fukui et al. (18), and then fixed at −10 to −15°C in 1% formalin. The cells were rinsed in TBS, pH 7.2, incubated for 30 min at 37°C with affinity-purified anti-myosin IB diluted in TBS containing 1% BSA, and rinsed in TBS. The rinsed cells were incubated in a 1:60 dilution of normal goat serum in TBS for 5 min at room temperature, then with FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated phalloidin for 30 min at 37°C, rinsed twice in TBS, and mounted in 90% glycerol in TBS containing 10 mg/ml of *p*-phenylenediamine adjusted to pH 8 with carbonate/bicarbonate buffer. The cells were observed and photographed using an inverted microscope (Carl Zeiss, Inc., Thornwood, NY) with phase-contrast or epifluorescence optics.

Purified plasma membranes were treated similarly except that all of the rinsing and staining procedures were done in suspension and the membranes were pelleted by centrifugation in an airfuge at 50,000 *g* for 10 min.

Electron Microscopy of Purified Plasma Membranes

Pelleted membranes were fixed at room temperature for 60 min in 3% glutaraldehyde, rinsed, and then postfixed for 30 min in 1% osmium tetroxide. Both fixatives were in 0.1 M sodium phosphate buffer, pH 6.8. The fixed pellets were dehydrated in a graded series of ethanol solutions, treated briefly with propylene oxide, and embedded in Epon. Thin sections were

Table I. Copurification of Myosin I and Plasma Membranes

Step*	Protein	ATPase activity	
	mg	*μmol/min*	*μmol/min/mg*
Homogenate	389	16.3	0.042
230 *g*			
Supernatant	389	15.9	0.041
Pellet	17	0.6	0.035
800 *g*			
Supernatant	322	11.3	0.035
Pellet	74	4.1	0.055
620 *g*			
Supernatant	21	1.1	0.051
Pellet	32	2.0	0.063
Plasma membranes	4.4	1.0	0.231

* See Materials and Methods for description of procedure.

1. *Abbreviation used in this paper*: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

examined and photographed in an electron microscope (LS410; Philips Electronic Instruments, Inc., Mahwah, NJ).

Results

Association of Myosin I with Purified Plasma Membranes

The distribution of myosin I during the fractionation procedure was followed by assaying for NH_4^+,EDTA-ATPase activity, which is diagnostic of this isozyme in *Acanthamoeba*. In the typical experiment shown in Table I, ~13% of the total myosin I was recovered in the crude plasma membrane fraction (before Percoll gradient centrifugation) and ~6% in the highly purified plasma membranes. About 1% of the total cell protein was recovered in the purified plasma membranes with an increase in specific activity of myosin I of about sixfold. The purity of the final plasma membrane fraction is illustrated in Fig. 1; no substantial contamination by other identifiable cellular structures was seen by electron microscopy. Clarke et al. (11) found that ~10% of the protein in similar plasma membrane preparations was derived from contaminating cytoplasmic protein and organelles, mostly accounted for by contractile vacuoles that do not have associated myosin I (see below). By immunofluorescence microscopy essentially all of the membrane fragments contained both actin and myosin I (Fig. 2). Myosin II was not detectable in the purified plasma membranes by immunofluorescence microscopy (data not shown).

The low recoveries of both plasma membrane and myosin I may have been due in part to the fragmentation of plasma membrane into pieces too small to be recovered in the low-speed pellets. For example, when an unfractionated cell homogenate was centrifuged for 2 h at 220,000 *g*, 75% of the myosin I ATPase activity was recovered in the pellet and only 25% in the supernatant. Moreover, ~75% of the myosin I ATPase activity that was discarded in the low-speed supernatants during the purification procedure was sedimented when the supernatants were recentrifuged at a higher speed. However, immunocytochemistry of formalin-fixed cells (see below) suggested that a substantial fraction of the myosin I is in the cytoplasmic compartment, so that the myosin I that was sedimented at high speed may have been associated with very small cytoplasmic vesicles or in the form of an actomyosin complex (40) and not membrane associated.

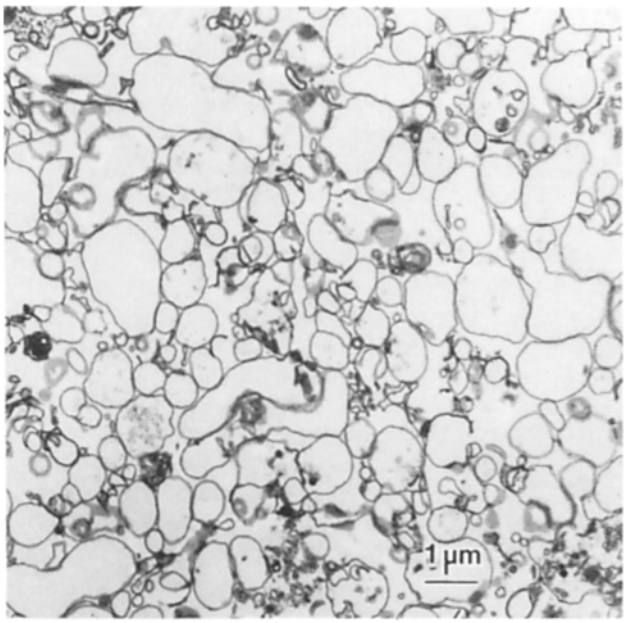

Figure 1. Electron micrograph of plasma membranes purified from *Acanthamoeba castellanii*. No significant contamination by other identifiable structures is seen. For experimental details see Materials and Methods.

