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Mobility of cytoplasmic and membrane-associated actin in living cells

(cytoskeleton/fluorescence/microfilament/motility/photobleaching)

YU-LI WANG*†, FREDERICK LANNI‡, PAUL L. MCNEIL*§, B. R. WARE‡, AND D. LANSING TAYLOR*§

*Cellular and Developmental Biology, Harvard University, 16 Divinity Avenue, Cambridge, Massachusetts 02138, and ‡Department of Chemistry, Syracuse University, Syracuse, New York 13210

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ABSTRACT **We have combined fluorescent analogue cytochemistry with fluorescence photobleaching recovery to measure the mobility of fluorescently labeled actin and other labeled test proteins microinjected into living amoebae. Bovine serum albumin, ovalbumin, and ribonuclease A have a cytoplasmic mobility, expressed as a diffusion coefficient, that is 1/2 to 1/3 of that observed in aqueous solution; 90% of the actin has a mobility 1/2 to 1/8 of that of G-actin in aqueous solution, and ≈10% of the actin has a mobility comparable to that of F-actin in aqueous solution. Therefore, no more than 10% of the actin in the cytoplasm of amoebae can exist as static filaments. Microinjection of phalloidin decreases the diffusion coefficient of the mobile component of cytoplasmic actin, and it also increases the low-mobility fraction to 50% but has no effect on the mobility of labeled ovalbumin. By comparing the mobility of actin in different parts of amoebae and by separating cytoplasm from plasmalemma-ectoplasm, we found the low-mobility fraction of actin to be enriched in the tail, along the plasmalemma-ectoplasm, and in contracted cytoplasm.**

A fundamental goal of the study of cell motility is to understand the molecular dynamics of specific cytoskeletal components in living cells. Because actin appears to play an important role in cell motility, it has received a great deal of attention in recent years (cf. 1–4). The detailed mechanisms of the assembly of actin filaments, the exchange of actin subunits, and the effects of specific actin-binding factors on the filament structure are being investigated *in vitro* (5–8). However, until similar measurements can be made in living cells, models explaining cell motility will remain speculative.

Fluorescent analogue cytochemistry, in which a cellular component is purified, labeled with a fluorescent probe, and then reintroduced as a functional analogue into living cells, provides a method for studying specific contractile proteins in living cells (9, 10). This technique (formerly referred to as molecular cytochemistry) has been used to define spatial and temporal variations in the distribution of actin and other cytoskeletal proteins (see ref. 11 for a review).

Fluorescence photobleaching recovery (FPR; refs. 12–14), in which the diffusion coefficients and mobile fractions of fluorescently labeled molecules are determined by measuring the return of fluorescence into a small photobleached region in the sample, was originally developed to measure the mobility of lipids and proteins in membranes. More recently, it has been used to measure the mobility of actin in solution and of bovine serum albumin and IgG in living cells (5, 15). Recent advances in instrument design have included the introduction of periodic pattern photobleaching (16) and modulation detection (17) to improve the accuracy and dynamic range of the measurements and to minimize possible artifacts due to adventitious bleaching in the monitoring beam and motion of the specimen.

We combine here the technique of fluorescent analogue cytochemistry and FPR to measure the mobility of actin in living amoebae. The free-living species *Amoeba proteus* and *Chaos carolinensis* were chosen because functional incorporation of fluorescent actin analogues and the distribution of actin in living amoebae has been well characterized (9, 18). In addition, cytoplasmic contractility can be regulated both in single cell models and in bulk cell-free extracts (19, 20). The intracellular free Ca^{2+} concentration and pH also have been measured (21, 22). Furthermore, several existing models of amoeboid movement provide a framework for functional interpretation of data from FPR measurements (see ref. 23 for a review).

MATERIALS AND METHODS

Solutions. Stabilization solution and contraction solution were prepared as described by Taylor *et al.* (19). Phalloidin (Boehringer-Mannheim) was 1 mM in 2% dimethyl sulfoxide, methylcellulose (Polysciences, #0846) was 4% (wt/vol) in appropriate culture solutions, and $LaCl_3$ was 0.1 mM in calcium-free culture solutions.

