

Abundance, Relative Gelation Activity, and Distribution of the 95,000-dalton Actin-binding Protein from *Dictyostelium discoideum*

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ABSTRACT We have studied the abundance, relative gelation activity, and distribution of the 95,000-dalton actin-binding protein in *Dictyostelium discoideum* amoebae. The 95,000-dalton protein was a prominent polypeptide as assessed using quantitative densitometry and radioimmunoassay. We estimated that this protein comprised ~1.2% of the protein in a soluble extract of amoebae. The molar ratio of the dimeric 95,000-dalton protein to actin in the soluble extract was 1:30. The apparent viscosities of actin mixtures with either the purified 95,000-dalton protein or the soluble extract were measured by falling ball viscometry in an attempt to assess the contribution of the 95,000-dalton protein to gelation of the soluble extract. The gelation of the soluble extract was significantly less than that expected from the contribution of the 95,000-dalton protein alone. Consequently, we questioned the validity of quantitative analyses of the contributions of specific actin-binding proteins to the gelation of cell extracts. The apparent distribution of the 95,000-dalton protein was observed in chemically fixed and extracted cells by immunofluorescence microscopy and compared with the distribution of cytoplasm and organelles visible using light microscopy. The 95,000-dalton protein was dispersed throughout the cytoplasm of fixed cells, was apparently excluded from prominent organelles, and displayed brightest fluorescence in regions of hyaline cytoplasm. These regions of hyaline cytoplasm that exhibited the brightest fluorescence were observed in the cortical region of rounded cells and in pseudopods of polarized cells. Thus, cell shape and polarity may also have influenced the apparent distribution of the 95,000-dalton protein observed by immunofluorescence microscopy. Study of the distribution of fluorescein-labeled ovalbumin injected into living cells supported the interpretation that the thickness of the cell and the distribution of organelles contributed to the apparent distribution of the 95,000-dalton protein observed in fixed cells using immunofluorescence microscopy. We suggest that the 95,000-dalton protein contributes to modulation of the consistency and contractility of the cytoplasm of *D. discoideum* amoebae, since it could cross-link actin filaments *in vitro* in a reversible process that was regulated by changes in the concentration of calcium and of protons, and since it was present in large quantity in the cytoplasm of these cells.

Characterization of the physical properties of cytoplasm is required for understanding cell shape and movement. The results of ultrastructural, rheological, and biochemical analyses indicate that cytoplasm is a viscoelastic substance composed in part of a network of actin filaments cross-linked by actin-binding proteins (35). Amoebae of the cellular slime

mold *Dictyostelium discoideum* has been useful for studying the consistency and contractility of cytoplasm. Gelation and contraction of soluble fractions derived from these cells are regulated by changes in the concentrations of Ca^{++} and H^+ in the physiological range (36, 11, 19).

A number of proteins may contribute to these changes in

the structure and contractility of cytoplasm of *D. discoideum* amoebae. Hellewell and Taylor (19) identified and partially purified a 95,000-dalton actin-binding protein that formed a gel when mixed with actin at low free $[Ca^{++}]$ but not at elevated free $[Ca^{++}]$. This protein was subsequently purified and characterized as a calcium- and pH-sensitive actin-binding protein with some properties similar to those of muscle α -actinin (15, 12). A 120,000-dalton protein has an actin-binding activity that is not affected by the free $[Ca^{++}]$ (9). Brown et al. (6) have isolated a 40,000-dalton protein that restricts the lengths of actin filaments in the presence of greater than micromolar free $[Ca^{++}]$. A 30,000-dalton protein has a calcium-sensitive actin-binding activity (19, 39; Brier and Taylor, unpublished results). A partially purified fraction from *D. discoideum* (39) exhibits an activity similar to that described for actin filament capping proteins (20, 24). The activity of this *D. discoideum* capping protein is not affected by micromolar calcium (39). Proteins that may regulate the number, length, and interactions of actin filaments are also present in many other cell types (31, 13, 22, 45).

The cellular functions of the various actin-binding proteins in the cytoplasm of *D. discoideum* amoebae are not known. In this study, we assess the potential role of the 95,000-dalton actin-binding protein by investigating its abundance, relative gelation activity, and distribution in cells. In addition, experiments that address the problems inherent in interpreting immunofluorescence images, and in quantifying the relative gelation activity of actin-binding proteins are discussed. A preliminary report of these results has been presented (16).

