MICROFILAMENTS IN *CHAOS CAROLINENSIS*

Membrane Association, Distribution, and Heavy Meromyosin Binding in the Glycerinated Cell

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INTRODUCTION

Recent ultrastructural studies indicate that 50–70 Å microfilaments are found in a variety of cell types (1, 10, 25, 27), some of which exhibit shape changes (6, 26), cell motility (4, 5, 17, 18), and cytoplasmic streaming (2, 3, 28). Biochemical investigators have isolated and purified an actin-like protein from *Physarum polycephalum* (2, 29–33), the amoebae of *Dictyostelium discoideum* (34), and the small *Acanthamoeba castellanii* (4, 7, 36–38). The isolated proteins from these primitive motile systems closely resemble muscle actin in several chemical, physical, and structural properties. One of the properties shared by the purified actin or actin-like proteins and the *in situ* 50–70 Å microfilaments is the capacity to form characteristic ATP-dissociable arrowhead structures when complexed with rabbit muscle heavy meromyosin (HMM) (1–4, 10, 25, 27, 34).

The present study utilized arrowhead formation by HMM and microfilaments in the giant amoeba, *Chaos carolinensis*, as a method for identifying and localizing thin filaments potentially functional in streaming or motility. The results represent the first *in situ* HMM tagging of thin filaments in any of the large, free-living amoebae. The results are in agreement with previous evidence for actin filaments in extracts of the large *Amoeba proteus* (3) and *in situ* in the small *A. castellanii* (4). The present work also includes the first evidence suggesting membrane association of actin filaments in the giant amoeba.

MATERIALS AND METHODS

Rabbit muscle myosin was isolated by a method[1] based on the procedure of Eisenberg and Moos (8). HMM was immediately prepared by the procedure of Lowey and Cohen (9), as modified by Pollard et al. (4). The stock solution of HMM was stored frozen after dialysis against 25% glycerol in 0.1 M KCl, 0.005 M $MgCl_2$, 0.006 M $NaPO_4$, pH 7.0 (standard salt) (10). The stock solution was diluted just before use to 2.0 or 0.6 mg HMM/ml with standard salt or with 0.1 M KCl.

Specimens of *C. carolinensis*, cultured in Marshall's medium (11), were glycerinated and reacted with HMM in a small Petri dish according to the procedure of Pollard et al. (4). Control amoebae were similarly glycerinated but reacted with standard salt buffer alone. (The Mg-ATP and $NaPP_i$ sets of control cells were also included and treated according to the specific protocol outlined by Pollard et al. [4].) Cells treated with HMM or with buffer were fixed 1 h in 3% glutaraldehyde-0.1 M sodium cacodylate buffer, pH 7.4, for 1 h. A brief 0.1 M sodium cacodylate rinse was followed by routine (graded ethanol series) dehydration, propylene oxide, and Epon embedding. Silver sections were stained with 2% aqueous uranyl acetate for 30 min at room temperature followed by Reynolds' lead citrate for 5–7 min at room temperature (12). Electron micrographs were taken on an AEI EM6B electron microscope at 50 or 60 kV with a 50 μm objective aperture. The stereo pair of electron micrographs was taken on a Philips 300 electron microscope at 60 kV with a 25 μm objective aperture. Both microscopes were calibrated with 0.500 μm latex spheres (Dow Chemical U. S. A., Membrane Systems Div., Midland, Mich., run no. LS-1029-E).

The response to glycerination was monitored by placing the cells on a cover slip under an agar overlay (13); glycerol solutions were pipetted directly onto the agar. The cells were observed by dark-ground or phase-contrast microscopy.

[1] Estes, J. Rensselaer Polytechnic Institute, Troy, N. Y. Personal Communication.

