Localization of Myosin IC and Myosin II in *Acanthamoeba castellanii* by Indirect Immunofluorescence and Immunogold Electron Microscopy

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Abstract. Polyclonal antisera have been raised against purified *Acanthamoeba* myosin II and to a synthetic 26 amino acid peptide that corresponds in sequence to the phosphorylation site of *Acanthamoeba* myosin IC. These antisera are specific for their respective antigens as determined by immunoblotting after SDS-PAGE of total cell lysates. By using the antisera, localization studies were performed by indirect immunofluorescence and by immunogold electron microscopy. Myosin II occurred in the cell cytoplasm and appeared to be concentrated in the cortex. Immunogold cytochemistry revealed at high resolution that myosin II is organized into rodlike filaments ~200 nm long. The antibody raised against the myosin IC synthetic peptide recognized both the plasma membrane and the membrane of the contractile vacuole. The plasma membrane staining was labile to treatment with saponin suggesting an intimate association of the myosin IC with membrane phospholipids. Immunogold cytochemistry with the antimyosin IC synthetic peptide showed that the myosin IC is closely associated with the membrane bilayer.

At least two distinct classes of myosin are contained in *Acanthamoeba castellanii* (see Korn et al., 1988, for review). Conventional myosin, also termed myosin II, consists of two identical heavy chains (175 kD) and two pairs of light chains (17.5 and 17 kD). The carboxyl-terminal halves of the heavy chains are associated to form a rodlike α-helical coiled-coil and the amino-terminal portions form two globular head domains. Assembly of myosin II into bipolar filaments is a property of the tail region of the molecule; the actin-activated Mg-ATPase activity is attributable to the globular head domains and is inhibited when three serine residues on each heavy chain are phosphorylated. Myosin I was first isolated from *Acanthamoeba* in 1973 (Pollard and Korn, 1973) and two isoforms of this enzyme were described in 1979 (Maruta et al., 1979). Myosins of class I display actin-activated Mg-ATPase activity which is dependent on the phosphorylation of a single serine (myosin IB) or threonine (myosin IA), located near the center of the subfragment 1–like domain of the molecule (Brzeska et al., 1989). Myosins I are monomeric, globular proteins that lack the α-helical tail present in myosins II and that can not self-assemble to form any higher oligomers under physiological conditions (Albanesi et al., 1985).

Myosin IA (140-kD heavy chain; 17-kD light chain) and IB (125-kD heavy chain; 27-kD light chain) have been localized in *Acanthamoeba* and compared to the distribution of myosin II (Gadasi and Korn, 1980; Hagen et al., 1986). Myosins I were found to be preferentially distributed near the plasma membrane while myosin II was located in the cytoplasm. The plasma membrane association of *Acanthamoeba* myosins I has been further characterized in two recent studies. Adams and Pollard (1989) showed that myosin I will bind to NaOH-extracted membranes isolated from *Acanthamoeba* and to pure lipids with an affinity sufficient for extensive binding in the cell. Miyata et al. (1989) have shown that myosin I copurifies with plasma membrane from *Acanthamoeba* and that this association can not be accounted for by binding of the myosin I to membrane-bound actin. Fukui et al. (1989) have localized myosin I, myosin II, and F-actin in *Dictyostelium* by indirect immunofluorescence. Myosin I was found at the leading edge of cells during directed chemotaxis and phagocytosis and of cells undergoing cytokinesis, while myosin II was localized in the posterior region of the cell cytoplasm of locomoting cells and in the contractile ring of dividing cells.

A third isoform of myosin I has recently been purified from *Acanthamoeba* and characterized (Lynch et al., 1989). Myosin IC has a single 130-kD heavy chain and a pair of 14-kD light chains. The myosin IC gene has been sequenced (Jung et al., 1987; misidentified in the original paper as the gene sequence for myosin IB, see Brzeska et al., 1989) and the phosphorylation site identified (Brzeska et al., 1989). The present study is concerned with the localization of myosin IC and comparison of its distribution with that of myosin II. To this end, an antibody has been raised against a synthetic 26 amino acid peptide that corresponds in sequence to the phosphorylation site of myosin IC: Tyr-Arg-Thr-Ile-Thr-Thr-Gly-Glu-Gln-Gly-Arg-Gly-Arg-Ser-Ser*-Val-Tyr-Ser-Cys-Pro-Gln-Asp-Pro-Leu-Gly-Ala, where Ser* is the phosphorylatable residue (Brzeska et al., 1989). Localization of myosin

IC and myosin II has been performed at both the level of the light microscope by indirect immunofluorescence and at the level of the electron microscope by immunogold cytochemistry. This is the first report of high resolution immunogold localization of myosin II in *Acanthamoeba* and of any myosin I in any cell.

Materials and Methods

Acanthamoeba castellanii (Neff strain) was grown in 1-liter culture flasks to a density of 10^6 cells ml^{-1}, as described by Pollard and Korn (1973). Alternatively, cells were grown on a plastic substrate in 750-ml culture flasks (Falcon Plastics, 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 observed to be much flatter than cells grown in suspension thus improving the resolution obtainable by immunofluorescence studies.

Two rabbit polyclonal antisera (C27073 and C27452) were raised against purified *Acanthamoeba* myosin II (purified by the method of Collins and Korn, 1981) and a rabbit polyclonal antiserum was raised against a synthetic 26 amino acid peptide corresponding to the phosphorylation site of myosin IC (Brzeska et al., 1989). The synthetic peptide was coupled to keyhole limpet hemocyanin (Calbiochem-Behring Corp., San Diego, CA) by a two-step glutaraldehyde coupling protocol (Harlow and Lane, 1988) and the conjugated protein-peptide was mixed 1:1 with Freund's complete adjuvant before immunization. Purified myosin II was subjected to SDS-PAGE, the heavy chain was excised from the gel after visualization in 4 M sodium acetate (Harlow and Lane, 1988) and homogenized into Freund's complete adjuvant (Difco Laboratories Inc., Detroit, MI). Female New Zealand white rabbits were primed at the age of 6 mo and received a minimum of two boosts with at least a 4-wk interval between injections. Antisera were collected 10–12 d after the final immunization. Affinity purifications of antisera were performed by adsorption to and elution from antigen bound to nitrocellulose strips (Smith and Fisher, 1984).