MATERIALS AND METHODS

Cells: *D. discoideum* amoebae, strain NC-4 (haploid), were grown on 2% nutrient agar with *Escherichia coli*, strain B/r, as a food supply. Cells were grown for 2 d, washed 3 times with Bonner's saline solution (BSS) (4) to remove any remaining bacteria, and placed on glass slides coated with poly-L-lysine (Sigma Chemical Co., St. Louis, MO). To prepare cells for orientation, *E. coli*-free suspensions of *D. discoideum* were developed on 2% non-nutrient agar for 8–12 h until aggregation streams were visible. These cells were then washed with BSS and placed on 24 × 15 mm poly-L-lysine-coated glass coverslips. Each coverslip was inverted and attached to a Zigmund chemotaxis chamber (49) such that a clearly marked area (1 × 24 mm) containing cells was aligned with a bridge separating two wells. One well was filled with 17 mM phosphate buffer containing 1 mM $CaCl_2$ (pH 6.1), and the second well contained this same buffer plus 2×10^{-6} M cyclic AMP, a chemoattractant for developing *D. discoideum* amoebae (4). Orientation was monitored using a light microscope and usually required ~25 min for a maximum response.

Preparation of IgG Reactive with the 95,000-dalton Protein: The 95,000-dalton actin-binding protein was purified from extracts of *D. discoideum* amoebae (strain AX-3) as described previously (15). A solution containing 0.3 mg of antigen diluted 1:1 with Freund's complete adjuvant (Difco Laboratories, Inc., Detroit, MI) was injected into the back and leg muscles of a rabbit. Blood was collected prior to immunization and once a week for 3 mo after immunization. Serum was stored at $-20^\circ C$.

IgG-containing fractions (preimmune and immune) were prepared by bringing the sera to 40% saturation in ammonium sulfate (Schwartz/Mann, Inc., Spring Valley, NY) at $4^\circ C$, and removing the precipitate by centrifugation at 12,000 g for 10 min. The pellets were resuspended in 10 mM KH_2PO_4 , 10 mM K_2HPO_4 , pH 7.0, dialyzed against this solution, and applied to columns of DEAE-cellulose (DE-52, Whatman Chemical Separation, Inc., Clifton, NJ) consisting of ~2.5 ml of bed vol/ml of serum, and equilibrated with the same solution. The preimmune and immune IgG were characterized by polyacrylamide gel electrophoresis (23) and stored at $-20^\circ C$.

Analysis of antigen-antibody interaction using immunodiffusion and immunoelectrophoresis was performed as described (46). Glass microslides were coated with 0.6% agarose-L (LKB Instruments, Inc., Rockville, MD) in 0.06 M barbital buffer (Sigma Chemical Co.), pH 8.6. Precipitin arcs were recorded photographically under dark-field illumination. The IgG-containing fraction

derived from samples of serum reactive with the 95,000-dalton protein is referred to as immune IgG or anti-95,000-dalton IgG.

Immunofluorescence Microscopy: Cells were fixed in 3.7% formaldehyde in 17 mM phosphate buffer (pH 7.1) for 20 min, washed with buffer, extracted with acetone for 5 min at $-20^\circ C$, and allowed to dry in air (34). Amoebae were treated with 10% goat serum to reduce nonspecific staining. IgG-containing fractions were diluted to 0.13 mg/ml in 17 mM phosphate buffer (pH 7.5), 100 mM NaCl, and 1% goat serum, and clarified for 20 min in an airfuge (Beckman Instruments, Inc., Palo Alto, CA). Cells were exposed to IgG for 45 min. at $37^\circ C$. After repeated washes to remove free IgG, 27 μg /ml of rhodamine-conjugated goat anti-rabbit IgG (Rh-GAR IgG) (Cappel Laboratories, Cochranville, PA) was applied to the cells for 30 min. at $37^\circ C$, in 17 mM phosphate buffer (pH 7.5) with 100 mM NaCl. Controls included reaction of *D. discoideum* amoebae with (a) IgG derived from preimmune serum followed by Rh-GAR IgG; (b) immune IgG mixed with purified 95,000-dalton protein (molar ratio of immune IgG to dimeric 95,000-dalton protein = 20:1) followed by Rh-GAR IgG; (c) Rh-GAR IgG alone; and (d) buffer alone to check for autofluorescence. Cells were observed with Nomarski optics (40× water immersion objective; NA, 0.75) and for rhodamine fluorescence (63× water immersion objective; NA, 1.2) on a Zeiss photomicroscope III. Images were recorded on Ilford XPI 400 film.