RESULTS

An actively streaming amoeba reverses flow in the pseudopods and forms a rounded cell within the next 10 min when placed in cold 5% glycerol-standard salt. During this time endoplasmic flow becomes slower and locally sporadic. When subsequently placed in 25% glycerol-standard salt, endoplasmic flow ceases. The cytoplasm appears homogeneous and is surrounded by a distinct hyaline region and refractile membrane. The 50% glycerol solution may induce brief, localized flow but the cell retains the appearance exhibited in 25% glycerol. The initial cell response of rounding up is characteristic of a mechanically or physically disturbed amoeba. The refractile membrane and homogeneous cytoplasm may reflect a physiological response to glycerol.

At the ultrastructural level the mitochondria, rough endoplasmic reticulum, and cytoplasmic vesicles are characteristically swollen and extracted in glycerinated cells. However, a clear-cut distinction between hyaline ectoplasm and vesicular cytoplasm is retained in the glycerinated, HMM-reacted amoeba (Figs. 1 and 2). The hyaline ectoplasm is characterized by the almost total absence of vesicles and thick filaments. The hyaline region and pseudopod-like projections are filled with arrowhead complexes characteristic of actin filaments tagged with HMM (1–4, 10, 25, 27, 34). The tagged thin filaments, which can be up to 0.5 μm long, are typically dispersed in random orientation but may also occur in parallel arrays (Fig. 2). Viewed at a higher magnification in Fig. 3, the filaments are seen to interact with HMM along their entire length (1, 14). Variation in thickness and density along the tagged filaments allows only an approximation of their average diameter (approximately 190 Å). The HMM interacts with each G-actin subunit in the filaments (1, 40); consequently, the density and overlap of material in thin sections permits measurement of arrowhead spacing only in selected instances. The average spacing is about 340 Å. These HMM arrowhead complexes do not occur in *Chaos* in the magnesium-ATP or sodium pyrophosphate controls (4).

Fig. 1 illustrates a distinctive feature of the vesicular cytoplasm: dense aggregates of tagged thin and of thick filaments appear among the organelles. Such aggregates occur in reticulate