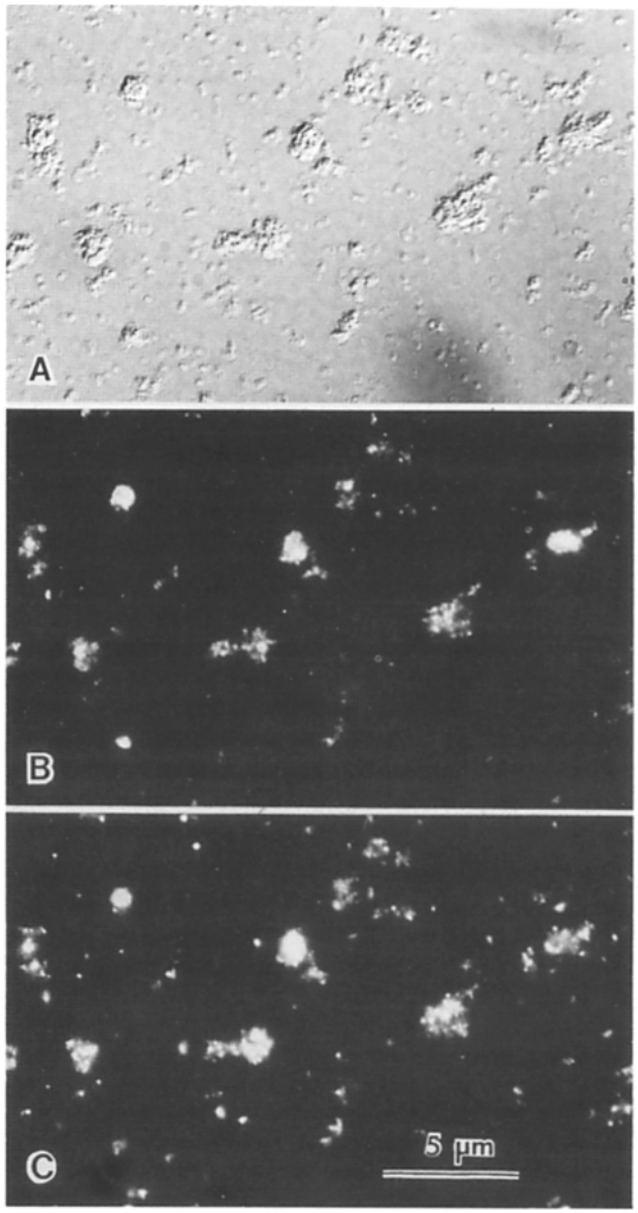

Figure 2. Demonstration by immunofluorescence microscopy that actin and myosin I were associated with the purified plasma membranes. Essentially every membrane profile detectable by differential interference contrast microscopy (*A*) was stained by anti-myosin IB/FITC-conjugated second antibody (*B*) and by rhodamine-phalloidin (*C*). Staining for myosin I was less intense than for actin, accounting for most of the apparent discrepancy between the two images. For experimental details see Materials and Methods.

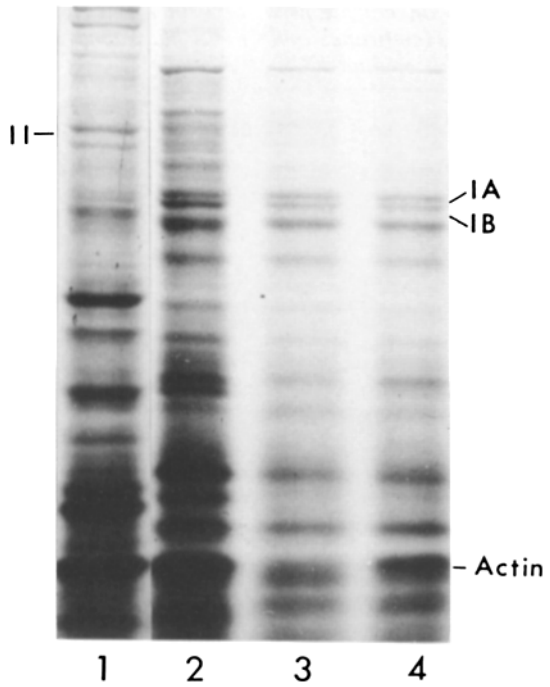

Figure 3. SDS-PAGE analysis of plasma membranes purified from *Acanthamoeba castellanii*. (Lane *1*) Total cell protein; (lane *2*) purified plasma membranes; (lane *3*) plasma membranes extracted with 0.6 M KI; (lane *4*) plasma membranes extracted with 0.6 M KCl plus 2 mM MgATP. The positions of the heavy chains of *Acanthamoeba* myosin II, IA, and IB and actin are shown. Gels (7% acrylamide) were quantified by scanning at 580 nm, and the data are shown in Table II. For clarity, the dye fronts of the gels shown in the figure have been removed, but the entire lanes were used for quantification as well as other gels using different percent acrylamide and different amounts of protein.