SDS-PAGE was performed by the method of Laemmli (1970) and immunoblot analyses were performed according to Towbin et al. (1979). FITC-conjugated goat anti-rabbit IgG and horseradish peroxidase-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). Myosins IA, IB, and IC were purified according to Lynch et al. (1990). All other chemicals were reagent grade.

Immunolocalization of Myosin IC and Myosin II

Immunolocalization was performed both at the level of resolution provided by the light microscope and the electron microscope. Identical fixation and permeabilization protocols were used for indirect immunofluorescence and preembedding immunogold electron microscopy. Fixation was with 3% formaldehyde and 0.25% glutaraldehyde in *Acanthamoeba* growth medium for 45 min at room temperature. Permeabilization was either with 0.1% saponin (Sigma Chemical Co., St. Louis, MO) for 10 min at room temperature (light permeabilization), 0.2% saponin for 15 min at room temperature (intermediate permeabilization), or 0.5% saponin for 45 min at room temperature (extensive permeabilization). Alternatively, simultaneous fixation and permeabilization was performed using 0.05% saponin, 0.05% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA), and 1.0% formaldehyde (Fisher Scientific Co., Fair Lawn, NJ) for 5 min at room temperature, followed by further fixation in 0.05% glutaraldehyde and 1.0% formaldehyde for 30 min. As a control for saponin permeabilization, cells were also permeabilized using 100% acetone at −20°C for 3 min after aldehyde fixation. Cells were washed twice in PBS, pH 7.4, between fixation and permeabilization, and after permeabilization. To reduce free aldehydes, cells were treated with sodium borohydride, 1 $mg \cdot ml^{-1}$ in PBS, for 10 min. After washing the cells twice in PBS, they were incubated in a buffer (blocking buffer: 1.0% BSA and 50 mM L-lysine in PBS, pH 7.4) designed to block nonspecific binding of antibodies. Incubations with primary antibodies (diluted 1:100 in blocking buffer to give a final concentration of 0.35 $mg \cdot ml^{-1}$ rabbit serum proteins) and with secondary FITC-conjugated antibodies (diluted 1:100 in blocking buffer to a final immunoglobulin protein concentration of 10 $\mu g \cdot ml^{-1}$) were for 1 h at 37°C. Incubations with the secondary gold-conjugated antibodies were either for 2 h at 37°C or overnight at 4°C. The cells were washed five times in PBS between antibody incubations and after the second antibody incubation.

For indirect immunofluorescence, cells were mounted in 50% glycerol in PBS and viewed with a Zeiss ICM 405 microscope equipped with phase-contrast and epifluorescence optics. For immunogold electron microscopy, cells were fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated through an alcohol series, and embedded in epon 812 (Electron Microscopy Sciences). Both fixatives were in 0.1 M phosphate buffer, pH 6.8. Gold or silver sections were cut with a diamond knife (Delaware Diamond Knives, Inc., Wilmington, DE) on an ultramicrotome (model MT 5000; Sorvall Instruments, Newton, CT) and viewed with a Philips LS410 electron microscope.

Results

Antibodies

The rabbit polyclonal antiserum raised against the 26 amino acid synthetic peptide corresponding to the phosphorylation site of *Acanthamoeba* myosin IC was clearly specific for myosin IC. Only myosin IC was recognized by the antiserum when the three native myosin I isoforms were dot-blotted onto nitrocellulose strips (Fig. 1 *A*), and only myosin IC reacted with the antiserum after SDS-PAGE and immunoblotting of the purified myosin I isoforms (Fig. 1 *A*). Marginal cross-reactivity of the antiserum with myosin IB was sometimes observed. In whole cell extracts analyzed by SDS-PAGE and immunoblotting, only a single protein band, which comigrated with purified myosin IC, was detected by the antibody (Fig. 1 *A*). Specific recognition of myosin IC in whole cell lysates could be observed after a 1:2,000 dilution of the polyclonal antiserum, which corresponds to a final protein concentration of 17.5 $\mu g \cdot ml^{-1}$. Preimmune serum, at dilutions of 1:100–1:800 (0.35 $mg \cdot ml^{-1}$ to 44 $\mu g \cdot ml^{-1}$), did not recognize any proteins in an *Acanthamoeba* whole cell lysate (data not shown). A single protein was also recognized by the antiserum when plasma membranes prepared by the method of Clarke et al. (1988) were analyzed by SDS-PAGE and immunoblotting (data not shown). Affinity purification of the antiserum with purified myosin IC did not affect the apparent specificity of the antibody as determined by SDS-PAGE and immunoblotting (data not shown).

Two rabbit polyclonal antisera have been raised against *Acanthamoeba* myosin II heavy chain. In whole cell extracts, both antisera recognized single protein bands that comigrated with purified myosin II heavy chain when analyzed by SDS-PAGE and immunoblotting (Fig. 1 *B*). Both specifically detected the heavy chain of myosin II even after a 1:4,000 dilution (8.75 $\mu g \cdot ml^{-1}$) of the antisera. Preimmune serum, at dilutions of 1:100–1:800 (0.35 $mg \cdot ml^{-1}$ to 44 $\mu g \cdot ml^{-1}$), did not recognize any proteins in an *Acanthamoeba* whole cell lysate.

