

Differential Localization of *Acanthamoeba* Myosin I Isoforms

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Abstract. *Acanthamoeba* myosins IA and IB were localized by immunofluorescence and immunoelectron microscopy in vegetative and phagocytosing cells and the total cell contents of myosins IA, IB, and IC were quantified by immunoprecipitation. The quantitative distributions of the three myosin I isoforms were then calculated from these data and the previously determined localization of myosin IC. Myosin IA occurs almost exclusively in the cytoplasm, where it accounts for ~50% of the total myosin I, in the cortex beneath phagocytic cups and in association with small cytoplasmic vesicles. Myosin IB is the predominant isoform associated with the plasma membrane, large vacuole membranes and phagocytic membranes and accounts for almost half of the total myosin I in the cytoplasm. Myosin IC accounts for a significant frac-

tion of the total myosin I associated with the plasma membrane and large vacuole membranes and is the only myosin I isoform associated with the contractile vacuole membrane. These data suggest that myosin IA may function in cytoplasmic vesicle transport and myosin I-mediated cortical contraction, myosin IB in pseudopod extension and phagocytosis, and myosin IC in contractile vacuole function. In addition, endogenous and exogenously added myosins IA and IB appeared to be associated with the cytoplasmic surface of different subpopulations of purified plasma membranes implying that the different myosin I isoforms are targeted to specific membrane domains through a mechanism that involves more than the affinity of the myosins for anionic phospholipids.

MYOSINS have been classified as type I or II depending on whether they possess one or two heavy chains, respectively (Korn and Hammer, 1988). Myosins II form bipolar filaments by self-association mediated by their long coiled-coil, α -helical tail domains. Single-headed myosins I (for recent reviews see Korn and Hammer, 1990; Pollard et al., 1991) have short globular tails and do not form filaments; examples, all from nonmuscle cells, include myosins I from amoebae (*Acanthamoeba castellanii* and *Dictyostelium discoideum*), intestinal brush border, and adrenal medulla (Barylko et al., 1992). Recent preliminary reports indicate that myosins I may be widespread in metazoan nonmuscle cells (Espreafico, E., R. Chaney, F. Spindola, M. Coehlo, D. Pitta, M. Mooseker, and R. Larson. 1990. *J. Cell Biol.* 111:167a; Li, D., and P. D. Chantler. 1991. *Biophys. J.* 59:229a; Bahler, M. 1990. *J. Cell Biol.* 111:167a; Atkinson, M. A. L., and D. M. Peterson. 1991. *Biophys. J.* 59:230a). Other nonmuscle myosins, e.g., the *MYO2* gene product in yeast (Prendergast et al., 1990), the murine dilute gene product (Mercer et al., 1991; also called 190 kD, Larson et al., 1988, 1990) and the protein encoded by the *Nina c* locus of *Drosophila melanogaster* (Montell and Rubin, 1988), although not yet fully characterized, may not meet the criteria of either type I or II myosins.

The *Acanthamoeba* myosins IA (heavy chain, 140 kD; light chain, 17 kD), IB (heavy chain, 125 kD; light chain, 27 kD) and IC (heavy chain, 130 kD; two light chains, 14 kD) are among the biochemically best characterized mono-

meric myosins (see Korn et al., 1988; Korn, 1991). The NH₂-terminal subfragment-1-like globular head (Jung et al., 1987; Brzeska et al., 1988) of each isoform contains the ATP-sensitive actin-binding site and the catalytic site; actin-activated Mg²⁺-ATPase activity is maximally expressed only after a single hydroxy amino acid in the head domain has been phosphorylated (Hammer et al., 1983; Brzeska et al., 1989) by myosin I heavy chain kinase. All three *Acanthamoeba* myosins I have a short, COOH-terminal tail with a membrane-binding domain and an additional, ATP-insensitive actin-binding site (Brzeska et al., 1988; Adams and Pollard, 1988; Lynch et al., 1989; for review, see Korn et al., 1988).

The biological roles of the *Acanthamoeba* myosins I are not yet defined, however, some insight into their functions can be obtained from their intracellular localizations. In the first such experiments, myosins IA and IB were thought to be localized near the plasma membrane at the level of resolution obtainable by immunofluorescence microscopy (Gadasi and Korn, 1980; Hagen et al., 1986; Miyata et al., 1989). Myosins IA and IB were also found associated with purified plasma membranes and shown to bind to salt-extracted membranes (Miyata et al., 1989), NaOH-extracted membranes (Adams and Pollard, 1989), and anionic synthetic phospholipid vesicles (Adams and Pollard, 1989). Later, myosin IC was shown to be localized to the plasma membrane and the contractile vacuole membrane by both immunofluorescence and immunoelectron microscopy (Baines

and Korn, 1990). *Acanthamoeba* myosin II, on the other hand, is present throughout the cytoplasm in the form of 200-nm minifilaments, is most concentrated in the cortex, but is absent from thin membrane extensions such as microspikes and filopodia (Baines and Korn, 1990). These results for *Acanthamoeba* are consistent with the finding that myosin I is at the leading edge and myosin II is in the posterior region of locomoting *Dictyostelium* (Fukui et al., 1989). We now report the more definitive localizations of *Acanthamoeba* myosins IA and IB in vegetative and phagocytosing amoebae at the level of resolution of immunoelectron microscopy and the quantitative distributions of the three *Acanthamoeba* myosin I isoforms among the several cell compartments in which one or more of them occurs.

Materials and Methods

Acanthamoeba castellanii (Neff strain) was grown either in 1-liter culture flasks to a density of 10^6 cells \times ml⁻¹, as described by Pollard and Korn (1973), on a plastic substrate in 750-ml culture flasks (Falcon, Cockeysville, MD), or on a glass substrate in 8-chamber Lab-Tek tissue culture slides (Nunc, Inc., Naperville, IL). Cells grown in contact with a substrate were much flatter than cells grown in suspension thus improving the resolution obtainable by immunofluorescence studies. Myosins IA and IB were purified by the method of Lynch et al. (1991).

Polyclonal Antibodies

Antimyosin IA was raised against a COOH-terminal 27-kD chymotryptic fragment of myosin IA heavy chain prepared by Dr. Thomas Lynch (Lynch et al., 1986). Antimyosin IB was raised against a 15-amino acid synthetic peptide Lys-Lys-Lys-Pro-Thr-Thr-Ala-Gly-Phe-Lys-Ile-Lys-Glu-Ser-Cys corresponding to residues 528–541 of myosin IC heavy chain (which is a region of 80% homology between myosins IC and IB) with a COOH-terminal cysteine residue (which makes the sequence more similar to that of myosin IB) added to facilitate conjugation to maleimide-activated keyhole limpet hemocyanin (Pierce Chemical Co., Rockford, IL). The conjugation was performed according to the manufacturer's instructions.

The conjugated peptide or chymotryptic fragment was mixed 1:1 (vol/vol) with Freund's complete adjuvant (Difco Laboratories, Detroit, MI) to form an emulsion before immunization. Female New Zealand white rabbits were primed at the age of 6 mos and received three additional boosts with at least 4 wk between injections. The antisera were collected 10–12 d after the final immunization. The antiserum raised against the 27-kD myosin IA chymotryptic fragment recognized myosin IA by immunoblot analysis of total amoeba proteins and an unidentified protein of ~50 kD. The cross-reacting antibody activity was removed by adsorbing the antiserum on nitrocellulose strips containing the 50-kD protein prepared by transfer from SDS-PAGE gels of total cell proteins (see Kulesza-Lipka et al., 1991).