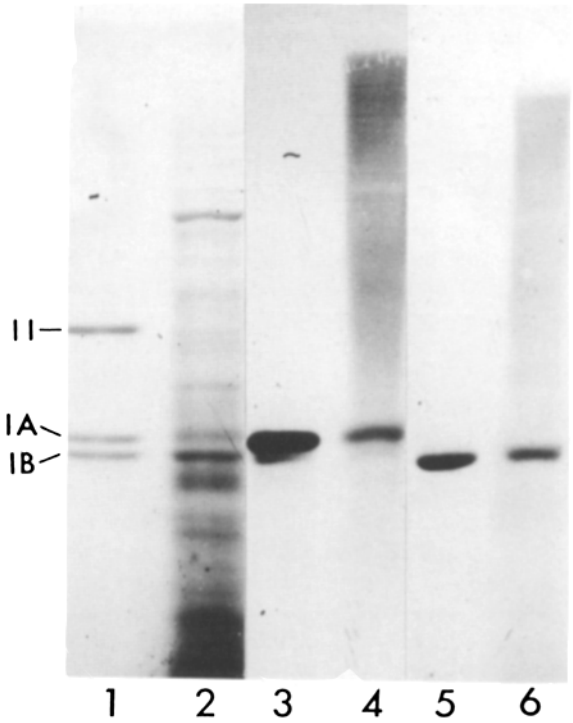

Figure 4. Identification of myosin IA and IB in purified plasma membranes by immunoblot analysis. (Lanes *1*, *3*, and *5*) A mixture of purified *Acanthamoeba* myosin II, IA, and IB and (lanes *2*, *4*, and *6*) purified *Acanthamoeba* plasma membranes were (lanes *1* and *2*) stained with Coomassie blue, (lanes *3* and *4*) immunoblotted with anti-myosin IA, and (lanes *5* and *6*) immunoblotted with anti-myosin IB. Immunoblots with anti-myosin II showed no reaction (not shown). The gels were 5% acrylamide; for other experimental details see Materials and Methods. For clarity only the upper portion of the gels are shown (actin and larger polypeptides); no reaction occurred on the lower portions of the blots.

From the results reported by Clarke et al. (11), a 30–40-fold increase in specific activity would be expected if all of the myosin I in the cell were associated with the plasma membrane. The lower value (about sixfold) that was consistently obtained in our experiments probably resulted from a combination of factors: (*a*) it is likely that not all of the myosin I is membrane associated (see below); (*b*) some of the membrane-bound myosin I probably dissociated during the isolation procedure; and (*c*) some of the myosin I that remained membrane associated may have lost its NH_4^+,EDTA-ATPase activity during the isolation procedure (see below).

SDS-PAGE analysis of purified plasma membranes is shown in Fig. 3, lane *2*, compared with total cell protein in lane *1*. The plasma membranes appear to be enriched in both myosin IA and IB. The presence of myosin IA and IB in the purified plasma membranes was confirmed by the immunoblots shown in Fig. 4. Myosin II was not detected in immunoblots of equivalent quantities of membrane (data not shown). Quantitative scans of Coomassie blue–stained plasma membrane fractions consistently showed a ratio of myosin IB to myosin IA of 1.5:2.5 (the pure proteins stained with identical intensity). In six preparations, myosin IA and IB accounted for 2.2 ± 0.09% of the protein by Coomassie blue stain but only 1.4 ± 0.12% of the protein by NH_4^+,EDTA-ATPase assay (protein concentrations were calculated from the specific activities of pure myosin IA and IB). Thus, based on Coomassie blue staining, myosin I was about nine- to tenfold enriched in the plasma membrane relative to the whole cell, which is somewhat closer to the enrichment reported for other plasma membrane markers (11) and could indicate that perhaps 25% of the myosin I in *Acanthamoeba* may be membrane associated.

Localization of Myosin I and F-Actin in Acanthamoeba

To test for the possibility that the myosin I had artifactually become associated with the plasma membranes during the purification procedure, the distributions of myosin I and F-actin in fixed cells were reexamined (Fig. 5). Rhodamine-phalloidin identified F-actin in microspikes and beneath the plasma membrane (Fig. 5, *C* and *F*). By immunofluorescence analysis (Fig. 5, *B* and *E*), myosin IB appeared to be more closely associated with the plasma membrane than the actin was. This was especially notable where membrane blebbing occurred (Fig. 5 *B*). Myosin I seemed not to be present in the microspikes (Fig. 5, *B* and *E*). Anti-myosin IB also diffusely stained the cytoplasm, consistent with the partial recovery of myosin I with the purified plasma membranes. However, very few of the many vacuoles that could be identified in the phase-contrast images (Fig. 5, *A* and *B*)