Indirect Immunofluorescence Localization of Myosin IC

Indirect immunofluorescence localization of myosin IC in cells after light saponin permeabilization clearly revealed fluorescent staining of the plasma membrane of at least 50% of the cells (Fig. 2 *D*). However, as shown by the phase-contrast image of the same field (Fig. 2 *C*), some cells were not fluorescent when light saponin permeabilization was used. Immunofluorescence staining after extensive saponin permeabilization showed an apparent redistribution of the

fluorescence to a single large vacuole or vesicle in the cytoplasm (Fig. 2 *F*). By careful study of phase-contrast images, the labeled vacuole was identified as the contractile vacuole (e.g., see Fig. 2, *G* and *H*); this was confirmed by electron microscopy (see later). The extent of permeabilization provided by saponin treatment could be controlled to yield intermediate states where the transition from exclusively plasma membrane staining to staining of the contractile vacuole could be observed (Fig. 2 *E*).

Cells that had not been treated with saponin, and were thus not permeable to the antibody, did not show any fluorescence above background (Fig. 2 *B*). Controls, in which the cells had been incubated in the presence of preimmune serum (Fig. 2 *J*), or in which the primary incubation with the antimyosin IC had been omitted (data not shown), also did not show any staining above background. To determine whether the observed fluorescence distribution was an artifact of the treatment with saponin, cells that had been made permeable by treatment with acetone at −20°C were stained with antimyosin IC. Cells permeabilized in this manner clearly showed fluorescence associated with the contractile vacuole but showed little or no staining of the plasma membrane (Fig. 2 *H*).

Immunogold Localization of Myosin IC

The localization of myosin IC by indirect immunofluorescence was confirmed and refined by immunogold cytochemistry. After light saponin permeabilization, gold particles were observed only in association with the plasma membrane (Fig. 3 *A*). The cytoplasm and contractile vacuoles of lightly permeabilized cells were not labeled with gold. The plasma membrane appeared to be labeled predominantly on its extracellular surface. However, the antigen recognized by the antimyosin IC can not normally be exposed on the external surface of the membrane since nonpermeabilized cells were never stained by the antibody (Fig. 2 *B*; data not shown for immunogold staining of nonpermeabilized cells).

Immunogold cytochemistry of intermediately permeabilized cells clearly demonstrated gold labeling of the contractile vacuole (Fig. 3, *F* and *G*). Labeling of the plasma membrane could also be observed in intermediately permeabilized cells with labeled contractile vacuoles (Fig. 3, *B* and *C*). It should be noted that although the plasma membrane labeling often occurred on the extracellular surface of the bilayer, it appeared to be associated with some extruded material (Fig. 3, *B* and *C*, *arrowheads*), which gave the membrane a wavy appearance and was most likely a result of

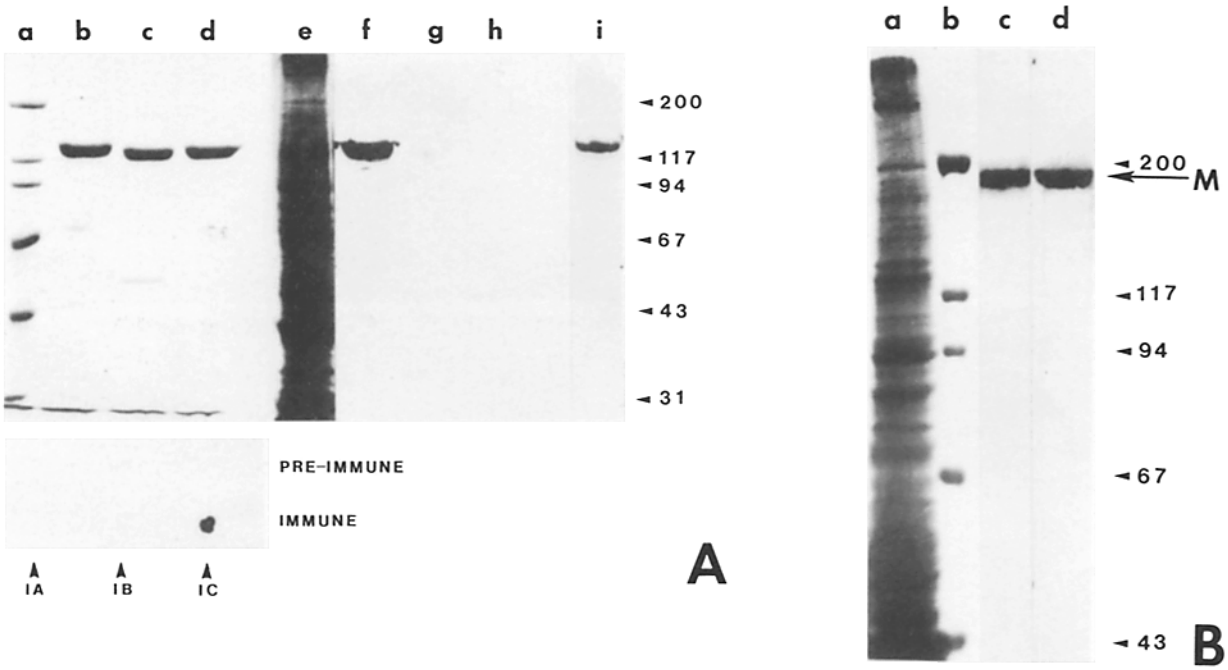
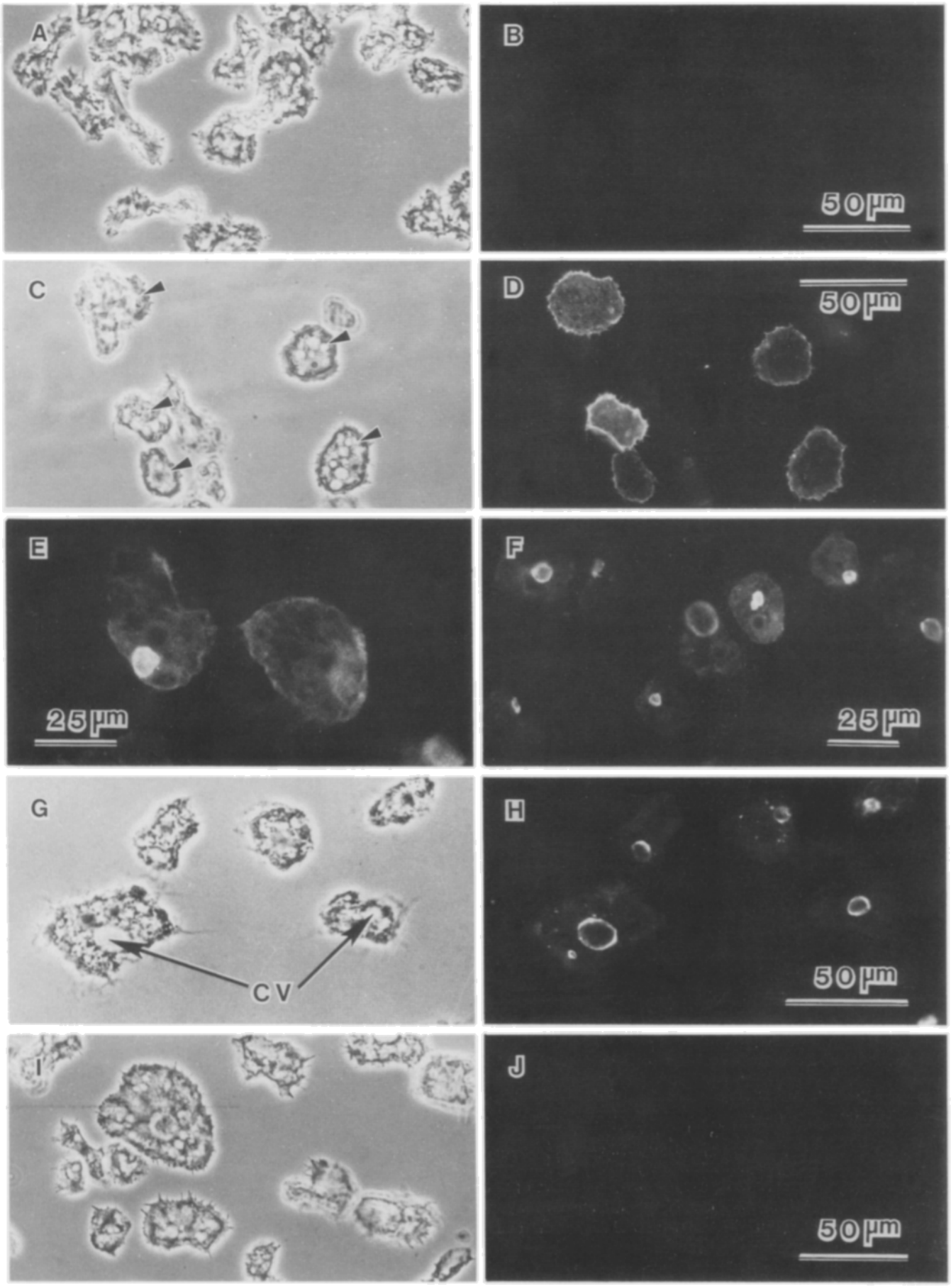