Immunolocalization of Myosins IA and IB

Four fixation and permeabilization protocols were used (Baines and Korn, 1990; Kulesza-Lipka et al., 1991): (a) Cells were fixed with 3% formaldehyde and 0.25% glutaraldehyde in growth medium for 45 min at room temperature and then permeabilized with 0.5% saponin for 30 min at room temperature; (b) cells were fixed and permeabilized simultaneously in the presence of 0.05% glutaraldehyde, 1% formaldehyde, and 0.05% saponin in growth medium for 5 min at room temperature followed by further fixation in 0.05% glutaraldehyde and 1% formaldehyde in growth medium at room temperature for 45 min; (c) cells were fixed as in (a) but were permeabilized by immersion in 100% acetone at -20°C; and (d) cells were fixed and permeabilized by immersion in methanol containing 1% formalin at -15°C for 10 min (protocols 3 and 4 were used for indirect immunofluorescence only since the morphological preservation was too poor for immunogold EM).

In all cases, cells were washed in PBS, pH 7.4, after fixation and again after permeabilization and treated with 1 mg \times ml⁻¹ of sodium borohydride in PBS for 20 min to reduce free aldehydes. To block nonspecific binding of antibodies, cells were incubated in 1.0% BSA, 50 mM L-lysine, 0.01% sodium ethylmercurithiosalicylate (Thimerosal; Fluka Chemical Corp., Ronkonkoma, NY) and 0.025% saponin in PBS, pH 7.4. Cells were incubated with the primary and secondary antibodies in the blocking buffer

with five washes with PBS between incubations. Rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR) staining was performed according to the manufacturer's instructions on cells fixed and permeabilized according to protocol 3 (see above).

Membrane Preparations

Plasma membranes were isolated by the method of Clarke et al. (1988) from cells grown to a density of $2 \times 10^6 \times$ ml⁻¹. The final pellet of purified membranes was suspended in 10 mM Tes (N-tris[Hydroxymethyl]-methyl-2-aminoethane-sulfonic acid), pH 6.9. All experiments were performed on freshly prepared plasma membranes. To remove peripherally associated membrane proteins, purified plasma membranes were extracted with either 0.6 M KCl or 0.6 M KI as described by Miyata et al. (1989). Plasma membranes ($300 \mu\text{g} \times \text{ml}^{-1}$) were incubated with 0.6 M KI and 30 μM sodium thiosulfate in 10 mM Tes, pH 6.9, for 2 h on ice, or with 0.6 M KCl in 10 mM Tes, pH 6.9, containing 2 mM MgATP, 0.6 mM PMSF, and 2 mM DTT for 15 min at room temperature. The mixtures were pelleted at 150,000 g for 30 min and the pellets resuspended in 10 mM Tes, pH 6.9.

For myosin binding studies, KI- and KCl-extracted plasma membranes (100–300 μg) and 10–30 μg of myosin IA or 10–30 μg of myosin IB were mixed in 1 ml of buffer containing 0.1 M KCl, 2 mM MgATP, 1% BSA, 2 mM DTT, 0.6 mM PMSF, and 10 mM Tes, pH 7.0, and incubated for 15 min at room temperature. The mixtures were pelleted at 150,000 g for 30 min and the pellets resuspended in 10 mM Tes, pH 7.0, containing 0.05% glutaraldehyde. Native plasma membranes were fixed the same way. Immunogold labeling was then performed on the fixed membranes in an identical fashion to whole cells except that brief sonication (30 s in small bath sonicator, 60 Hz, 40 W; Heat Systems-Ultrasonics, Inc., Plainview, NY) was necessary to disperse the membranes after each pelleting to ensure that the entire membrane population was available to the primary and secondary antibodies.

Immunoprecipitation and Quantification of Myosins

Quantitative immunoprecipitations of myosin I isoforms from solubilized total amoeba proteins were performed using formalin-fixed, heat-treated *S. aureus* (Immuno-Precipitin; GIBCO BRL, Gaithersburg, MD) after the method of Kessler (1981), as modified by Hammer et al. (1984), except that the amoebae were not labeled with [³⁵S]methionine. For precipitation of myosins IA and IB, the antisera described in this paper were used; for precipitation of myosin IC, the antiserum described by Baines and Korn (1990) was used. Cell lysates for immunoprecipitation were prepared as recommended by Harlow and Lane (1988). Total cellular myosin IB and IC were most effectively solubilized by SDS-denaturation lysis (2% SDS, 50 mM Tris, pH 7.5) while myosin IA was more effectively extracted by RIPA-detergent lysis (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5). Total extraction of myosin I from residual insoluble cellular material was confirmed by immunoblotting. Before immunoprecipitation, SDS was removed by dialysis or the lysate was diluted 1:20 in PBS, pH 7.2, containing 1% BSA. Briefly, immunoprecipitation was performed as follows. The lysate was precleared by addition of preimmune serum to a final dilution of 1:5 followed by immunoprecipitation of the IgG by addition of an excess of *S. aureus* (Immuno-Precipitin stock solution at 500 mg \times ml⁻¹; 1.0 mg binds 10.7 μg IgG). Antibodies were then added to a final dilution of 1:10 which was found to be sufficient to immunoprecipitate all the myosin I. An excess of Immuno-Precipitin was added and was spun down through a 1 M sucrose cushion. The Immuno-Precipitin pellet was washed twice in PBS, pH 7.2, and resuspended in SDS-PAGE sample buffer. The method of Hammer et al. (1984) used two washes in high salt; this was found to remove a small proportion of the bound myosin I and was therefore avoided. After every wash and pelleting samples of supernatant were taken, concentrated by Amicon-30 centrifugation (Amicon Corp., Danvers, MA) (~10-fold), and tested for the presence of myosin I by immunoblotting to ensure that no myosin I had been lost. Myosins I were quantified by gel-scanning after SDS-PAGE. Purified myosins IA, IB, or IC were electrophoresed on SDS-PAGE in amounts of 0.1, 0.5, 1.0, or 2.0 μg per lane and scanned by densitometry to obtain a standard curve.

Quantification of Antibody Titer

Antibody titers were quantified by a solid phase antibody capture immunoassay after the protocol of Harlow and Lane (1988). Purified myosin I was bound to PVC in a 96-well microtiter plate (Falcon Plastics, Cockeysville, MD), antibody was added in a dilution series, and the captured antibody was quantified by binding of a second HRP-coupled anti-immunoglobulin antibody with 3',3',5',5'-tetramethylbenzidine as substrate.

Other Materials and Methods

SDS-PAGE was performed after the method of Laemmli (1970) and immunoblot analyses were performed according to Towbin et al. (1979). FITC-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-rabbit IgG were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Goat anti-rabbit IgG antibodies conjugated to gold particles were obtained from Janssen Life Sciences Products (Beerse, Belgium). All other chemicals were reagent grade.

Results

Antibody Characterization

Antimyosin IA diluted 1:8,000 ($4 \mu\text{g} \times \text{ml}^{-1}$ of total serum protein) detected $1 \mu\text{g}$ of myosin IA with the mid-point of the titration curve falling at dilutions of $\sim 1:2,000$ (data not shown). By immunoblot analysis against the purified enzymes, this antiserum, at dilutions of 1:800 or greater, recognized only myosin IA, but, at dilutions $< 1:500$, marginal cross-reactivity with myosins IB and IC was detected (Fig. 1, lanes a-c, anti-MIA). At 1:800 dilution, the antiserum detected only myosin IA in total cell extracts (Fig. 1, lane e).

Antimyosin IB detected $1 \mu\text{g}$ of myosin IB at dilutions $> 1:5,000$ ($2 \mu\text{g} \times \text{ml}^{-1}$ of IgG) with the midpoint of the titration curve falling at $\sim 1:1,750$ (data not shown). This antiserum recognized myosin IB with much higher affinity than myosin IC (Fig. 1, lanes a-c, anti-MIB), even though it was raised against an amino acid sequence adjacent to the actin-binding site of myosin IC. At 1:400 dilution, this antiserum detected both myosin IB and IC (data not shown) but only myosin IB was detected at antiserum dilutions greater than 1:500 (Fig. 1, lane f).