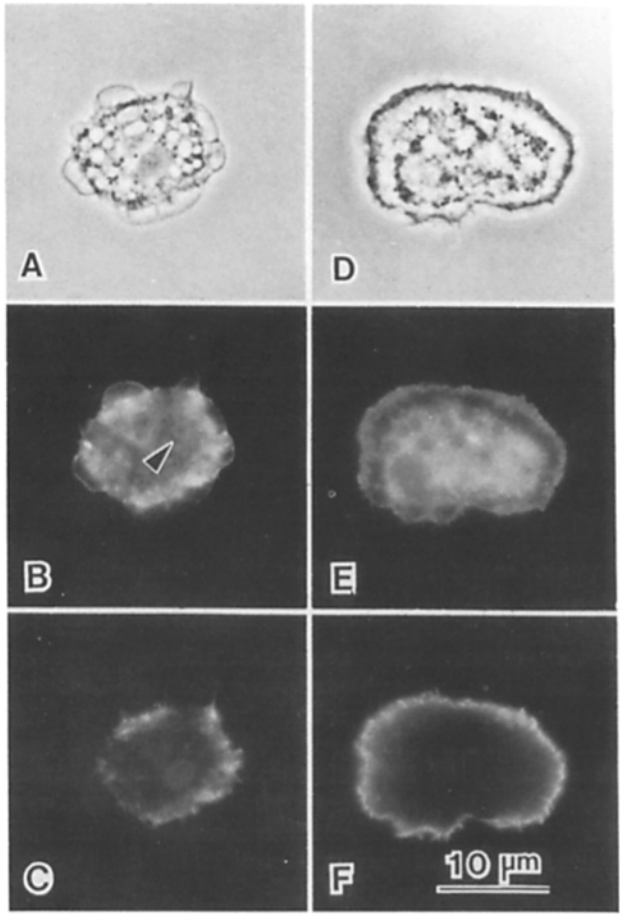

Figure 5. Localization of myosin I and actin in *Acanthamoeba castellanii*. (*A* and *D*) Phase-contrast micrographs of two cells; (*B* and *E*) fluorescent images of the same cells stained with affinity-purified anti-myosin IB and FITC-conjugated second antibody; (*C* and *F*) fluorescent images of the same cells double-stained with rhodamine-phalloidin. Almost all of the large vesicles are nonfluorescent (*E*) but occasionally, and never more than one per cell, an unidentified fluorescent vesicle was seen (*B, arrow*). The profile shown in *B* is of a damaged cell in which the plasma membrane has separated from the cortex. This was selected to illustrate the association of myosin I with the plasma membrane, but the intracellular fluorescence in this cell is less uniform than was usually observed.

were stained by the myosin IB antiserum (or by rhodamine-phalloidin). Contractile vacuoles were never stained with myosin IB antiserum. Occasionally, the myosin IB antiserum did stain an unidentified vesicle-like profile (see Fig. 5 *B*), but this was a rare event. Typically there were >20 large vacuoles per cell and the myosin IB antiserum usually stained none, and never more than one, per cell. The stained structures may have been invaginations of the plasma membrane or newly formed phagocytic vesicles. Unambiguous surface invaginations were always labeled by phalloidin and often by myosin IB antiserum. However, these data do not rule out the possibility that myosin I may also be associated with some of the numerous vesicles (40% of the total cell membrane) below 0.5 μm in diameter that could not be resolved in these immunofluorescent images.

Table II. Relative Concentrations of Myosin I and Actin in Purified Plasma Membranes and in Plasma Membranes After Extraction with KI and KCl

Plasma membranes	Protein	Actin*		Myosin IA plus IB*	
	%	%	*nmol/mg*	%	*nmol/mg*
As isolated (native)	100	100	1.4	100	0.13
KI extracted‡	43	15	0.48	53	0.16
KCl extracted‡	55	37	0.95	47	0.11

* The units are nanomoles of actin and myosin per milligram of plasma membrane protein. The total protein in the plasma membrane fractions was determined colorimetrically. Actin and myosin values were calculated from scans of Coomassie blue-stained SDS-PAGE using different percents of acrylamide so that all of the polypeptides were resolved. The values are for the plasma membranes before and after extraction.
‡ After extraction with 0.6 M KI or with 0.6 M KCl plus 2 mM MgATP.

How Is Myosin I Bound to the Isolated Plasma Membranes?

Because actin accounts for ~6% of the protein of the purified plasma membranes (Table II), we first considered the possibility that myosin I was associated with the membranes through F-actin. However, as described below, several lines of experimental evidence argued against this being the principal mechanism of binding.

When plasma membranes were extracted with 0.6 M KI, 85% of the actin and only 47% of the myosin I were removed, as shown by SDS-PAGE (Fig. 3, lane *3*, and Table II). These observations were confirmed by immunoblots with anti-myosin IB antibodies and anti-actin antibodies (data not shown). Attempts to remove a greater fraction of the membrane-associated actin by repeated extraction with 0.6 M KI (even with sonication), by dialysis against low ionic strength buffers (even including EDTA), by treatment with DNase I, or by using two or more of these procedures sequentially were all unsuccessful. As shown in Fig. 3, many other proteins were essentially totally extracted, indicating that the actin was almost certainly completely accessible to the KI. The molar ratio of myosin I to actin subunits increased from a value of about 1:10 in the purified plasma membranes to about 1:3 in the KI-extracted membranes (Table II). Although the KI-extracted plasma membranes retained sufficient actin to bind the myosin I, differential extraction of the actin seems an unlikely result if all of the myosin I were bound to the plasma membrane through actin.

As described in the introduction, myosin I has an ATP-sensitive and an ATP-insensitive actin-binding site (29); binding of F-actin to both sites is weakened by elevated ionic strength. The K_D values for the binding of purified myosins IA and IB to purified F-actin in solutions containing 0.1 M KCl and 2 mM MgATP were found to be 10 and 40 μM, respectively. At low ionic strength, in the presence or absence of MgATP, the K_D value for both isoenzymes is <1 μM (3). With this information, it was possible to test whether the affinity of myosin I for the purified plasma membrane was consistent with their association being mediated by F-actin.