Figure 1. Analysis by SDS-PAGE and immunoblotting of purified myosins I, myosin II, and *Acanthamoeba* whole cell lysate with antibodies raised against the myosin IC synthetic peptide (*A*) and against the myosin II heavy chain (*B*). (*A*) Lanes *a–e*, SDS-PAGE gel stained with Coomassie blue; (*a*) molecular mass markers in kilodaltons (top to bottom: muscle myosin heavy chain, β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase); (*b*) purified myosin IA (2 μg); (*c*) purified myosin IB (2 μg); (*d*) purified myosin IC (2 μg); (*e*) *Acanthamoeba* total cell protein (100 μg). Lanes *f–i*, immunoblots stained with antimyosin IC synthetic peptide; (*f*) purified myosin IC; (*g*) purified myosin IB; (*h*) purified myosin IA; (*i*) total cell protein. Below the SDS-PAGE gel tracks of purified myosin I are the corresponding dot-blots, showing that only purified myosin IC is recognized by the antibody after dot-blotting the native myosin I isozymes (1 μg each) onto nitrocellulose. (*B*) Lanes *a* and *b*, SDS-PAGE of (*a*) total cell protein, and (*b*) molecular mass standards in kilodaltons (top to bottom: muscle myosin heavy chain, β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin), stained with Coomassie blue. Lane *c*, immunoblot of total cell proteins stained with polyclonal anti-myosin II antiserum C27073; lane *d*, immunoblot of total cell proteins stained with polyclonal antimyosin II antiserum C27452. *M*, the position of the *Acanthamoeba* myosin II heavy chain.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saponin permeabilization (see Discussion). Labeling was also observed on the cytoplasmic side of the plasma membrane (Fig. 3, *B* and *C*, *arrows*).

In some cells, the contractile vacuole consisted of several compartments. When the contractile vacuole was divided into two large vacuoles, both were labeled by the antibody-gold complex. The spongiome, consisting of the numerous small vesicles that surround the vacuoles (see Patterson, 1980, for review), was not labeled by the antibody (Fig. 3 *G*). There was no gold labeling of phagocytic or pinocytic vacuoles, cytoplasmic vesicles, mitochondria, nuclei, or the cytoplasm.

In control experiments, preimmune serum was substituted in place of the antimyosin IC serum; although background staining of the cytoplasm was observed in this instance, no specific labeling of the plasma membrane or contractile vacuole occurred (Fig. 3 *D*). Quantification of the distribution of the myosin IC gold label and comparison with the distribution of gold label after treatment with pre-immune serum are shown in Table I. The quantitation clearly shows a reduction in the amount of gold label associated with the plasma membrane after increased saponin permeabilization (5.3 particles/μm vs. 1.2 particles/μm). The amount of gold label associated with the membrane of the contractile vacuole (3.0 particles/μm) was of the same order as that associated with the plasma membrane. To compare the quantity of gold label associated with the cell membranes with the quantity of gold in the cytoplasm, the data for the membranes were recalculated as the number of particles per unit area, taking the width of the plasma membrane as 10 nm. Thus the plasma membrane had 530 particles/μm^2 after light permeabilization and 120 particles/μm^2 after intermediate permeabilization, and the membrane of the contractile vacuole had 300 particles/μm^2 after intermediate permeabilization. There was no association of gold particles with either the plasma membrane or the contractile vacuole membrane after treatment with preimmune serum. The number of gold particles in the cytoplasm was essentially the same for both immune and preimmune sera (5.9 particles/μm^2 vs. 5.4 particles/μm^2). It should be noted that $\sim$50% of the gold particles occurred in clusters of more than four particles. These clusters represented specific binding of the antibody about the antigenic site since no clusters of gold particles could be observed after treatment with preimmune serum.