Cultures and Manipulations of Cells. Amoebae were cultured as described for *A. proteus* (24) and *C. carolinensis* (25). For production of single-cell models, cells were ruptured by suction applied through micropipettes to the plasma membranes. This allowed cytoplasm to flow away from the membrane (19). Cells ruptured into the stabilization solution extrude cytoplasm, which is viscoelastic but nonmotile. The stabilized cytoplasm contracts to a fraction of the original volume upon addition of the contraction solution (19). Fibrils were stretched from stabilized cytoplasm with a microneedle.

J744.1 macrophage-like cells were grown *in vitro* on microscope slides as described (26).

Fluorescent Labeling of Proteins. Actin purified from rabbit skeletal muscles was labeled with 5-(iodoacetamido)fluorescein (AF-actin; Molecular Probes, Plano, Texas) as described (27). Ovalbumin, bovine serum albumin, and ribonuclease A (Sigma) were labeled with fluorescein isothiocyanate as described (28). The molar ratios of bound fluorescein to protein for actin, bovine serum albumin, ovalbumin, and ribonuclease A were, respectively, 0.7–0.8, <0.1, 1.85, and 0.3. Labeled proteins were injected at 3–5 mg/ml.

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Abbreviations: FPR, fluorescence photobleaching recovery; AF-actin, 5-(iodoacetamido)fluorescein-labeled actin.

† New address: Department of Molecular and Cellular Biology, National Jewish Hospital and Research Center, Denver, Colorado 80206.
§ New address: Department of Biological Sciences, Center for Fluorescence Research, Carnegie–Mellon University, Pittsburgh, Pennsylvania 15213.

FPR Measurements. Laser optics provided light for fluorescence excitation and photobleaching. A coarse diffraction grating (Ronchi ruling) was substituted for the microscope illuminator field stop so that the specimen was illuminated by a series of parallel stripes. The maximal intensity used for fluorescence excitation was 0.08 W/cm^2, measured at the specimen (λ, 488 nm; objective, ×16). For photobleaching, the peak intensity was typically increased for 100 msec to 270 W/cm^2. The dissipation of the pattern, as a result of diffusive motion of labeled molecules, was subsequently measured by translating the grating back and forth and recording the modulation of the fluorescence signal (Fig. 1). The details of this periodic photobleaching method have been described elsewhere (17).

A grating with 100 lines per 2.5 cm and ×6.3, ×16, and ×40 objective lenses produced an illumination pattern with a periodicity of 74.7, 29.5, or 11.8 μm, respectively. With the ×6.3 objective, the field was large enough to make whole-cell measurements on *C. carolinensis*. The ×16 objective was used for whole-cell measurements on *A. proteus* and for local measurements on *C. carolinensis*. The ×40 objective was used for measurements on extruded cytoplasm and small cellular processes where the required depth of field was small. For each objective lens used, an upper limit on the range of observable mobilities was set by the finite response time of the instrument.