Extraction of myosin I from isolated plasma membranes as a function of KCl concentration in the presence and absence of 2 mM MgATP is shown in Fig. 6. As measured by the solubilization of NH_4^+,EDTA-ATPase activity, very little myosin I was extracted, even in the presence of MgATP,

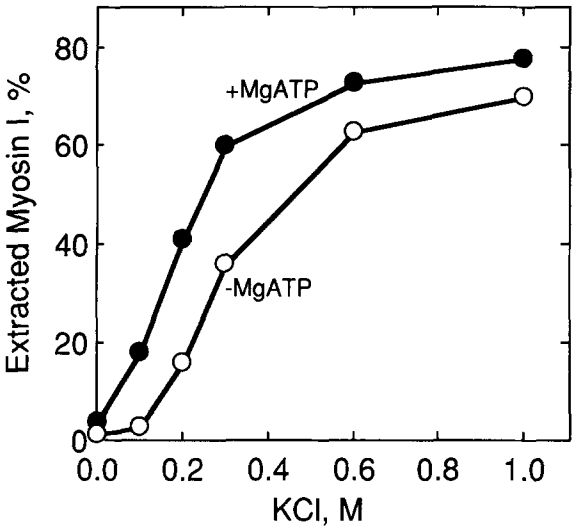

Figure 6. Extraction of myosin I from purified *Acanthamoeba* plasma membranes by KCl. The amount of myosin I extracted at different concentrations of KCl in the presence and absence of 2 mM MgATP was determined by assaying for NH_4^+,EDTA-ATPase activity. The data were normalized to the sum of the ATPase activity recovered in the supernatant and pellet fractions; total recovery was between 80 and 90% in all experiments.

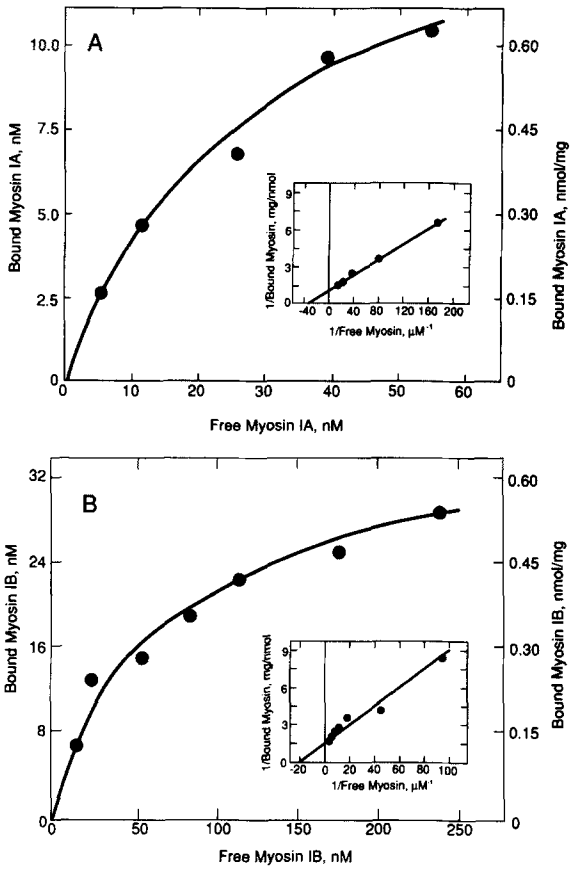

Figure 7. Binding of exogenously added myosin I to KI-extracted *Acanthamoeba* plasma membranes. Increasing concentrations of purified mysoin IA (*A*) or IB (*B*) were added to KI-extracted plasma membranes in 0.1 M KCl plus 2 mM MgATP and 1% BSA, the membranes were pelleted, and the amounts of free and bound myosin were determined by assaying supernatant and pellet fractions for NH_4^+,EDTA-ATPase activity. (*Insets*) Double-reciprocal plots of the same data to estimate the capacity and affinity of the binding. For additional experimental details see Materials and Methods. Data at higher concentrations of myosin IA are not shown because nonspecific binding obscured the hyperbolic curve due to specific binding.

until ~0.2 M KCl was reached, and maximal solubilization (~80%) required ~0.6–1 M KCl. If the myosin I were bound to the plasma membranes exclusively through association with F-actin, essentially all of the myosin I should have been solubilized by 0.1 M KCl and 2 mM MgATP, as the concentrations of both actin and myosin I in these experiments were much less than the K_D values (10 and 40 μM) for the binding of myosins IA and IB to F-actin.

Less myosin I appeared to be extracted from the plasma membranes by 0.6 M KCl when analyzed by SDS-PAGE than when analyzed by NH_4^+,EDTA-ATPase activity. For example, by SDS-PAGE analysis, 47% of the myosin I remained in the plasma membrane after extraction by 0.6 M KCl and 2 mM MgATP (Table II). Extractions of myosin IA and IB were similar (Fig. 3, lanes *2* and *4*). The results obtained by SDS-PAGE were confirmed by immunoblot analyses (data not shown) and, thus, probably were not due to the presence of other polypeptides migrating at the positions of the myosin I heavy chains. The differences between the enzymatic (80% extracted) and electrophoretic assays (50% extracted) can be reconciled if some of the plasma membrane–bound myosin I were denatured and, thus, enzymatically inactive and also less soluble than the native enzyme. If this were so, the results of the enzyme assays would be more truly reflective of the affinities of native myosins IA and IB for plasma membranes. An alternative explanation, that some of the myosin I was trapped inside closed vesicles and therefore inaccessible to the ATPase assay, is improbable for the following reasons: (*a*) as seen in Fig. 3, many proteins were extracted essentially quantitatively by 0.6 M KCl (it is highly unlikely that all of these were on the outside of closed vesicles); (*b*) at least 85% of the unextracted vesicles were permeable to BSA and immunoglobulins when prepared for immunoelectron microscopy (data not shown); and (*c*) identical results were obtained when the ATPase assays were carried out in the presence of 0.5% saponin or 0.2% Triton X-100, neither of which affected the activity of purified myosin I.