Localization of Myosin II

Indirect immunofluorescence and immunogold cytochemistry were also used to localize myosin II in *Acanthamoeba*. Identical results were obtained with the two polyclonal antisera C27073 and C27452. Indirect immunofluorescence revealed a distribution of myosin II similar to that observed in previous studies (Gadasi and Korn, 1980; Hagen et al., 1986). Myosin II occurred throughout the cell but appeared to be concentrated in the cell cortex (Fig. 4). Cells exhibiting mitotic figures showed myosin II in the cleavage furrow (Fig. 4, *arrowheads*). Immunogold cytochemistry confirmed the localization of myosin II at high resolution (Fig. 5). Interestingly, the gold label appeared to be distributed in the form of filamentous, or rodlike structures (Fig. 5, *arrows*). These have a length of $\sim$200 nm (most clearly illustrated in Fig. 5 *B*) and are consistent with myosin II in *Acanthamoeba* being present in the form of the octameric minifilaments as characterized in vitro (Sinard and Pollard, 1989; Sinard et al., 1989). Myosin II was not observed to be associated with the plasma membrane (Fig. 5), cytoplasmic vesicles, or the nucleus. Myosin II was not present in close apposition to the contractile vacuole membrane (Fig. 5 *C*). Quantification of the myosin II gold label (Table II) documents the absence of significant labeling of membranes and reveals that 87% of the gold particles in the cytoplasm were associated to form clusters consisting of more than eight particles. These clusters were not observed after treatment with preimmune serum.

Discussion

The immunofluorescence and immunogold localization data presented here show that myosin IC is associated with the plasma membrane, as has been previously reported for myosins IA and IB (Gadasi and Korn, 1980). Two further observations, however, were unexpected: firstly, the apparent association of myosin IC with the membrane of the contractile vacuole; secondly, the apparent distribution of the myosin IC on the external surface of both the plasma membrane and the membrane of the contractile vacuole. Also, the dependency of the contractile vacuole staining on the extent of saponin permeabilization and the loss of plasma membrane staining after extensive saponin treatment require explanation.

The latter observations can be accounted for by consideration of the properties of saponin as a detergent. Extensive permeabilization of the cell will allow better penetration of the antibody (thus resulting in staining of the contractile vacuole), but will also extract phospholipids from the plasma membrane. If myosin I were closely associated with the lipid bilayer (Miyata et al., 1989; Adams and Pollard, 1989), extraction of the plasma membrane lipids by extensive saponin permeabilization could result in a concomitant loss of myosin IC. A very light saponin permeabilization, on the other hand, could be optimal for staining of plasma membrane-

Figure 2. Indirect immunofluorescence with antimyosin IC synthetic peptide and FITC-linked goat anti-rabbit Ig. (*A* and *B*) Nonpermeabilized cells treated with 0.35 mg·ml^{-1} antimyosin IC. (*A*) Phase contrast, (*B*) immunofluorescence. (*C* and *D*) Lightly permeabilized cells (0.1% saponin for 10 min) treated with 0.35 mg·ml^{-1} antimyosin IC. (*C*) Phase contrast, (*D*) immunofluorescence. Not all the cells observed by phase contrast were stained by the antibody; those that were stained are indicated by arrowheads. (*E*) Cells treated with 0.35 mg·ml^{-1} antimyosin IC after intermediate permeabilization (0.2% saponin for 15 min). (*F*) Cells treated with 0.35 mg·ml^{-1} antimyosin IC after extensive permeabilization (0.5% saponin for 45 min). (*G* and *H*) Cells permeabilized by 100% acetone and treated with 0.35 mg·ml^{-1} antimyosin IC. (*G*) Phase contrast, (*H*) immunofluorescence. (*I* and *J*) Cells treated with 0.35 mg·ml^{-1} preimmune serum after intermediate permeabilization. (*I*) Phase contrast, (*J*) immunofluorescence. *CV*, contractile vacuole.