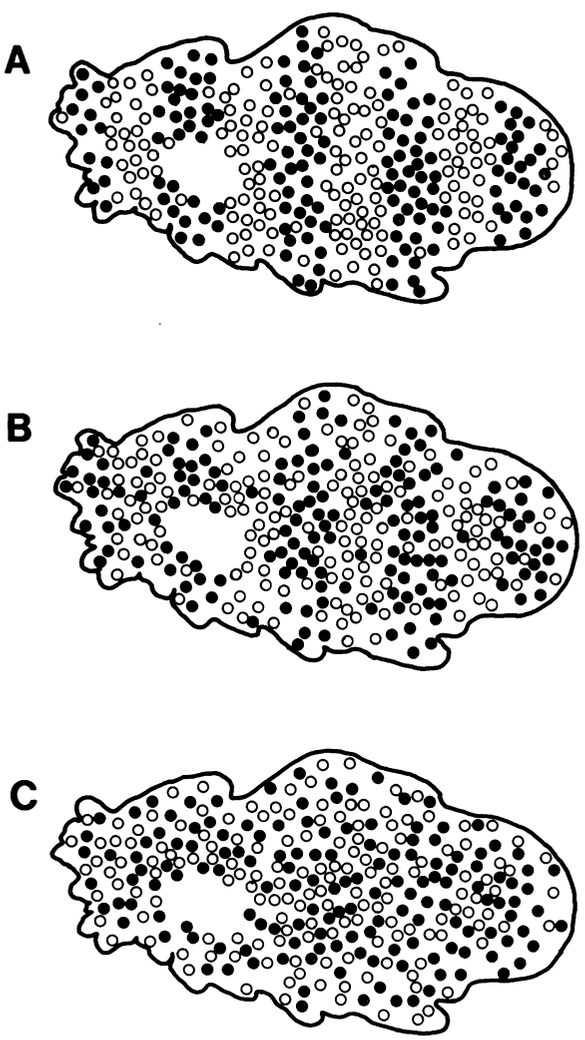

FIG. 1. Diagrammatic representation of bleaching and dissipation of banded pattern. (*A*) An amoeba immediately after photobleaching. (*B*) During recovery. (*C*) After recovery. ○, Fluorescent molecules; ●, bleached molecules.

The limits for the ×6.3, ×16, and ×40 lenses were, respectively, 7×10^{-6}, 1×10^{-6}, and 1.4×10^{-7} cm^2/sec. The fluorescence recovery due to diffusion of labeled particles of higher mobility than those limits would be essentially complete before a record could be obtained.

The initial distribution of fluorescence in cells was often highly inhomogeneous because of irregular cell shapes or large nonfluorescent cytoplasmic inclusions, such as vacuoles. This decreased the accuracy with which an immobile fraction could be determined. Persistence of the fluorescence modulation signal could be due both to immobile materials and to inhomogeneities in unknown proportion. In such cases, only an upper limit on the percentage of molecules with low mobility could be determined. Accurate determinations of immobile or low-mobility fractions were performed by using data records that did not show a high fluorescence-modulation level prior to photobleaching. The initial modulation level was minimized by rotating the specimen.

A. proteus and *C. carolinensis* maintained normal morphological characteristics after three successive bleaches, but further photobleaching sometimes altered the appearance of the cell. Therefore, all FPR measurements were limited to no more than three bleaches per cell. None of the reported parameters was observed to vary systematically with successive bleaches.

Patterns bleached in rapidly locomoting cells often moved out of the detection field during measurements. To avoid this problem, cells with relatively low rates of cytoplasmic streaming were selected for direct measurements. Cells with relatively high rates of streaming were either slowed by immersion in a solution of methylcellulose, or they were immobilized by immersion in a medium containing La^{3+}. Cells in 4% methylcellulose showed a decreased rate of streaming but retained their elongate appearance despite the constraints of the viscous fluid. On the other hand, amoebae immersed in media containing La^{3+} stopped locomoting within 2 min. Some cells maintained their elongate appearance in the presence of La^{3+}, whereas other cells became rounded upon the treatment. Neither La^{3+} nor methylcellulose is toxic to amoebae, and both have fully reversible effects on amoeboid movement (29, 30).

RESULTS

Diffusion coefficients of a variety of test proteins in whole *A. proteus* were measured by FPR. Photobleaching recovery of labeled ovalbumin, bovine serum albumin, and ribonuclease A was consistent with a single and highly mobile species (Fig. 2). As shown in Table 1, the diffusion coefficients of these proteins ranged from 2.2×10^{-7} to 6.0×10^{-7} cm^2/sec and, thus, are only 1/2 to 1/3 of the corresponding values in aqueous solution. Similar results were obtained from untreated cells and from cells treated with methylcellulose or La^{3+}. FPR measurements also were performed on labeled ovalbumin injected into a macrophage-like cell line, J744.1 (26). The diffusion of ovalbumin in J744.1 cells was slower than in amoebae and showed more than one component: 80–90% of the ovalbumin in J744.1 cells had a diffusion coefficient of $3.2–3.5 \times 10^{-8}$ cm^2/sec, whereas 10–20% of the ovalbumin had a diffusion coefficient of $<1 \times 10^{-9}$ cm^2/sec (Table 1).