Indirect Immunofluorescence

Myosin IA was diffusely localized throughout the cell (Fig. 2 b). Through-focusing, however, revealed a slight increase in the fluorescent signal in a broad band beneath the plasma membrane corresponding to the cell cortex (Fig. 2 b, arrowheads). The plasma membrane did not appear to be specifically labeled. In contrast, myosin IB was highly concentrated at the plasma membrane (Fig. 2 d). Myosin IB was also associated with digestive vacuoles (Fig. 2 d, arrowhead), which were easily distinguished from contractile vacuoles both by EM and by double labeling with antibodies to myosin IC. Actin was present in all regions that contained myosin IA or IB (Fig. 2 i).

Because immunoblot analysis had demonstrated that high concentrations of antimyosin IB cross reacted with purified myosin IC (see previous section), the possibility that myosin IC was contributing to the fluorescence attributed to myosin IB was assessed by monitoring the fluorescence of the contractile vacuole which is known to contain a high concentration of myosin IC (Baines and Korn, 1990). The contractile vacuoles were labeled with antimyosin IB at dilutions $< 1:5$ but not with antiserum dilutions $> 1:20$; plasma membranes, however, were still fluorescent at antiserum dilutions $> 1:1,000$ (data not shown). Thus, over the range of antibody dilutions used in the current study (1:50 to 1:500), antimyosin IB would be expected to detect only myosin IB. In similar control experiments, antimyosin IA at dilutions of 1:20 to 1:1,000 did not label the plasma membrane or the contractile vacuole membrane confirming that, at the concentrations

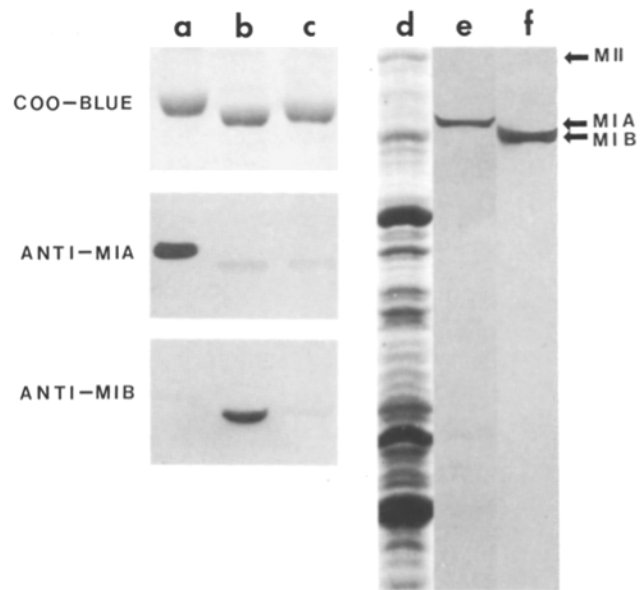


Figure 1. Characterization of antimyosin IA and antimyosin IB by immunoblotting against purified myosins and whole cell extracts. (Lanes a-c) SDS-PAGE of $2 \mu\text{g}$ of purified myosins IA (lane a), IB (lane b), and IC (lane c) stained with Coomassie blue (Coo-blue), immunoblotted with antimyosin IA diluted 1:500 ($66 \mu\text{g} \times \text{ml}^{-1}$ of total serum protein) (anti-MIA), and immunoblotted with antimyosin IB diluted 1:500 (anti-MIB). (Lanes d-f) SDS-PAGE of *Acanthamoeba* extract ($100 \mu\text{g}$ of protein) stained with Coomassie blue (lane d), immunoblotted with a 1:800 dilution of antimyosin IA (lane e), and immunoblotted with a 1:800 dilution of antimyosin IB (lane f).

used in this study, this antiserum did not detect either myosin IB or myosin IC.

The immunofluorescent staining patterns for myosin IA and IB observed in the current study are consistent with earlier localization studies (Gadasi and Korn, 1980; Miyata et al., 1989) even though the images differ slightly. Both earlier studies used antibodies which had significant cross-reactivity. For this reason, the fluorescent images for both myosin IA and IB showed association with the plasma membrane and the cytoplasm. In fact, quantitative immunogold cytochemistry performed in the present study (see below) has established that there are similar amounts of myosin IB and IA in the cytoplasm, although the fluorescent images in Fig. 2 give the impression that almost all myosin IB was associated with the plasma membrane. This is because myosin IB is ~ 60 -fold more concentrated at the plasma membrane than in the cytoplasm and the intensity of indirect immunofluorescence reflects relative concentrations, not absolute quantities.

Immunogold Electron Microscopy

The observations made by indirect immunofluorescence were confirmed and refined at the higher resolution obtainable by immunoelectron microscopy. Myosin IA was detected throughout the cell (Fig. 3 a). While gold particles were more concentrated at the plasma membrane, the major portion of gold was cytoplasmic (Table I) with a slightly higher concentration of myosin IA in the cell cortex (data not shown). Myosin IA was also associated with small cytoplas-