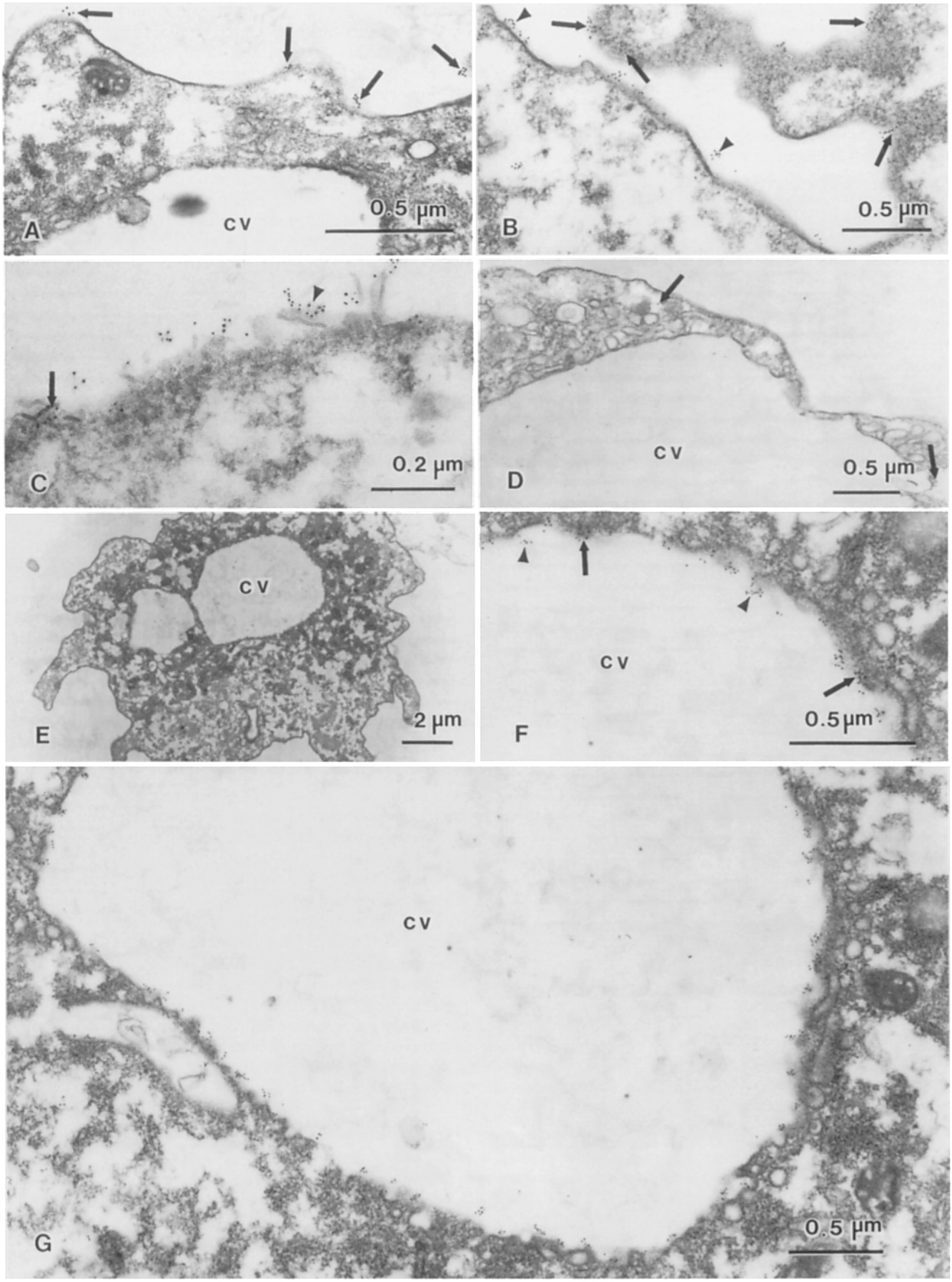
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Table I. Immunoelectron Microscopic Localization of Acanthamoeba Myosin IC

	Particles/μm	Particles/μm^2	In clusters of more than four particles	On inner face	Total particles	Total area
			%	%	μm^2	
Light permeabilization						
Plasma membrane						
Immune	5.2 ± 0.8	530	54.5 ± 17	35.3 ± 16	689	1.3
Preimmune	0.001	0.1	0	0	1	9.4
Intermediate perm.						
Plasma membrane						
Immune	1.2 ± 0.3	120	43.6 ± 8	29.2 ± 11	1,885	15.3
Preimmune	0	0	0	0	0	10.3
Contractile vacuole						
Immune	3.0 ± 0.4	300	60.2 ± 12	25.4 ± 7	1,022	3.4
Preimmune	0	0	0	0	0	3.1
Cytoplasm						
Immune		6.3 ± 5.1	0		377	60
Preimmune		5.5 ± 1.7	0		748	136

These data were derived from measurements performed on a minimum of 10 cells from 3 different sample preparations. For statistical analysis, each cell was treated as a single sample and a mean was calculated for each. The numbers given in the table represent a mean of means ± σ_{n-1}. The area (μm^2) of the membrane compartments included gold particles contained in a zone 10 nm on either side of the membrane. Membrane area was calculated by membrane length × 10 nm.

associated myosin IC but insufficient to allow penetration of the antibody to the cell interior. It should be noted that *Acanthamoeba* plasma membranes have a high ratio of sterol to phospholipid (0.98 mol/mol) (Korn and Olivecrona, 1971). Since saponin permeabilizes cells through an interaction with membrane sterols, their high sterol content should make *Acanthamoeba* plasma membranes particularly susceptible to saponin permeabilization. Although saponin is thought to interact with membrane sterols to form globular micelles, each with a central pore, the pores are unlikely to be the route of antibody entry into the cell since they are only ~8 nm in diameter (Lucy and Glauert, 1964; Seeman, 1974). Instead, it is likely that antibody entry occurs where saponin-sterol micelles disorganize and disrupt the bilayer (Ohtsuki et al., 1978).

For accurate localization studies, the antibodies must be able to permeate the cytoplasm freely once past the plasma membrane. Aldehyde fixation, however, cross-links proteins in the cytoplasm forming a matrix that may prevent the free passage of macromolecules (Ohtsuki et al., 1978). Thus, free penetration of macromolecules through the cytoplasm after aldehyde fixation probably depends on removal of a portion of the cytoplasmic constituents (Ohtsuki et al., 1978). It is likely that light saponin permeabilization is sufficient to allow the antibodies to pass through the plasma membrane but insufficient to allow further penetration that is prevented by cross-linked proteins in the cortical region of the cell. Extensive saponin permeabilization, however, would extract a large enough proportion of detergent-soluble cytoplasmic proteins to allow penetration of the antibodies throughout the cell.

Previous localization (Gadasi and Korn, 1980; Miyata et al., 1989; Hagen et al., 1986) of myosin IA and IB did not reveal any association of these isozymes with the contractile vacuole. It should be noted that fluorescence staining of the contractile vacuole was observed in the present study using identical conditions to those described in the previous localization studies. Thus, myosin IC and myosins IA and IB may be differentially localized in *Acanthamoeba*.

The contractile vacuole is a membrane-bound organelle responsible for the maintenance of a constant cytoplasmic water potential (see Patterson, 1980, for review). It fills (during diastole) passively and then expels water (during systole) by active contraction. Recent proposals for the function of myosins I (Fukui et al., 1989; Adams and Pollard, 1989) suggest a role in membrane dynamics, for example, at the leading edge of a motile cell. Vacuolar contraction involves dynamic movements of the membrane and thus the contractile vacuole may also be an appropriate location for a myosin I.