Binding of Purified Myosin I to Plasma Membranes

To determine the affinity and capacity of membranes for myosin I, purified myosins IA and IB were incubated with plasma membranes in 0.1 M KCl and 2 mM MgATP, and the membranes were then sedimented by centrifugation. The original mixture, supernatant and pellet were then assayed for myosin I by NH_4^+,EDTA-ATPase activity. In the first experiments, KI-extracted membranes were used to minimize the possibility that the exogenous myosin I would bind to the membranes through association with actin. The assay was not affected by the presence of endogenously bound myosin I because, although KI did not extract all of the endogenous myosin (see Fig. 3, lane *3*, and Table II), KI did inactivate the myosin so that it did not contribute to the ATPase assays.

As shown in Fig. 7, both myosin IA and IB bound to KI-extracted plasma membranes in a saturable manner as is

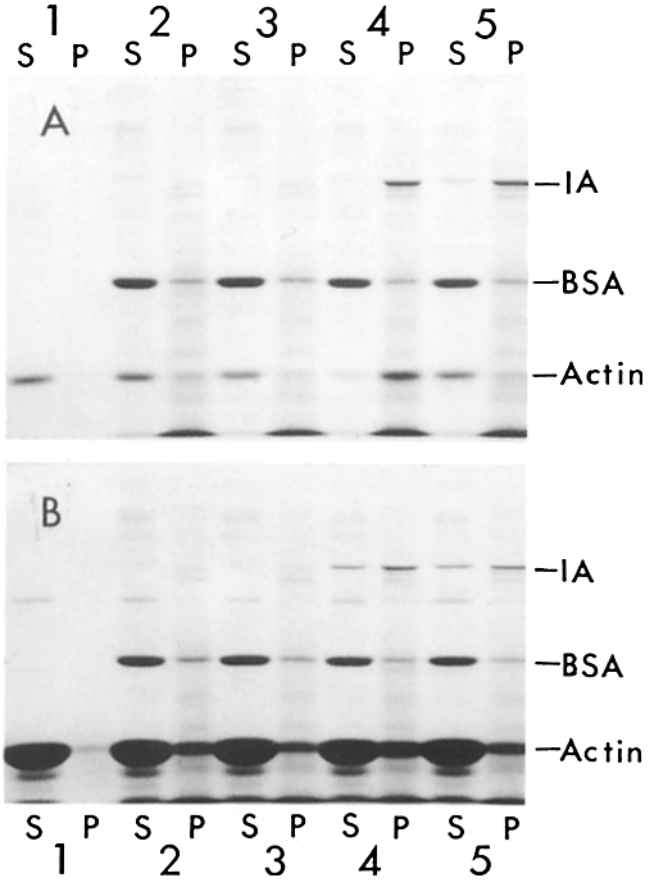

Figure 8. Binding of exogenously added F-actin to plasma membranes with and without exogenously bound myosin I. KI-extracted membranes were incubated without myosin I or with saturating concentrations of myosin IA in 0.1 M KCl and 2 mM MgATP containing myosin I heavy chain kinase and 1% BSA as described in Fig. 7. The membranes were pelleted, resuspended, and then incubated for 15 min at 20°C with either 0.12 μM F-actin (*A*) or 3.3 μM F-actin (*B*) in the absence or presence of 2 mM MgATP. All tubes contained BSA to inhibit nonspecific binding. The membranes were then pelleted, and the supernatants and pellets analyzed by SDS-PAGE (7% acrylamide). (Lane *1*) F-actin alone; (lanes *2* and *3*) membranes without exogenous myosin IA and with added F-actin; (lanes *4* and *5*) membranes with exogenous myosin IA and added F-actin. (Lanes *2* and *4*) Without MgATP; (lanes *3* and *5*) with MgATP. Supernatants (*S*) and pellets (*P*) are indicated. The positions of myosin IA heavy chain, BSA, and actin are marked. For additional details see Materials and Methods.

most clearly evidenced by the linear double reciprocal plots. The concentrations of myosin IA and IB required for half-maximal binding were 30 and 50 nM, respectively. These values are three orders of magnitude lower than the measured K_D values for the binding of pure myosins IA and IB to F-actin (10 and 40 μM, respectively). At saturation, the KI-extracted membranes bound 0.85 nmol of exogenously added myosin IA and 0.65 nmol of myosin IB per milligram of membrane protein (Fig. 7). This is about four to five times more than the myosin I content of the purified plasma membranes before and after extraction by KI (~0.13 and 0.16 nmol/mg, respectively; Table II) and about twice the amount of myosin I that could have been bound to the residual actin in the KI-extracted membranes (~0.48 nmol/mg; Table II). Binding

Table III. MgATP-dependent Binding of Exogenous F-Actin to KI-extracted Plasma Membranes with and without Bound Exogenous Myosin I

Conditions	Actin sedimented*		
	F-Actin	F-Actin + PM‡	F-Actin + PM‡ + MI§
	%‖	%	%
−MgATP	0	12	79
+MgATP	0	7	12

* The data were calculated from scanning two gels such as that shown in Fig. 8 *A*.
‡ KI-extracted plasma membranes.
§ Myosin IA.
‖ Percent of total.

did not occur in 0.2 mM KCl plus 2 mM MgATP or in 0.6 M KCl, in the presence or absence of MgATP, consistent with the observation that endogenous myosin I was almost totally extracted under these conditions (Fig. 6). In the absence of KCl, nonspecific, nonsaturable binding occurred.