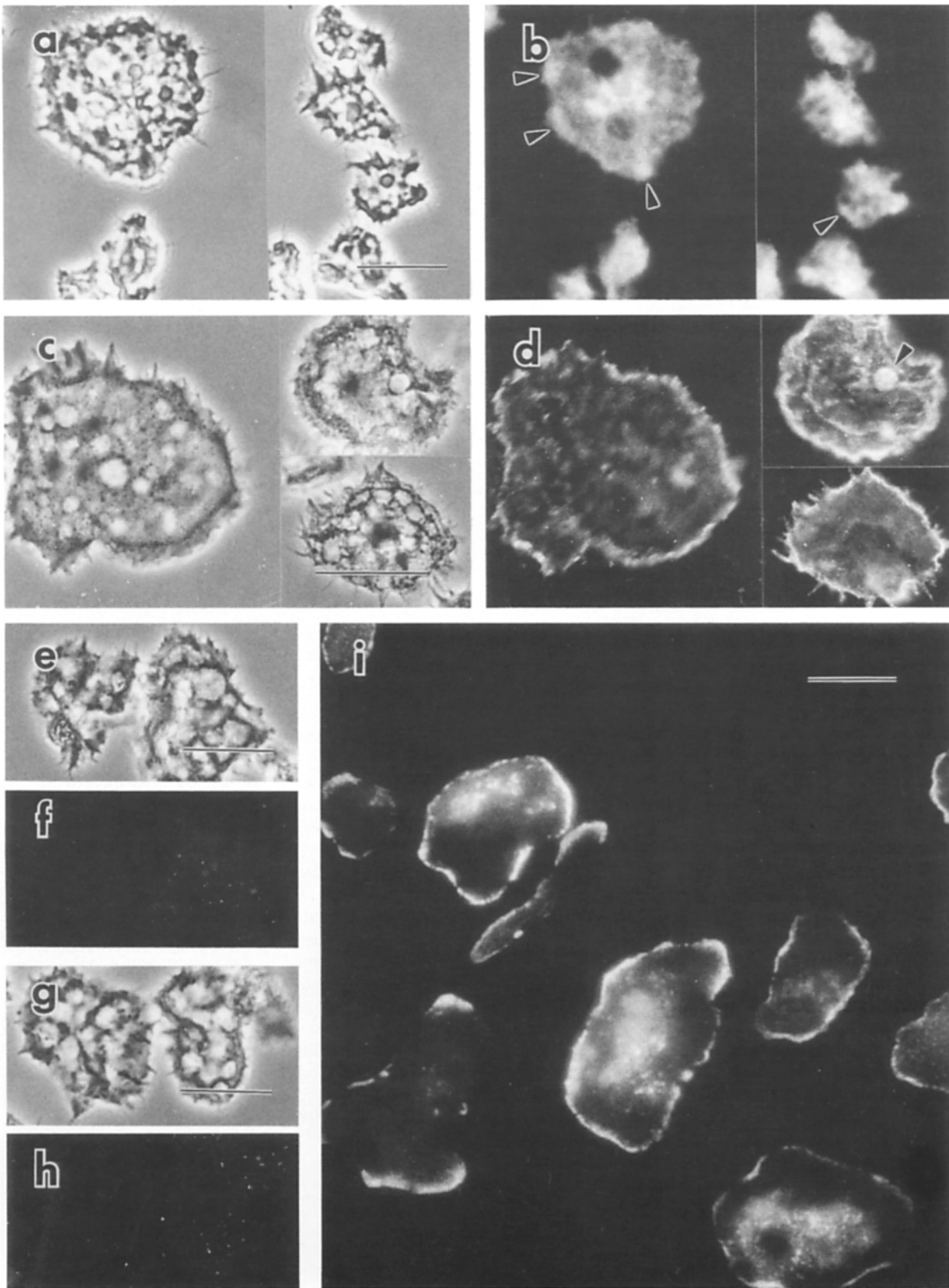


Figure 2. Indirect immunofluorescence localization of myosin IA and IB in vegetative *Acanthamoeba*. Phase contrast (*a*, *c*, *e*, and *g*) and fluorescence (*b*, *d*, *f*, *h*, and *i*) micrographs of cells stained with either antimyosin IA (1:50 dilution) (*a* and *b*), antimyosin IB (1:50 dilution) (*c* and *d*), antimyosin IA preimmune serum (1:50 dilution) (*e* and *f*), or antimyosin IB preimmune serum (1:50 dilution) (*g* and *h*) and FITC-coupled goat anti-rabbit IgG. Rhodamine-phalloidin labeling of cells has also been included to show the distribution of F-actin (*i*).

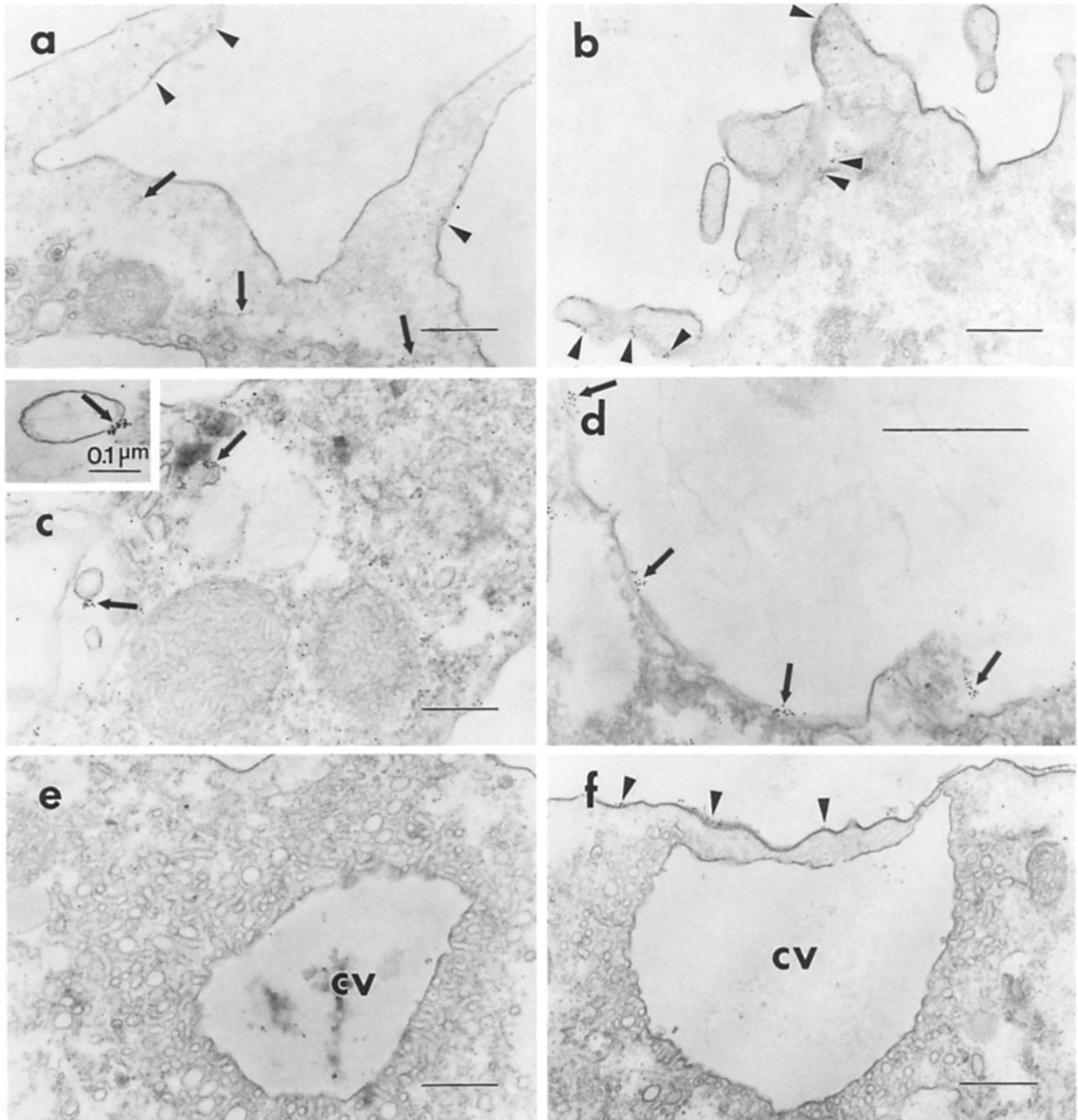


Figure 3. Immunogold electron microscopic localization of myosin IA and myosin IB. Saponin-permeabilized cells (protocol 1, see Materials and Methods) were stained with antimyosin IA (1:50 dilution) (*a*, *c*, and *e*) or antimyosin IB (1:50 dilution) (*b*, *d*, and *f*) followed by goat anti-rabbit IgG coupled to 5-nm gold. (*a* and *b*) Thin sections of cell cortex and plasma membrane stained with antimyosin IA (*a*) or antimyosin IB (*b*); myosin IA was present in the cell cortex (*a*, arrows); and associated with the plasma membrane (*a*, arrowheads); myosin IB was associated only with the plasma membrane (*b*, arrowheads) and was excluded from the cortex. (*c*) Association of myosin IA with small cytoplasmic vesicles (arrows, see also open arrow in Fig. 4 *e*). (*d*) Association of myosin IB with the membrane of a large internal vacuole (arrows). (*e* and *f*) Lack of any association of myosin IA (*e*) or myosin IB (*f*, arrowheads identify plasma membrane labeling) with the membrane of the contractile vacuole (cv). Bars, 0.5 μ m.

Fixation and permeabilization followed protocol 4 for myosin I and protocol 3 for actin (see Materials and Methods). Fluorescence due to myosin IA was diffuse throughout the cytoplasm with local concentrations in the cell cortex (*b*, arrowheads) while fluorescence due to myosin IB followed the cell outline, including acanthopods and filopodia, indicative of close association with the plasma membrane. Occasional digestive vacuoles were also stained (*d*, arrowhead). Bars, 25 μ m.