The possibility must also be considered, however, that the

Figure 3. Immunogold staining of saponin permeabilized cells with antimyosin IC synthetic peptide and goat anti-rabbit IgG coupled to 5- or 10-nm gold. (*A*) A lightly permeabilized cell treated with 0.35 mg·ml^{-1} antimyosin IC and 10-nm gold. Only the plasma membrane was labeled by the antibody (*arrows*). (*B* and *C*) A cell treated with 0.35 mg·ml^{-1} antimyosin IC and 5-nm gold, after intermediate permeabilization. Gold labeling occurred both on the external (*arrowheads*) and internal (*arrows*) faces of the plasma membrane. The disruption of the plasma membrane by saponin can be observed in *C*. (*D*) A cell treated with 0.35 mg·ml^{-1} preimmune serum after intermediate permeabilization. Neither the plasma membrane nor the membrane of the contractile vacuole were recognized by the serum; occasional gold particles were observed in the cytoplasm (*arrows*). (*E–G*) A cell treated with 0.35 mg·ml^{-1} antimyosin IC after intermediate permeabilization showing the entire cell (*E*) and the contractile vacuole at higher magnifications (*F* and *G*). Gold labeling occurred on both the external (*arrowheads*) and internal (*arrows*) faces of the membrane.

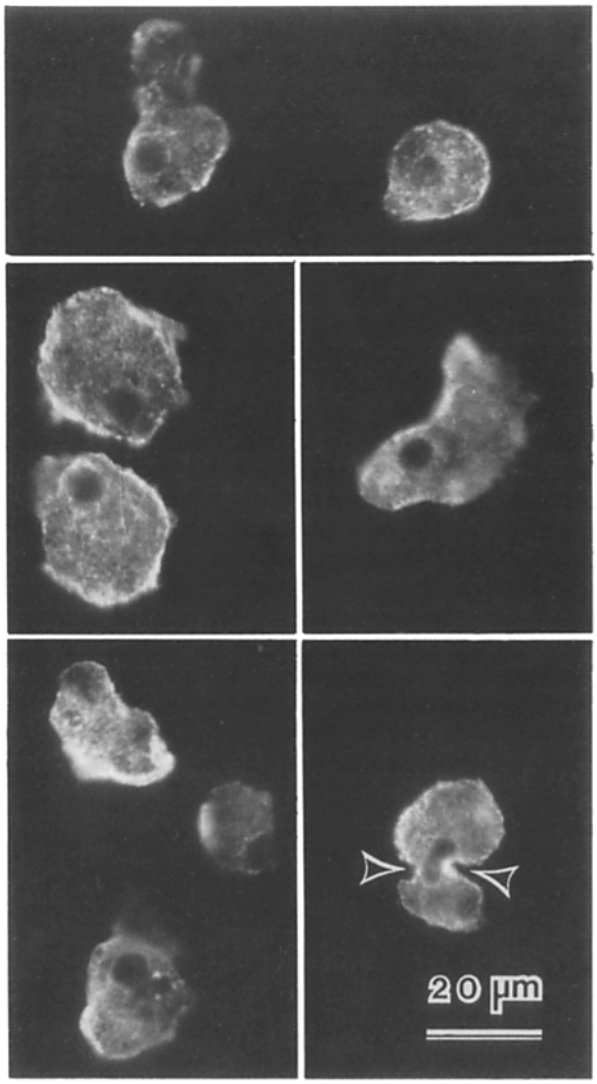

Figure 4. Indirect immunofluorescence with antimyosin II (C27073) and FITC-linked goat anti-rabbit Ig. The myosin II was concentrated in the cell cortex of vegetative cells and in the contractile ring of dividing cells (*arrowheads*).

contractile vacuole staining was due to a cross-reaction of the antimyosin IC antibody with shared epitopes on another protein. Three lines of evidence argue against this. (*a*) The antibody recognizes only a single band of the correct molecular mass for myosin IC heavy chain on immunoblots of whole cells and plasma membrane preparations. It is unlikely that the antibody cross-reacts with a protein in the native state but does not recognize the same protein after SDS denaturation as the antibody was raised against a synthetic peptide that would be expected to have minimal tertiary structure. (*b*) A monoclonal antibody raised against native myosin I recognizes the contractile vacuole (Yonemura, S., and T. D. Pollard, personal communication). (*c*) The distribution of gold particles after staining with antimyosin IC is identical on the membrane of the contractile vacuole and the plasma membrane. An attempt was made in the course of this study to purify contractile vacuole membranes and look for the association of myosin IC with the purified membranes. Although myosin IC did copurify with the contractile vacuole membranes, no preparation was obtained that was sufficiently free from plasma membrane contamination to allow differential analysis.

The observed bias of immunogold staining for the external surface of the plasma and contractile vacuole membranes requires discussion. The quantified data (see Table I) indicate that 65–75% of the gold particles associated with the plasma membrane or the membrane of the contractile vacuole were located on the external (noncytoplasmic) face of the membrane. If myosin IC interacts with actin, as seems most likely, it presumably does so at the cytoplasmic surface of the membrane. Also, the antibody used in these experiments recognizes the phosphorylation site of myosin IC. The myosin I heavy chain kinase is presumably located within the cell and thus the phosphorylation site on the myosin I heavy chain would be expected to be at the cytoplasmic surface of the membrane. Finally, it should be emphasized that the antigen recognized by the antimyosin IC is not normally exposed on the cell surface since nonpermeabilized cells are not stained by the antibody (Fig. 2 *B*). For these reasons, we believe that the apparent predominant external location of myosin IC is an artifact of the saponin permeabilization. In a study of the

Table II. Immunoelectron Microscopic Localization of Acanthamoeba Myosin II

	Particles/μm	Particles/μm²	In clusters of more than eight particles	Total particles	Total area
					μm²
Plasma membrane					
Immune	0.1 ± 0.1	10	0	145	15.2
Preimmune	0.01	1.0	0	14	10.8
Contractile vacuole					
Immune	0.01	1.2	0	15	12.5
Preimmune	0.01	0.7	0	7	9.9
Cytoplasm					
Immune		251 ± 52	89.6	4,454	18
Preimmune		4.5 ± 1.7	0	433	96

Data derived as for Table I.