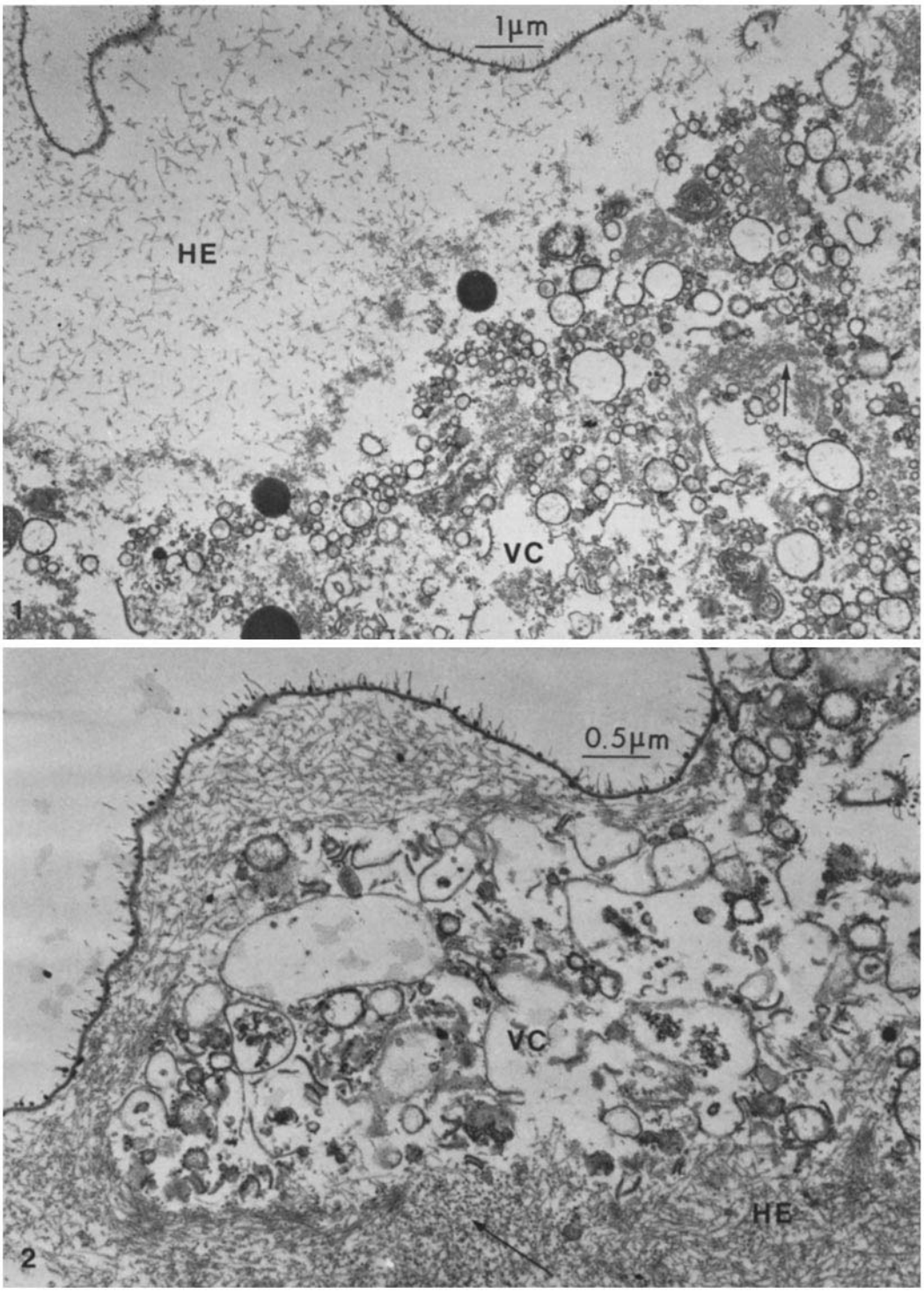

FIGURE 1 A portion of a glycerinated, HMM-reacted (2 mg/ml) *C. carolinensis*. Note the clear distinction of hyaline ectoplasm (*HE*) from vesicular cytoplasm (*VC*). Tagged thin filaments predominate in the hyaline region. Aggregates of thick and tagged thin filaments (arrow) fill the vesicular cytoplasm. × 10,300.

FIGURE 2 A region of an amoeba, treated as in Fig. 1, showing dense packing of HMM-tagged thin filaments in the hyaline ectoplasm (*HE*). These filaments are in random orientation or parallel arrays; one group appears in cross section (arrow). The vesicular cytoplasm (*VC*) is more thoroughly extracted than in Fig. 1. × 20,500.