Table I. Immunoelectron Microscopic Localization of Myosin I Isoforms

Compartment	Myosin IA	Myosin IB	Myosin IC*
Plasma membrane			
Particles/ μm (preimmune)	3.0 ± 1.6 (0.03)	15.4 ± 3.3 (0.08)	5.2 ± 0.8 (0.001)
Particles/cell	1×10^5	5.3×10^5	1.8×10^5
% on outer surface	30	81	70
Large vacuole membrane			
Particles/ μm (preimmune)	0 (0)	22.6 ± 8.5 (0)	7.4 ± 7.0 (0)
Particles/cell	0	6×10^5	2×10^5
Contractile vacuole membrane			
Particles/ μm (preimmune)	0 (0)	0 (0)	3.0 ± 0.4 (0)
Particles/cell	0	0	4.7×10^3
Cytoplasm			
Particles/ μm^2 (preimmune)	44 ± 17 (2.5 ± 1.7)	11 ± 4.0 (4.0 ± 2.0)	6.3 ± 5.1 (5.5 ± 1.7)
Particles/cell	1.4×10^6	2.4×10^5	2.7×10^4

These data were derived from measurements performed on a minimum of 10 cells taken from three different preparations of which those in Fig. 3 are illustrative. For statistical analysis a mean was calculated for each cell; the values reported are means of means ($\pm \sigma_n$). Gold particles falling within a 10-nm zone on either side of the middle of a membrane bilayer were included as part of the membrane compartment. The number of gold particles per μm of membrane or μm^2 of cytoplasm (after correcting for the amount of labeling, if any, by preimmune sera) were converted to total number of particles in the cell using morphometric data and assuming a constant section thickness of 75 nm. The values for one cell from Bowers et al. (1981) are: plasma membrane area, $2,590 \mu\text{m}^2$, cytoplasmic volume, $2,540 \mu\text{m}^3$ and large vacuolar membrane system area, $2,032 \mu\text{m}^2$. A contractile vacuole membrane area of $118 \mu\text{m}^2$ was calculated from diameters measured in the current study assuming that fully filled vacuoles were spherical.

* Data from Baines and Korn (1990).

mic vesicles with diameters $<1 \mu\text{m}$ (Fig. 3 c). Typically, the gold particles formed rod-shaped clusters perpendicular or parallel to the membranes of the small vesicles (Fig. 3 c, arrows).

Again in agreement with the immunofluorescence data, myosin IB was predominantly but not exclusively associated with the plasma membrane at the higher resolution of immunoelectron microscopy (Fig. 3 b; Table I). Myosin IB was not associated with small cytoplasmic vesicles but was associated with the membranes of some but not all large interval vacuoles with diameters $>1 \mu\text{m}$ (Fig. 3 d). Those vacuole membranes that were labeled had $\sim 50\%$ more particles per $1 \mu\text{m}$ than the plasma membrane (Table I). The contractile vacuole was not labeled by antibodies to either myosin IA (Fig. 3 e) or IB (Fig. 3 f).

Table II. Cell Distributions of Acanthamoeba Myosin I Isoforms

Compartment	IA	IB	IC
	Total amount of isoform, ng/ 10^6 cells (pmol/ 10^6 cells)		
	37 (0.24)	176 (1.2)	50 (0.32)
Percent of total isoform in cell			
Plasma membrane	7	39	44
Large vacuole membrane	0	44	49
Contractile vacuole membrane	0	0	1
Cytoplasm	93	17	6
Percent of total myosin in compartment			
Plasma membrane	3	73	24
Large vacuole membrane	0	76	24
Contractile vacuole membrane	0	0	100
Cytoplasm	51	44	5

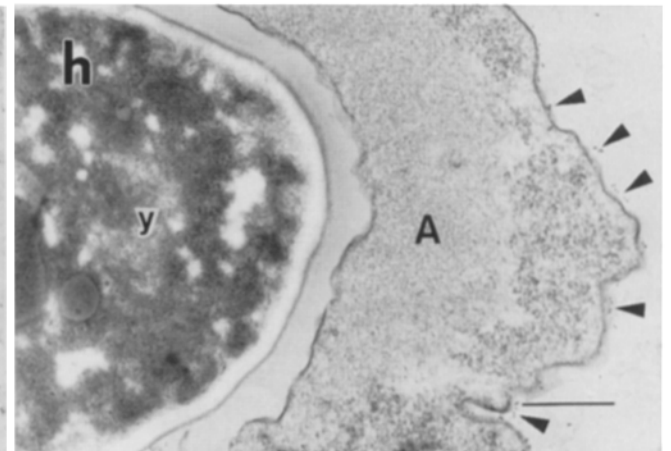
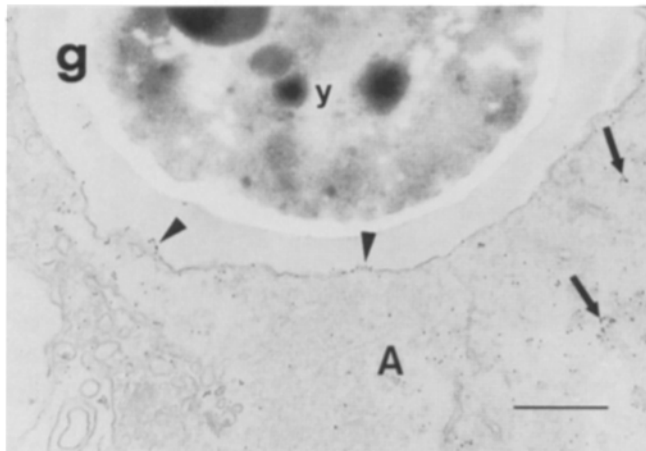
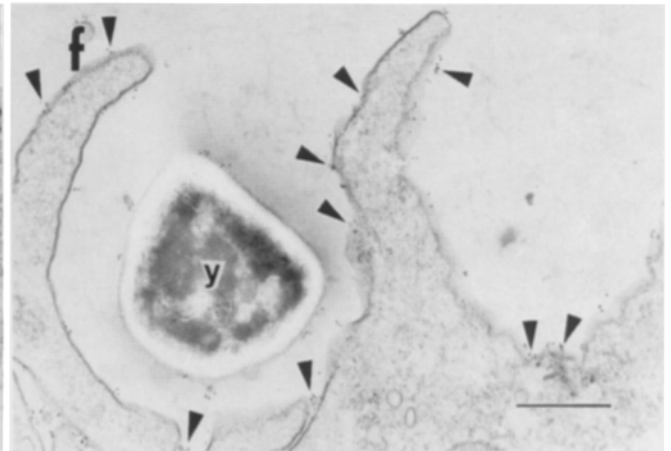
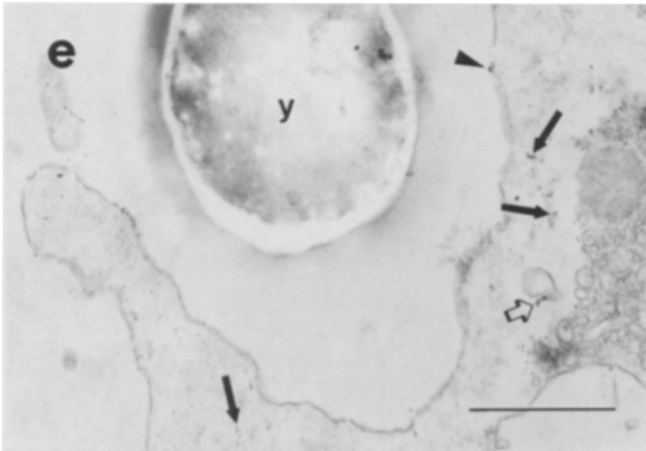
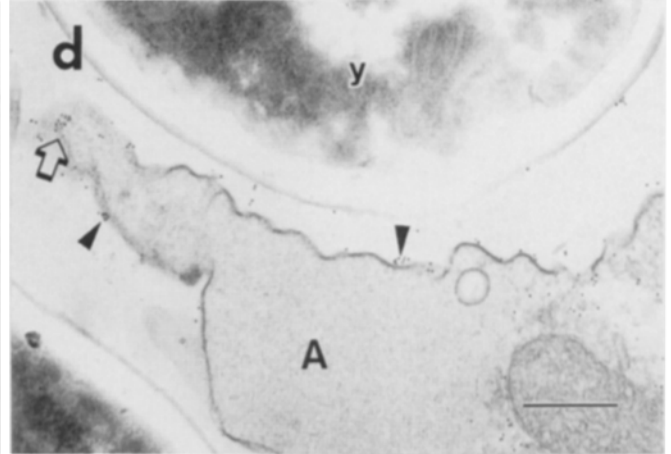
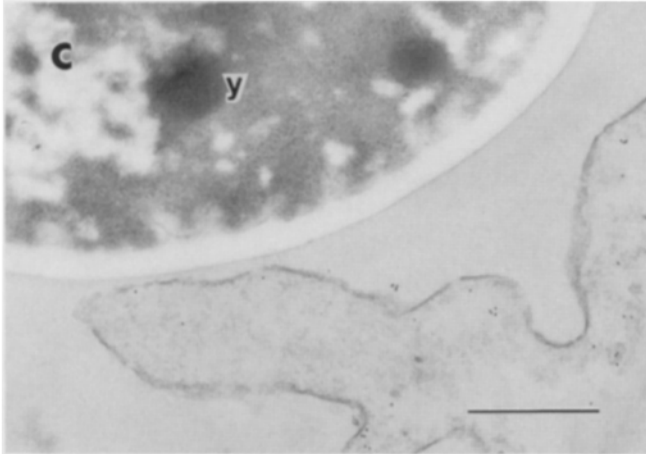
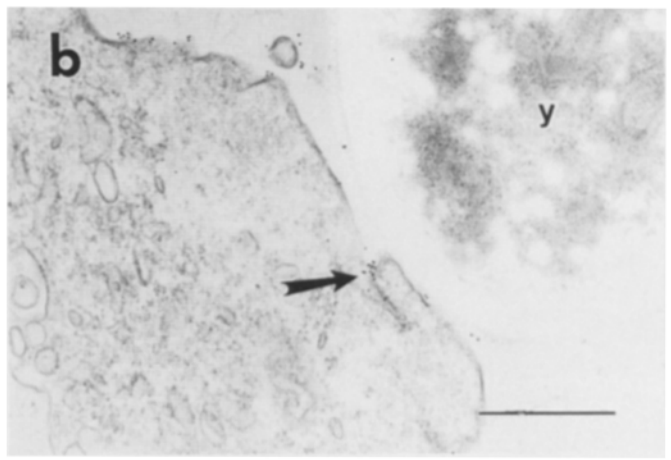
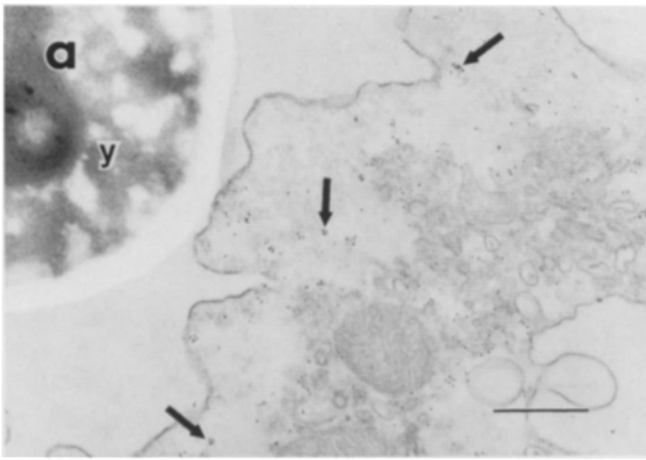
The total amount of each isoform was determined by quantitative immunoprecipitation of each isoform from a solution of total cell proteins by isoform-specific antibodies and quantitative immunoblotting following SDS-PAGE (see Materials and Methods for details). The percent distributions of each isoform among the cell compartments were calculated from the data in Table I. The percentage contribution of each isoform to the total myosin I within each compartment could then be calculated from the two previous data sets.