Binding studies were also carried out using myosin IA and KCl-extracted plasma membranes, which contained more endogenous actin and less endogenous myosin I than KI-extracted membranes (see Fig. 3, lane *4*, and Table II). An apparent K_D of 20 nM and a binding capacity of ~0.73 nmol/mg of membrane protein were obtained. These values are essentially the same as those that were obtained for the binding of myosin IA to the KI-extracted membranes, again indicating that the binding of exogenous myosin I was not through actin. In all of the experiments, the sum of the NH_4^+,EDTA-ATPase activities of the membranes and purified myosin I assayed separately, the activity of the mixture of the two before centrifugation, and the sum of the activities of the membrane pellet and supernatant after centrifugation were the same. Thus, there was no indication of a change in specific activity when myosin I was bound to membranes.

Binding of F-Actin to Membrane-associated Myosin I

The experiments just described indicated that purified myosin I can bind to isolated plasma membranes with high affinity through saturable binding sites that do not involve actin. In that case, the actin-binding sites on the membrane-bound myosin I might be available to bind exogenous F-actin. To test this, the amount of F-actin sedimenting at low speed was determined in the absence and presence of KI-extracted membranes with or without bound myosin IA, and in the presence and absence of MgATP. The results obtained using an actin subunit-to-myosin I ratio of 2.4:1 are illustrated in Fig. 8 and quantified in Table III. Under the conditions of centrifugation that were used, F-actin alone did not sediment (Fig. 8 *A*, lane *1*). When the F-actin was incubated with KI-extracted membranes in the absence of MgATP, only 12% of the actin sedimented with the membranes (Fig. 8 *A*, lanes *2*), and this was reduced to 7% by the addition of MgATP (Fig. 8 *A*, lanes *3*). When the F-actin was incubated with KI-extracted plasma membranes that had previously been incubated with exogenous myosin IA, 79% of the F-actin pelleted with the membranes in the absence of MgATP (Fig. 8 *A*, lanes *4*), but only 12% pelleted in the presence of MgATP (Fig. 8 *A*, lanes *5*). These results provide clear evidence that the MgATP-sensitive actin-binding site of myosin

I was still available to bind F-actin when exogenous myosin I was bound to the plasma membranes. Because little F-actin was bound to the membrane-associated myosin in the presence of MgATP, the data also suggest that the MgATP-insensitive actin-binding site of myosin I was unable to interact with actin when the myosin was bound to the membranes.

When a higher actin-to-myosin I ratio of 100:1 was used, again very little of the F-actin pelleted in the absence of membranes (Fig. 8 *B*, lanes *1*), more in the presence of membranes (Fig. 8 *B*, lanes *2* and *3*), and still more when exogenous myosin IA had been bound to the KI-extracted membranes before the addition of the actin (Fig. 8 *B*, lanes *4*). The latter binding was inhibited in the presence of MgATP (Fig. 8 *B*, lanes *5*). These results were similar to those obtained at the lower actin-to-myosin I ratio.

However, at the high actin-to-myosin I ratio (100:1), the F-actin solubilized ~45% of the membrane-associated myosin I, both in the presence (Fig. 8 *B*, lanes *5*) and absence of MgATP (Fig. 8 *B*, lanes *4*). If the association of myosin I and plasma membranes were a simple equilibrium reaction this could be explained simply by dissociation of the myosin I–membrane complex due to binding of free myosin I to F-actin in solution. Dissociation of membrane-bound myosin I was not induced by the presence of as much as 10 mg/ml of BSA in the absence of F-actin. The high concentration of added F-actin required to dissociate the myosin I from the membrane provides additional circumstantial evidence that myosin I was not bound to the membrane principally through membrane-associated F-actin.

Similar results were obtained when F-actin was mixed with native, purified plasma membranes containing their own complement of myosin I, but without additional, exogenous myosin I. When 0.15 μM F-actin was used, ~62% and ~19% of the actin was sedimented with plasma membranes in the absence and presence of MgATP, respectively, and no detectable myosin was solubilized. With 3 μM F-actin, however, ~10–13% of the endogenous membrane-bound myosin I was solubilized, both in the absence and presence of MgATP.