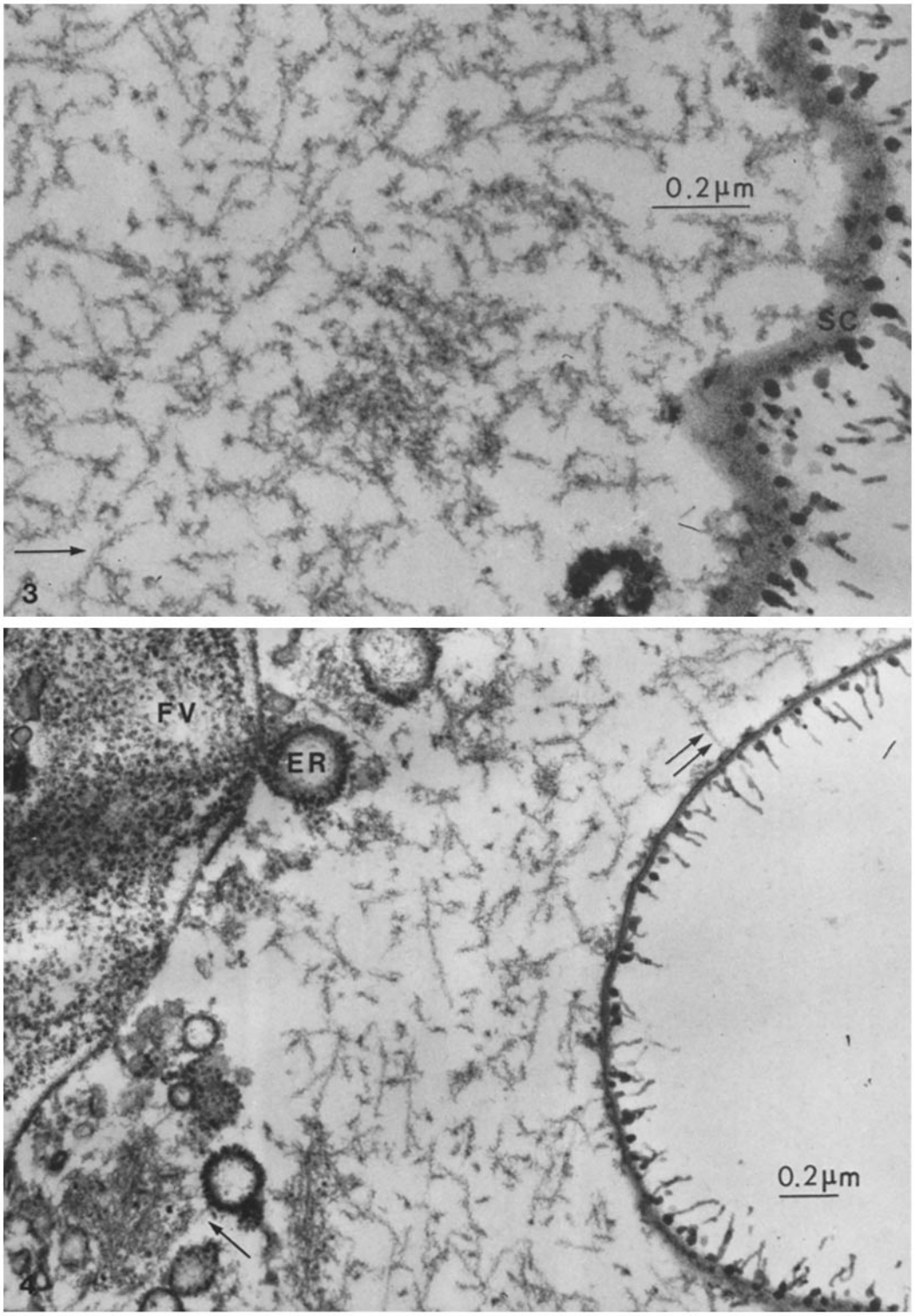

FIGURE 3 A high magnification view of HMM-tagged filaments in the hyaline region. Arrow denotes filament exhibiting unidirectional polarity of the HMM arrowheads. The continuous layer of the surface coat (*SC*) is cut tangentially. The usual filamentous exterior layer appears to be partially clumped, probably as a result of the glycerination procedure. × 74,000.

FIGURE 4 A high magnification view of the hyaline region and vesicular cytoplasm. Note the association of HMM-tagged actin filaments with cell organelles (single arrow) and the plasma membrane (double arrows). A large food vacuole (*FV*) and endoplasmic reticulum (*ER*) appear among the cytoplasmic organelles. × 44,100.