Quantification of Myosin I Isoform Distributions

To quantify the amount of each myosin I isoform associated with each cell compartment, it was necessary first to determine the amount of each isoform in the cell. This was done using immunoprecipitations of total cell proteins by excess antibodies to separate, and scanning of Coomassie blue-stained SDS-PAGE gels to quantify, the three myosin I isoforms (see Materials and Methods). The amounts of each isoform are shown in Table II. The total amount of the three myosin I isoforms was $263 \text{ ng}/10^6$ cells ($1.7 \text{ pmol}/10^6$ cells assuming an average molecular mass for myosins I of 155 kD) or $\sim 0.27\%$ of the total cell protein, in reasonable agreement with the values of $291 \text{ ng}/10^6$ cells ($1.9 \text{ pmol}/10^6$ cells) and 0.2% of total cell protein estimated from the $\text{K}^+\text{EDTA-ATPase}$ activity of a whole cell lysate (see Lynch et al., 1991).

From these data and the data in Table I, the myosin I composition of each compartment could be calculated (Table II). Myosin IA occurs almost entirely in the cytoplasm, however, because there is much more myosin IB than IA in the cell, the relatively small amount of total myosin IB that is in the cytoplasm accounts for a substantial fraction of the total cy-

Figure 4. Immunogold electron microscopic localization of myosin IA and myosin IB in phagocytosing *Acanthamoeba*. Saponin-permeabilized amoebae (protocol 1) that had phagocytosed yeast for 2 min were labeled with either antimyosin IA diluted 1:50 (a, c, e, and g) or antimyosin IB diluted 1:50 (b, d, f, and h) followed by goat anti-rabbit IgG coupled to 5-nm gold. (a and b) Thin sections of initial contacts formed between yeast (y) and the cell surface labeled with antimyosin IA (a) or antimyosin IB (b). Although myosin IA was present in clusters in the cortex in the region of contact (a, arrows), no specific associations within this region were observed. In contrast, myosin IB was seen to be accumulated at the precise site of contact (b, arrow). (c and d) Thin section of pseudopods extending to engulf yeast. Myosin IB was concentrated at the tips of the pseudopods (d, open arrow) and was associated with the nascent phagocytic cup membrane (d, arrowheads) whereas there was no discernible increase in the amount of myosin IA in these regions (c). (e) Phagocytic cups were associated with clusters of myosin IA in the adjacent cortex (closed arrows), the plasma membrane (arrowhead) and attached to small vesicles (open arrow). (f) Myosin IB can be seen at the plasma membrane of two adjacent phagocytic cups (f, arrowheads) (the yeast was lost from one of the cups during preparation). (g and h) Nascent phagosomes had myosin IA associated with the phagosome membrane (g, arrowheads) and in the adjacent actin-rich region (g, arrows), while myosin IB was absent from the actin-rich region (h, A) and the phagosome membrane (h) but associated with the plasma membrane (h, arrowheads). Bars: (a-d and f-h) $0.5 \mu\text{m}$; (e) $1.0 \mu\text{m}$.