The kinetics of the recovery of AF-actin in *A. proteus* consisted of two components (Fig. 2). About 90% of the actin was highly mobile, both in untreated cells and in cells treated with methylcellulose or La^{3+}. The diffusion coefficient of this component was $2.0–4.0 \times 10^{-7}$ cm^2/sec for cells in media containing La^{3+} and was $1.1–2.5 \times 10^{-7}$ cm^2/sec for untreated cells or cells in 4% methylcellulose (Table 2). These diffusion coefficients are only 1/2 to 1/8 of that of G-actin in aqueous solution

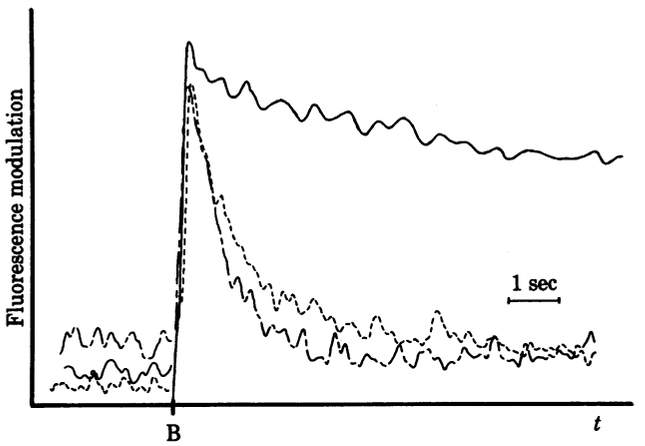

FIG. 2. Kinetics of recovery after photobleaching, recorded by using modulation detection (17). The curves represent the measured magnitude of the alternating current component (extent of modulation) in the photocurrent. A ×16 objective and ruling of 100 lines per 2.5 cm were used with Zeiss epifluorescence optics. This combination produces a striped pattern in the specimen with a period of 29.5 μm. Bleaching occurred at position B on the trace. —·—, fluorescein isothiocyanate-labeled bovine serum albumin; ---, AF-actin; ——, AF-actin with phalloidin.

(5). The remaining AF-actin (10–20%) was of low mobility, with a diffusion coefficient of $<1 \times 10^{-8}$ cm^2/sec (Table 2), which was comparable to the diffusion coefficient of F-actin *in vitro* (5).

The AF-actin data indicated that only ≈10% of the actin in the cytoplasm of amoebae had a mobility comparable to that of F-actin *in vitro*. Therefore, the question arose: can stable F-actin in living cells be detected by the FPR technique? To answer this question, we injected the toxin, phalloidin, into amoebae previously injected with AF-actin. Phalloidin induces actin polymerization and stabilizes F-actin *in vitro* and *in vivo* (31, 32). Upon microinjection of phalloidin, the amoeba cytoplasm contracted into a dense and highly fluorescent knot (18). The contraction was accompanied by a decrease in the mobility of AF-actin (Fig. 2 and Table 2). As a result, ≈50% of the actin in phalloidin-injected cells showed a low diffusion coefficient ($D < 1 \times 10^{-9}$ cm^2/sec). In contrast, the mobility of labeled ovalbumin was not affected by subsequent injection of phalloidin, indicating that the decrease in the mobility of AF-actin after injection of phalloidin was not simply due to changes in cytoplasmic viscosity.

Two approaches were used to identify the subcellular location

Table 1. Diffusion coefficients of globular proteins at 25°C

Protein	M_r	Diffusion coefficient × 10^7, cm^2/sec: Water	Diffusion coefficient × 10^7, cm^2/sec: *A. proteus* cytoplasm*
Ribonuclease A	14,000	13.6	3.2–6.0
G-actin	43,000	9.2	see Table 2
Ovalbumin	45,000	8.9	2.2–5.3†
Bovine serum albumin	68,000	6.8	3.3–4.6

*The range of values are obtained from untreated cells, cells in 4% methylcellulose, and cells treated with $LaCl_3$.

†This range includes cells doubly injected with ovalbumin and phalloidin. Ovalbumin injected into J744.1 cells shows a major diffusing component (80–90%) in the D range of 3.2–3.5 × 10^{-8} cm^2/sec and a minor diffusing component (10–20%) with a D of $<1 \times 10^{-9}$ cm^2/sec.