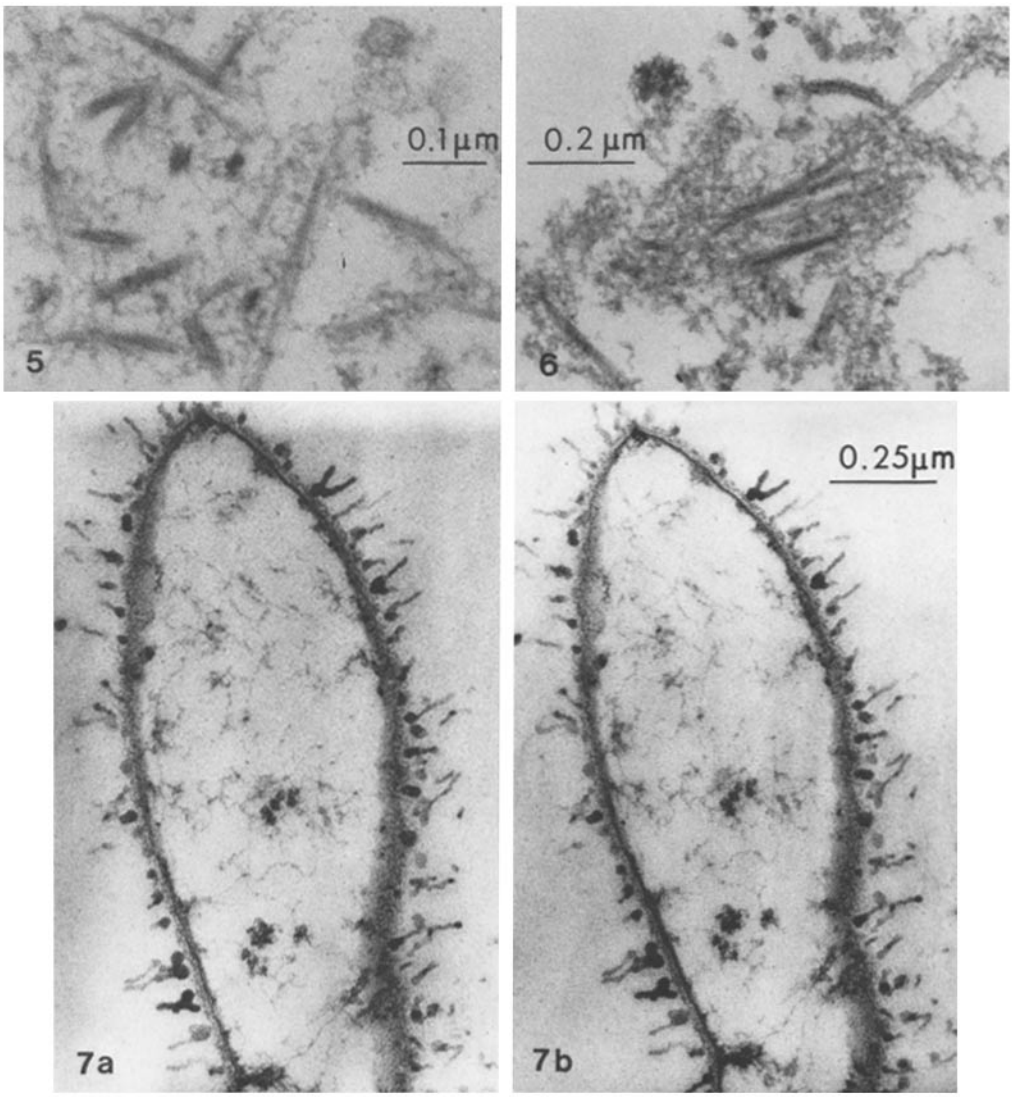

FIGURE 5 Thick filaments in a glycerinated control cell. Note the taper, occasional bare regions, and thin projections. × 108,600.

FIGURE 6 Thick filaments in HMM-reacted cell. The filament taper and bare central regions are still apparent. HMM appears to aggregate with the thin projections. × 70,400.

FIGURES 7 *a* and *b* A pair of stereo micrographs of a glycerinated buffer control cell. The thin, untagged 70 Å filaments typically occur in reticular or network arrays in the hyaline ectoplasm of control cells. The stereo pair illustrates the continuity between the plasma membrane and the thin filaments. In this image some filaments appear to be associated with aggregated material at the plasma membrane. (*a*), +6°; (*b*) −6°. × 55,550.

or parallel arrays; they may also appear at the boundary between the hyaline region and the vesicular cytoplasm (Figs. 1 and 4). In Fig. 4 (arrow) aggregates containing both thick and thin filaments appear to interconnect with various cell organelles. The extreme dispersal of organelles by glycerination, however, allows only infrequent observations of such filament-organelle associations.