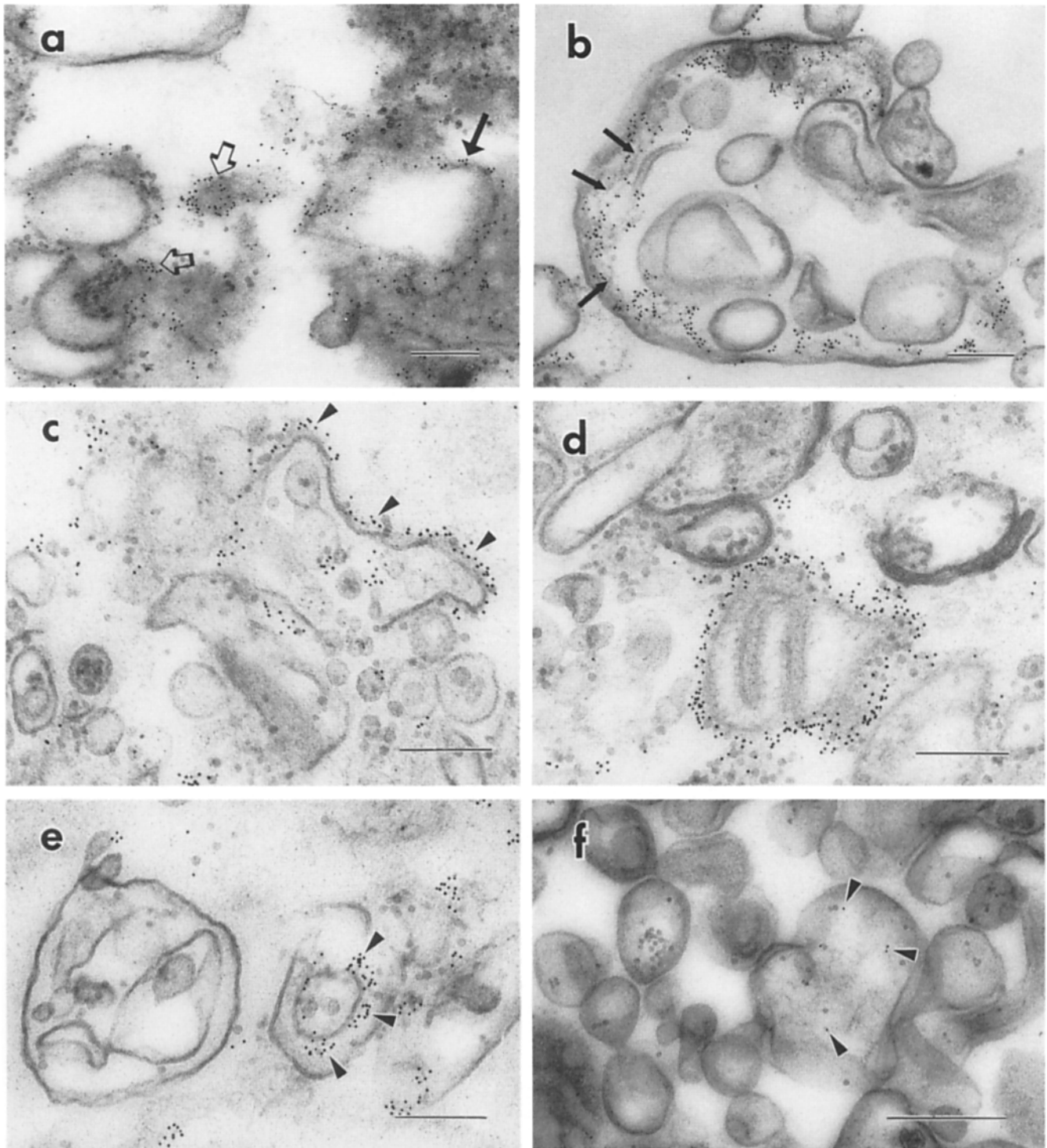


Figure 5. Immunogold electron microscopic localization of myosin IA and IB in purified native plasma membranes and reconstituted membranes. (*a* and *b*) Purified membranes labeled with antimyosin IA or antimyosin IB at 1:50 dilution followed by anti-rabbit IgG coupled to 5-nm gold (permeabilization was not necessary). Myosin IA was associated with small vesicles (*a*, open arrows) and short membrane sheets (*a*, closed arrow) while myosin IB was associated with large sheets of membranes (*b*). In all cases only one side of the membrane was labeled. (*c–e*) Purified membranes were extracted with 0.6 M KI, incubated with either purified myosin IA (*c* and *e*) or myosin IB (*d*) at a concentration of 0.45 nmol of myosin I per mg of membrane protein, washed and labeled with either antimyosin IA or antimyosin IB. Exogenous myosin IA bound to one side of a subpopulation of membranes (*c*, arrowheads) and vesicles, which could lie within unlabeled vesicles (*e*, arrowheads). Exogenous myosin IB bound to one side of a subset of membranes (*d*). (*f*) Purified membranes labeled with preimmune serum showing random gold particles (arrowheads) that were not associated with the membranes. Bars: (*a–e*) 0.2 μm ; (*f*) 0.5 μm .

toplasmic myosin I. Myosins IB and IC are largely localized to the plasma membrane and large vacuolar membrane system, and account for almost all of the myosin I in that membrane system; myosin IB is the dominant isoform in this compartment. A small fraction of the total myosin IC accounts for all of the myosin I in the contractile vacuole membrane.

The quantitative data in Table II do not reveal some features that were discernible from careful examination of the immunoelectron micrographs. For example, a much smaller fraction of the gold particles representing myosin IA was on the outer surface of the plasma membrane than those representing myosins IB and IC (Table I). As discussed previously (Baines and Korn, 1990), the appearance of gold particles on the outer cell surface of permeabilized cells (the enzymes are not detectable in nonpermeabilized cells) probably reflects their redistribution due to the disruption of the lipid bilayer by the permeabilizing agent. That myosin IA remains predominantly on the inner plasma membrane surface during permeabilization suggests that it may be more internally localized than myosins IB and IC and possibly not directly associated with the plasma membrane. This interpretation is supported by the fact that, although higher concentrations of saponin extract most of the myosin IC (Baines and Korn, 1990) and IB (data not shown) from the cells, myosin IA remains largely in place (data not shown). Myosin I heavy chain kinase, which is predominantly cytoplasmic, is also stable to saponin extraction (Kulesza-Lipka et al., 1991).

Phagocytosis

Three stages of phagocytosis were investigated: (a) initial contact between the ingested particle and the plasma membrane (Fig. 4, a and b); (b) formation of the phagocytic cup (Fig. 4, c-f); and (c) completion of phagocytosis and the formation of an internal phagosome (Fig. 4, g and h). Myosin IA did not appear to be specifically involved at any of these stages (Fig. 4, a, c, e, and g) with the possible exception of a slight increase in concentration of myosin IA in the cell cortex just beneath the phagocytic cup (Fig. 4 e) and perhaps a twofold increase in concentration in the membrane of the phagocytic cup compared with other regions of the plasma membrane (data not shown). Myosin IB, on the other hand, was associated with the membrane at all three stages of phagocytosis (Fig. 4, b, d, and f) with at least a twofold increase in concentration relative to other regions of the plasma membrane at the tips of extending pseudopods (Fig. 4 h) and at the site of first contact between the plasma membrane and the object to be phagocytosed (Fig. 4 b). Some phagocytic cups and phagosomes, however, had no membrane-associated myosin IB (Fig. 4 h).

Immunogold Cytochemistry of Purified Membranes

To investigate further the nature of the membrane association of myosins I, immunogold cytochemistry was performed on samples of purified *Acanthamoeba* membranes. Antimyosin IA and antimyosin IB appeared to label different subpopulations of membranes. Antimyosin IA was associated with small membrane vesicles (Fig. 5 a) that accounted for ~9% of the total membrane profile (Table III) while antimyosin IB labeled predominantly large sheets of membrane (Fig. 5 b) that accounted for ~30% of the membrane profile (Table

Table III. Distributions of Myosin IA and IB in Isolated *Acanthamoeba* Plasma Membranes and of Myosin IA and IB Added to KI-extracted Membranes

	% Membrane labeled	Particles/ μm
Myosin IA		
As isolated	8.5	21.5 \pm 7.3
Re-bound	12.5	83.4 \pm 24.2
Myosin IB		
As isolated	31.0	31.8 \pm 13.2
Re-bound	39.0	123.7 \pm 29.7

These data were derived from measurements made on a minimum of 100–200 μm of membrane from two different preparations of which those in Fig. 5 are representative. Each section was treated as a separate sample and a mean of means calculated.