Table 2. Diffusibility at 25°C of AF-actin incorporated into amoebae

Condition	High-mobility component $D \times 10^7$, cm^2/sec	Low mobility fraction, %
	A. proteus	
Intact Amoebae		
In culture medium or 4% methylcellulose	1.1–2.5	<10
Treated with $LaCl_3$	2.0–4.0	10–20
Injected with phalloidin	0.2	50
	C. carolinensis	
Intact Amoebae		
Tips of pseudopods treated with $LaCl_3$	2.5	<10
Tips of pseudopods in 4% methylcellulose	5	<20
Tails in 4% methylcellulose	1.7	50
Tails treated with $LaCl_3$	—	100
Extruded Cytoplasm		
In ≈10 nM free Ca^{2+}	0.5–1.5	0
In ≈1 μM free Ca^{2+}	0.6–3.0	30–35
Isolated Plasmalemma-Ectoplasm in ≈10 nM free Ca^{2+}	0.5–1.1	45–85

of the actin with low mobility. First, measurements by FPR were carried out with a high-power objective in small regions of the tips and the tails of *C. carolinensis*. AF-actin showed relatively high mobility at the tips of pseudopods (D = 2.5–5.0 × 10^{-7} cm^2/sec, Table 2). In contrast, 50–100% of the actin showed low mobility in the tail ($D < 1 \times 10^{-8}$ cm^2/sec, Table 2).

In a second approach, plasmalemmas were mechanically separated from the cytoplasm of single *C. carolinensis* preinjected with AF-actin (18, 19). The plasmalemma-ectoplasm of ruptured amoebae contained 45–85% low-mobility actin ($D < 3 \times 10^{-9}$ cm^2/sec, Table 2). The extruded cytoplasm, on the other hand, contained predominantly actin of higher mobility (D = 0.5–1.5 × 10^{-7} cm^2/sec, Table 2). Similar diffusion coefficients were obtained from fibrils oriented from the stabilized cytoplasm with a microneedle. However, when the stabilized cytoplasm was subsequently rinsed with the contraction solution (free $Ca^{2+} \approx 10^{-6}$ M), a large low-mobility fraction of actin was observed (30–35% with $D < 1 \times 10^{-8}$ cm^2/sec, Table 2). Single cell models from *A. proteus* gave results consistent with those from *C. carolinensis*.

DISCUSSION

The diffusion coefficients reported in this study are tracer diffusion coefficients defined for the distance specified by the spacing of the pattern in the sample. We have not observed a distance dependence of the measured tracer diffusion coefficients, but it is to be expected that slight differences might be observed by varying the spacing or the orientation of the pattern because of the internal structure of the cytoplasmic matrix. However, none of the conclusions of this report should be limited by the restriction of interpreting our data in terms of the tracer diffusion coefficient.

Our FPR measurements of microinjected ovalbumin, bovine serum albumin, and ribonuclease A indicate that soluble test proteins diffuse rapidly in the cytoplasm of amoebae. Because

only a single diffusing component was observed, the mobility of these proteins cannot differ greatly in cytoplasmic domains such as endoplasm and ectoplasm. In addition, because the diffusion coefficients are only 1/2 to 1/3 of those in water, the cytoplasm of amoebae cannot be more than 2 to 3 times more viscous than water. However, the cytoplasm of amoebae is highly viscoelastic as measured by micromanipulative and optical methods (4, 33). Therefore, the structure of the cytoplasm in amoebae could be formed by crosslinked filaments that hinder only the diffusion of large probes but not the diffusion of small protein molecules. It should be interesting to determine the limit of permeation in the cytoplasm using probes of different sizes.

The two discrete diffusion coefficients of labeled ovalbumin in J744.1 cells are both significantly lower than the single component in amoebae. In a previous study, the diffusion coefficient of labeled bovine serum albumin in the cytoplasm of fibroblasts was measured by FPR as $\approx 1/70$ of that in water (15). Because the measurements were limited to components with diffusion coefficients of $< 4 \times 10^{-8}$ cm^2/sec, it is not certain if there were also a second diffusive component, above the detection limit, in fibroblasts. The significant differences in protein mobility between amoebae and small cultured cells probably reflects different structural properties of their cytoplasm. For example, the cytoplasm of amoebae does not contain significant numbers of microtubules or intermediate filaments. Because the diffusion coefficient of bovine serum albumin in fibroblasts increases after colchicine treatment, one may conclude that a combination of various cytoskeletal structures could inhibit the diffusion of soluble molecules (15). In addition, differences in the abundance of intracellular membranes also could affect the diffusion of soluble molecules.