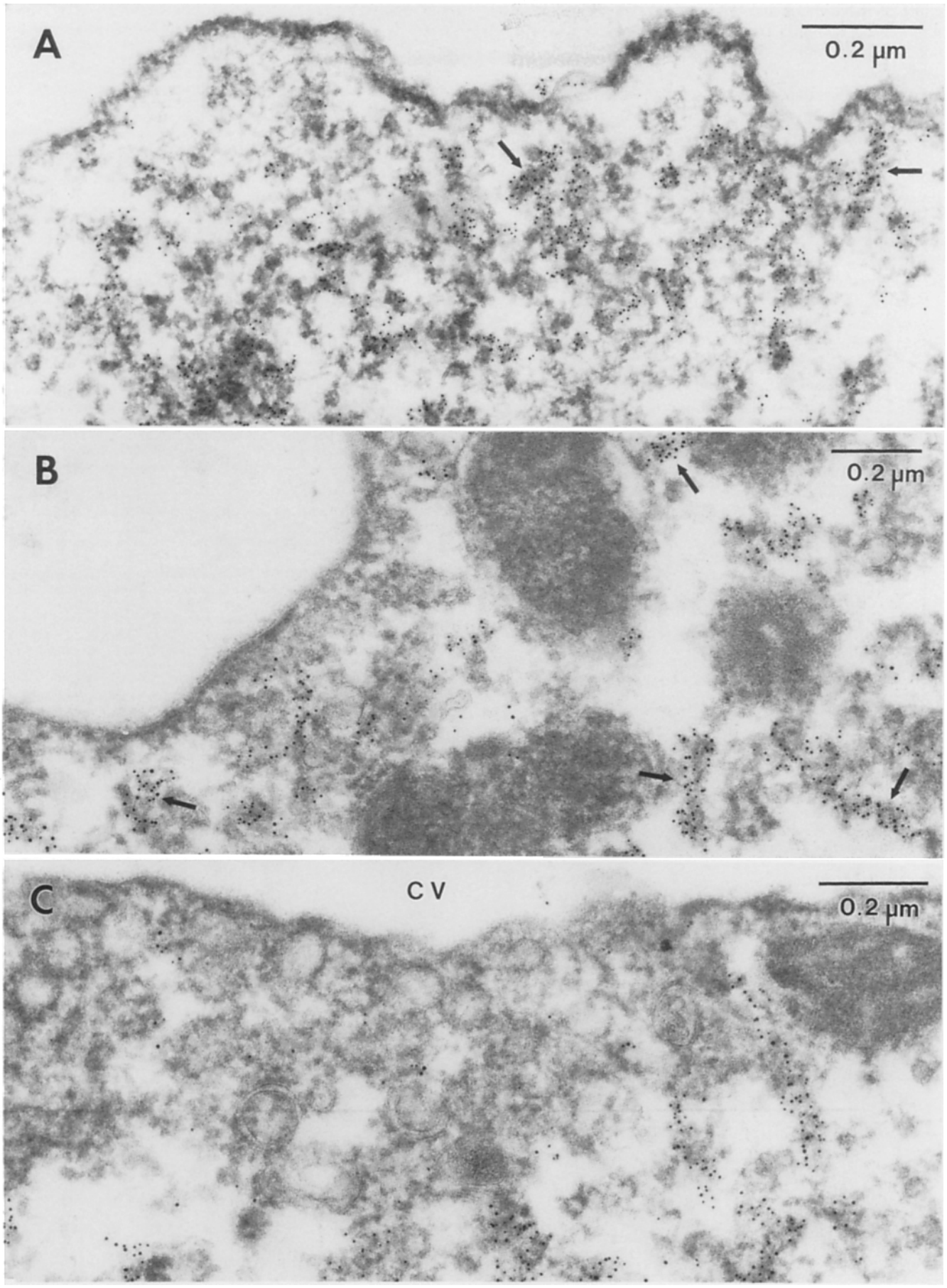

Figure 5. Immunogold staining of an extensively permeabilized cell (0.05%, saponin at the same time as fixation, see Materials and Methods) with 0.35 $mg \cdot ml^{-1}$ antimyosin II heavy chain (*A* and *C*, C27073; *B*, C27452) and goat anti-rabbit IgG coupled to 5-nm gold. Myosin II appeared to be organized into rodlike filaments present throughout the cytoplasm (*A* and *B*, *arrows*) but absent from the plasma membrane (*A*), vesicle membranes (*B*), and membrane of the contractile vacuole (*C*).

entry of macromolecular tracers into cells after aldehyde fixation and saponin permeabilization, Ohtsuki et al. (1978) noted that saponin induced membrane extrusions which were probably caused by local expansions of the phospholipid bilayer. Similar extrusions were observed in the present study and were often the site of antimyosin IC immunogold labeling (Fig. 3 *C*); these protrusions were not labeled in cells incubated with preimmune serum (data not shown) or antibody to myosin II (Fig. 5). Thus, we conclude that myosin IC is very closely associated with the membrane of the contractile vacuole and the plasma membrane, and is highly labile to treatment with detergent.

The localization of myosin II observed in the present study is in agreement with previous studies (Gadasi and Korn, 1980; Hagen et al., 1986). Additionally, the high resolution immunogold localization of myosin II reveals an organization into rodlike filaments ~200 nm long. This is compatible with recent data showing that in the ionic conditions expected to be present in the *Acanthamoeba* cytoplasm, myosin II self-associates in vitro into octameric minifilaments of that size (Sinard and Pollard, 1989; Sinard et al., 1989).

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