III). The density of labeling of those membrane profiles that were labeled was somewhat higher for myosin IB than for myosin IA (Table III). Both antibodies labeled only the cytoplasmic surface of the membranes, identified by the presence of associated actin filaments (the amorphous material on the labeled side of the membranes in Fig. 5 b, arrows). It should be emphasized that the identification of different subpopulations of membranes by morphological criteria is unreliable since all the membranes look similar (except contractile vacuole derived vesicles, see Clarke et al., 1988) and, therefore, the main evidence that myosins IA and IB were associated with different populations of membranes was that myosin IB labeled at least threefold more membrane (see Table III).

To investigate further the apparent differential localizations of myosins IA and IB, isolated membranes were depleted of endogenous myosin I (and other extrinsically associated membrane proteins) by treatment with 0.6 M KI (Miyata et al., 1989) which removed ~50% of the myosin I and ~85% of the actin. Highly purified myosin IA or IB was added to the extracted membranes at concentrations sufficient to give half-maximal binding (0.45 nmol \times mg⁻¹ of membrane protein) (see Miyata et al., 1989) and the samples were processed for immunogold cytochemistry using either antimyosin IA or antimyosin IB to detect the bound myosin I. The exogenously added myosins IA and IB appeared to bind to the same subpopulations of membranes with which their endogenous counterparts had been associated (Fig. 5, c-e; Table III). The exogenous myosin IA and IB also were bound only to one side of the membranes (Fig. 5, c-e), presumably the original cytoplasmic side. Very similar results were obtained when the membranes were extracted with 0.6 M KCl, which removed up to 80% of the endogenous myosin I (data not shown; Miyata et al., 1989). When myosin I was added to intact cells, no binding was detectable (data not shown), consistent with the observation that myosin I reassociates only with the cytoplasmic surface of salt-stripped membranes.

The purified membranes used for these studies were prepared as described by Clarke et al. (1988). Although the membranes shown in Fig. 5 appear to be less homogeneous than those obtained by Clarke et al. (1988) and Miyata et al. (1989), they were actually indistinguishable before immunogold labeling (data not shown). However, immunogold labeling involves addition of substantial amounts of protein (1% BSA as a blocking agent as well as the antibodies) and

repeated brief sonications to disperse membrane pellets after each wash (see Materials and Methods) which make the final material appear to be less homogeneous than the initial purified membrane preparation.

Discussion

The data presented in this paper establish the differential localizations of the three known isoforms of myosin I in *Acanthamoeba*. Myosin IA occurs almost exclusively in the cytoplasm where it accounts for ~50% of the total myosin I and is also associated with small cytoplasmic vesicles. Myosin IB is the predominant isoform associated with the plasma membrane and large vacuole membranes and accounts for almost half of the total myosin I in the cytoplasm. Myosin IB, however, is absent from the actin-rich cortical region immediately beneath the plasma membrane, the region where myosin IA is most abundant. Myosin IC accounts for a substantial fraction of the total myosin I associated with the plasma membrane and large vacuole membranes and is the only myosin I isoform associated with the contractile vacuole membrane. From previous work, it is known that 30% of the total myosin I heavy chain kinase is bound to the plasma membrane and 70% is cytoplasmic (Kulesza-Lipka et al., 1991) and that 100% of the myosin II is cytoplasmic (Baines and Korn, 1990).

The calculations on which the quantification depends assume equal access of all antibodies to all compartments, equal retention of all myosin isoforms during permeabilization and equal binding of antibodies to the myosin in each compartment. The reasonableness of these assumptions, in general, was supported by multiple experiments with each antibody using mild, intermediate and extensive permeabilization (Baines and Korn, 1990, and data not shown). If anything, myosins IB and IC were more readily extracted from the plasma membrane compartment than myosin IA which would lead to an overestimate of the amount of myosin IA in the plasma membrane; even so, myosin IA is the least abundant of the three isoforms in this compartment.

Also, as shown in this paper, both antimyosin IA and antimyosin IB labeled purified membranes *in vitro*. Control experiments (not shown) established that antimyosin IB bound to myosin IB that was cross-linking actin filaments *in vitro* with a molar ratio of bound antibody/myosin IB of 2:1. Thus, the relatively small amounts of myosin IA detected in the plasma membrane compartment and of myosin IB detected in the cell cortex did not result from an inability of the respective antibodies to bind to myosin in these compartments.

The presence of myosin IA associated with the cytoplasmic surface of plasma membranes and small cytoplasmic vesicles suggests a relationship between these two membrane systems. Myosin IA might possibly have a role in pinching off small vesicles from the plasma membrane and/or be responsible for the transport of these small vesicles to and/or from the plasma membrane. A somewhat similar association of brush border myosin I with small cytoplasmic vesicles in undifferentiated enterocytes of adult chickens has been reported (Drenckhahn and Dermietzel, 1988). In addition, the high concentration of myosin IA in the cytoplasmic cortex suggests that it may be the major myosin I isoform mediating cortical contraction.

On the other hand, the high concentration of myosin IB in plasma membranes and at the tips of advancing pseudopods suggests that it may be the primary isoform responsible for myosin I-mediated surface extensions. As the membranes of large internal vacuoles and phagocytic vesicles are derived from the plasma membrane, it is not surprising that myosin IB has a similar concentration in all these membranes. However, not all internal vacuoles nor all phagocytic cups were labeled by antibodies to myosin IB. It is possible, therefore, that myosin IB is only transiently associated with the membrane of phagocytic cups, phagosomes, and internal vacuoles, for example perhaps during their formation from the plasma membrane, resulting in populations both with and without myosin I.

Myosin IC, although present in the plasma membrane, is not associated with the membranes of phagocytic structures (Baines, I. C., and E. D. Korn, unpublished observations). As previously documented (Baines and Korn, 1990), its specific association with the contractile vacuole, which contains neither myosin IA nor IB, implies a specific role for myosin IC in the functioning of this organelle.

The specific associations of myosins IA and IB with subpopulations of membranes in the isolated plasma membranes is consistent with their specific associations with different cytoplasmic membrane systems (each probably derived from the plasma membrane) *in situ*. Moreover, whereas myosin IB is rather uniformly distributed in the plasma membrane (see Figs. 3 *f* and 4, *f* and *h*), myosin IA (Fig. 3 *a*) and myosin IC (Baines and Korn, 1990) occur in clusters. This is consistent with myosin IB being associated with the membranes of large internal vacuoles while myosin IA is associated with much smaller cytoplasmic vesicles that might be related to small, specialized regions of the plasma membrane.

It is instructive that only the cytoplasmic surface of isolated plasma membranes was labeled by antimyosin IA and antimyosin IB, that exogenously added myosins IA and IB bound only to one side of KI-extracted membranes and that the endogenous and exogenously added myosins IA and IB seemed to be specifically associated with the same subpopulations of membranes. These observations suggest that specific membrane proteins may be involved in the targeting of each myosin I isoform to specific membrane domains even though, as shown by Adams and Pollard (1989), myosin I can bind to vesicles of acidic phospholipids *in vitro*. The putative myosin I-binding proteins are likely to be integral membrane proteins as they were resistant to extraction by KI.

We thank Dr. Thomas Lynch for providing the antimyosin IA, Mr. Thomas Olszewski for culturing cells on glass and plastic substrates, and Dr. Blair Bowers for advice on microscopy.

Received for publication 21 April 1992 and in revised form 1 July 1992.

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