The interpretation of our results with AF-actin is based on the assumption that the microinjected actin analogue can incorporate functionally into cellular pools of endogenous actin. Although it is difficult to demonstrate such functional incorporation directly, a number of observations indicate that at least under some experimental conditions, microinjected AF-actin shows properties identical to those of endogenous actin. For example, intracellular bundling and aggregation of injected AF-actin can be induced by microinjection of phalloidin or excess free Ca^{2+} (9, 18). In addition, when cells are ruptured in the stabilization solution, a fraction of AF-actin, but not labeled ovalbumin, remains associated with the stabilized cytoplasm and the plasmalemma-ectoplasmic ghost (9, 18). In the present study, changes in mobility of AF-actin are observed when isolated cytoplasm is subjected to changes in free Ca^{2+} concentration, indicating that microinjected AF-actin can respond to cytoplasmic messengers.

Previous studies of cellular actin have been limited primarily to electron microscopy and immunofluorescence techniques, which yield little information about the dynamics of actin in the cell. These static images could give a false impression that actin exists predominantly in a stable filamentous form. By combining fluorescent analogue cytochemistry and the FPR technique, we have identified a high percentage of mobile actin in the cytoplasm. This high percentage of mobile actin can be explained in at least two ways. First, most cytoplasmic actin could be present in monomeric or oligomeric forms (or both), and structures in the cytoplasm could be formed by only a small fraction of actin. This explanation is supported by several biochemical observations. The formation of gel structures *in vitro* requires only low concentrations of F-actin and crosslinking factors (4, 8). In addition, a significant fraction of actin in cell extracts is present in the nonfilamentous form (34, 35). A number of proteins are known to affect the assembly state of actin, including profilin, which stabilizes actin in the monomeric form, and gelsolin, which converts F-actin into short fragments or oligomers (see ref. 8 for a review). A large excess of unpolymerized or oligomeric actin would ensure the availability of actin subunits when polymerization at specific sites is required, as possibly during rapid and frequent redirection of amoeboid locomotion.

Alternatively, a large percentage of actin in the cytoplasm may be present in a polymerized but highly dynamic form, and its high mobility might be caused, therefore, not by thermal diffusion but by energy-dependent movements of actin filaments. Such a mechanism could involve rapid exchange of actin subunits or interactions with myosin, or both. One of the possible mechanisms of actin subunit exchange, head-to-tail polymerization, could result in effective translocation of filaments when coupled to constant association with anchorage sites (36). It is also possible that actin filaments interact constantly with myosin and undergo constant translocation. This latter possibility is supported by the results from cytoplasm isolated in the stabilization solution, where actin remains at least temporarily in the filamentous form (19) yet still exhibits a mobility higher than that of F-actin in solution.

To distinguish between the above two explanations, it will be necessary to determine the size distribution of actin-containing structures in the cytoplasm. One possible approach is to combine fluorescent analogue cytochemistry and fluorescence polarization techniques to measure rotational freedom, which is less sensitive to directional filament movements and could yield more definitive information about the size of actin-containing structures.

The plasmalemma-ectoplasmic ghosts of amoebae were associated with actin of relatively low mobility. If this result represents the normal state of living cells, then it is possible that a distinct fraction of cellular actin is stably associated with the membrane. The result also suggests that the membrane-to-cytoplasm (surface-to-volume) ratio could affect the measured percentage of mobile actin in intact cells. For example, in cultured cells that have a higher membrane/cytoplasm ratio than amoebae, a significantly higher percentage of low-mobility actin would be observed.

The low mobility of actin in the tail of amoebae could reflect active cytoplasmic contraction. This explanation is consistent with the presence of fibrillar actin-containing structures in the tail cortex (18, 37) and with the presence of free Ca^{2+} in the tail sufficient to induce contraction (21). The presence of myosin filaments in the tail region also has been reported (37). Alternatively, it is possible that cytoplasmic streaming depletes preferentially a mobile fraction of actin, leaving behind only the fraction that is stably associated with the membrane. The absence of low-mobility actin in the tips, on the other hand, suggests that either there is little contractile activity there, or that contraction in tips is highly transient or localized.

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