The thick filaments, appearing in both HMM-

reacted and control cells, range from 160 to 280 Å in diameter; the average diameter is 220 Å. Their tapered shape, irregular projections, and, occasionally, bare central zones (Figs. 5 and 6) strongly resemble those of the negatively stained thick filaments isolated from striated muscle (1). The thick filaments in glycerinated *Chaos* are also the same in appearance, size, and maximum length (0.5 μm) as those found in *A. proteus* extracts (16) and in Alcian blue-treated or glutaraldehyde-fixed *Chaos* (17, 18). The effect of 5 mM $MgCl_2$ (in the standard salt buffer in this experiment) on the aggregation of myosin into thick filaments in *Physarum* has been reported by Nachmias (19); ion-induced aggregation of myosin also occurs in smooth muscle (20). The appearance of the thick filaments in *Chaos* and their occurrence in the presence of glycerol and $MgCl_2$ strongly suggest that these filaments are, in fact, myosin.

Fig. 4 illustrates a consistent observation: the tagged actin filaments appear to be associated with the plasma membrane of the giant amoeba. Membrane association of thin, untagged filaments is also frequently observed in the glycerinated control cells. Figs. 7 *a* and *b*, a stereo pair, illustrate the continuity between filaments, or filaments with aggregated material, and the plasma membrane. Thin sections of unglycerinated, routinely fixed cells also show an apparent association of thin filaments with the plasma membrane (21; manuscript in preparation). An association of actin filaments with isolated plasma membrane has also been reported in the small *A. castellanii* (22). The random orientation of filaments in *Chaos* makes it difficult to determine the direction of polarity of the tagged filaments with respect to the plasma membrane. Where arrowheads are distinct along a filament, the polarity is unidirectional (Fig. 3, arrow).

DISCUSSION

The definitive evidence for the existence of actin in primitive motile systems is derived from the characterization of purified actin-like proteins isolated from *P. polycephalum* (2, 29–33), *D. discoideum* amoebae (34), and *A. castellanii* (4, 7, 36–38). Muscle actin and these primitive cell actins share the following characteristics (36): (*a*) polymerization of G-actin monomers into fibrous F-actin, with accompanying ATP hydrolysis; (*b*) complexing with rabbit muscle myosin to form actomyosin which exhibits a viscosity drop and recovery on the addition of ATP in high ionic strength solutions; and (*c*) activation of muscle myosin Mg-ATPase at low ionic strength. In addition, the sedimentation constants, molecular weights, and amino acid compositions of the primitive cell actins are similar to those of muscle actin. The F-actin filaments, from muscle actin (1, 23) and from these primitive systems, have a double helical, beaded ultrastructure; the purified F-actin also binds rabbit HMM to form ATP-dissociable arrowhead structures (2, 4, 34). Purified actin, isolated thin filaments (1), and *in situ* thin filaments (10) of muscle all form arrowhead complexes. Similarly, purified F-actin and *in situ* thin filaments of *A. castellanii* form these characteristic arrowhead structures with rabbit HMM (4).

The present results show that thin filaments in *C. carolinensis*, identified as actin by *in situ* HMM binding, occur both in the vesicular cytoplasm, where streaming normally occurs, and in the hyaline ectoplasmic layer in apparent association with the plasma membrane. In the vesicular cytoplasm the tagged thin filaments occur in association with thick filaments which may be myosin aggregates. Myosin-like proteins are found in both *Physarum* (14, 15, 19, 32, 33, 39) and *Acanthamoeba* (35); in contrast to *Physarum* (19), the myosin-like protein isolated from *Acanthamoeba* does not aggregate as thick filaments. Membrane association of thin filaments is also reported in *Acanthamoeba* isolated membranes (22) and in motile vertebrate cells (5). In some cells filament-membrane association may occur in the region of cell-substratum attachment and may function in cell locomotion (5, 24). Such filaments may also occur on the entire cell periphery and function in shape determination (5). In the glycerinated amoeba, membrane-associated actin filaments may provide a means for pseudopod retraction in a disturbed, rounding-up cell.

The clear-cut evidence for actin in amoebae and the probable existence of both myosin and membrane-associated filaments are three features essential to a sliding filament contractile system functional in both cytoplasmic streaming and in cell motility.

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