Discussion

The data in this paper confirm and extend previous evidence (19, 20) for the association of myosin I with the plasma membrane of *Acanthamoeba*. Localization by immunofluorescence has demonstrated that myosin I is more closely associated with the plasma membrane than is the cortical actin, and highly purified plasma membrane fragments were found uniformly to contain myosin I which was enriched five- to tenfold relative to its concentration in the whole cell homogenate. The immunofluorescence data also indicated that myosin I is diffusely distributed in the cytoplasm with no apparent association with any membranous structures discernible by phase-contrast microscopy (the cytoplasmic myosin I may be bound to F-actin [40]). These observations are consistent with recent evidence that myosin I is associated with the plasma membrane of *Dictyostelium discoideum* amoebae as well as occurring diffusely in the cytoplasm unassociated with any detectable structures (Fukui, Y., T. J. Lynch, H. Brzeska, and E. D. Korn, manuscript submitted for publication). In the experiments with *Dictyostelium*, the membrane-associated myosin I was found to be highly concentrated in specific regions of the plasma membrane—at the leading edges of pseudopodia and lamellipodia of locomoting cells and of cells undergoing cytokinesis and at the sites of formation of phagocytic cups. Myosin I was undetectable elsewhere in the *Dictyostelium* plasma membranes and was notably absent from the rear of locomoting cells and the contractile ring of dividing cells, where myosin II is highly concentrated (43; Fukui, Y., T. J. Lynch, H. Brzeska, and E. D. Korn, manuscript submitted for publication). In the present studies, the plasma membrane–associated myosin I of *Acanthamoeba* was found to be more generally distributed around the cell periphery, perhaps because these cells were not as highly polarized as *Dictyostelium* amoebae undergoing either cAMP-induced chemotaxis, cell division, or phagocytosis.

The absence of any direct evidence in our study for myosin I being associated with any membranes other than the plasma membrane is not necessarily inconsistent with the report by Adams and Pollard (1) that vesicles of unknown origin, phagosomes, and several membrane fractions contain bound myosin I. As stated earlier, it is possible that myosin I is associated with cytoplasmic vesicles too small to have been resolved in our experiments or with phagosomes newly formed from the plasma membrane (26, 41, 42) which might be expected to contain membrane-associated myosin I. We have found that purified phagocytic vesicles do, in fact, contain measurable myosin I, but in a much lower amount than plasma membranes (data not shown), and, as mentioned, in *Dictyostelium* amoebae, the plasma membrane at the site of formation of the phagocytic cup is particularly enriched in myosin I (Fukui, Y., T. J. Lynch, H. Brzeska, and E. D. Korn, manuscript submitted for publication). In addition, at least some of the membrane vesicles studied by Adams and Pollard (1) might have been vesiculated fragments of plasma membranes.

Actin, of course, is a major component of the purified *Acanthamoeba* plasma membrane fraction (11, 25, 35), and myosin I has a high affinity for F-actin. For several reasons, however, we think that most of the membrane-associated myosin I is not bound primarily through F-actin. One reason is that 0.6 M KI extracted significantly more actin than myosin I from the plasma membrane fraction. Perhaps more convincing, a significantly higher concentration of KCl (0.6–1 M) was required to solubilize the myosin I than would be expected from the measured affinities of myosin I for F-actin under the conditions used. And, certainly, the binding data for purified myosin I added to plasma membranes were inconsistent with the properties of the myosin I–F-actin interaction. First, the same amount of myosin I bound to KI-extracted plasma membranes as to KCl-extracted membranes even though the former had less than half as much associated actin. Second, the amount of myosin I that bound to KI-extracted membranes was four to five times more than could be accounted for by the residual actin content of the membranes, assuming a 1:1 stoichiometry between actin subunits and myosin I. Third, the concentration of myosin I required for half-maximal binding to the plasma membranes (30–50 nM) was about three orders of magnitude less than the K_D for the binding of myosin I to F-actin (10–40 μM) under the same ionic conditions (0.1 M KCl, 2 mM MgATP).

We do not know at this time how either endogenous or exogenous myosin I binds to the *Acanthamoeba* plasma mem-

brane, but, from the data just discussed, neither the MgATP-sensitive actin-binding site in the NH_2-terminal end of the heavy chain nor the MgATP-insensitive actin-binding site in the COOH-terminal end of the heavy chain seems to be involved. However, the MgATP-insensitive actin-binding site of membrane-associated myosin I appeared not to be able to bind added F-actin, suggesting that this site might have been sterically blocked or, possibly, that this site bound with high affinity to an actin-like, integral membrane protein. Finally, our data are not inconsistent with the observations by Adams and Pollard (Adams, R. J., and T. D. Pollard, manuscript submitted for publication) that raise the possibility of saturable binding of myosin I to membrane lipids.

As mentioned in the introduction, myosin I isozymes have been shown to be present in *Dictyostelium* (15) and in the microvilli of intestinal brush border (10, 12–14). In the latter, myosin I (the 110-kD protein–calmodulin complex) seems to cross-link actin filament core bundles to the plasma membrane (for review see 34) and, thus, possibly to move one relative to the other. Because of the specific location of the plasma membrane-associated myosin I of *Dictyostelium*, the suggestion was made (Fukui, Y., T. J. Lynch, H. Brzeska, and E. D. Korn, manuscript submitted for publication) that actomyosin I produces a projectile force that leads to the formation of pseudopodia, lamellipodia, and phagocytic cups in these amoebae. We suggest that myosin I has a similar role in the motile activities of *Acanthamoeba* (endocytic channels, for example, show colocalization of F-actin and myosin I [data not shown]) and possibly continues to serve a motile function for the phagocytic vesicles (1) that are formed by internalization of phagocytic cups. We do not yet know whether the plasma membrane-associated myosin I of *Dictyostelium* and *Acanthamoeba* moves actin filaments relative to the plasma membrane and/or, by virtue of its two actin-binding sites, moves one actin filament relative to another while being anchored in the plasma membrane. In addition to the myosin I that is probably directly associated with the plasma membrane, there is likely also to be myosin I–cross-linking actin filaments in the submembranous cortex (perhaps this is the myosin that is extracted by KI and KCl from the purified plasma membrane fraction) which could contribute to the "projectile" activity.

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