Controls for Interpreting Immunofluorescence: Fluorescein-labeled ovalbumin (FTC-ovalbumin) was microinjected into living vegetative *D. discoideum* amoebae (strain AX-3). The cells were washed in 17 mM phosphate buffer containing 1 mM $CaCl_2$, pH 6.1, and were allowed to attach to poly-L-lysine-coated slides. Preparation of the labeled protein and microinjection were performed as previously described (42, 44, 1). The distribution of fluorescence intensity was recorded with a silicon-intensified target camera (RCA 1030H, RCA Electro-Optics & Devices, RCA Solid State Div., Lancaster, PA) and a $\frac{3}{4}$ in videotape recorder (Nippon Electric Co., Elk Grove Village, IL). Observation of fluorescence distribution of this soluble protein reveals the effects of pathlength and accessible volume on the fluorescence of fluorescently-labeled functional proteins (41, 42, 44).

Viscometry: The apparent viscosity of various concentrations of the chromatographed extract of *D. discoideum* was measured in the presence of 0.8 mg/ml of rabbit muscle F-actin using the falling ball technique (25, 15). The measurements were made in the presence of 20 mM PIPES, pH 6.8, 1 mM $MgCl_2$, 1 mM ATP, 5 mM EGTA, 0.25 mM $CaCl_2$, and 50 mM KCl. Actin was purified from an acetone powder of rabbit skeletal muscle (33), stored, and prepared as previously described (17) except that the actin was polymerized for 1 h in the presence of 2 mM $MgCl_2$ and 50 mM KCl. The extract was mixed with F-actin on ice, drawn into 100- μl glass capillaries, and held at $28^\circ C$ for 1 h before determining the apparent viscosity. Results are the average of triplicate determinations. Apparent viscosity was estimated using calibration curves generated by glycerol solutions of known viscosity as previously described (17). A semiquantitative characterization of the consistency of these non-Newtonian solutions was obtained using the falling ball technique.

Soluble extracts of *D. discoideum* amoebae (strain AX-3) were prepared as previously described (15). To establish the ionic composition of the extract to be used for viscometry, 10 ml of extract was chromatographed on a column (2.6 × 10 cm) of Sephadex G-25 (Sigma Chemical Co.) equilibrated in 20 mM PIPES, 50 mM KCl, 5 mM EGTA, 0.25 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.1 mM ATP, and 0.02% sodium azide, pH 6.8. This solution maintains a free $[Ca^{++}]$ of $\sim 2.7 \times 10^{-8}$ M, assuming a K_{app} for Ca^{++} /EGTA buffers of $1.95 \times 10^6 M^{-1}$ at pH 6.8 (2). The polypeptide composition of the chromatographed extract was not detectably different from that of the extract, as assessed using quantitative densitometry.

Western Transfer Technique (Electrophoretic Blotting): The homogenate, soluble extract, or purified 95,000-dalton protein from *D. discoideum* was resolved by SDS PAGE in small gels (5 × 8 cm) composed of linear gradients of 5–10% polyacrylamide (23). Polypeptides in the gel were electrophoretically transferred to a sheet of nitrocellulose paper (BA 83, Schleicher & Schuell, Inc., Keene, NH) by modifying the method of Towbin et al. (43), which was recommended by K. Lawley and D. Branton (Harvard University, Cambridge, MA). The gel and nitrocellulose sheet were placed between layers of filter paper (no. 1 Whatman filter paper, Whatman Chemical Separation, Inc.) and sponge-immersed in 20 mM Tris, 150 mM glycine, 10% methanol, and 0.1% SDS, and subjected to a voltage gradient of ~ 25 V/cm for 2 h. The nitrocellulose paper was transferred to 8 ml of 5% bovine serum albumin (BSA) in 0.15 M NaCl for 1 h. The paper was immersed for 1 h in 8 ml of the same solution containing 2 μg of $[^{125}I]$ IgG reactive with the 95,000-dalton protein. The IgG was labeled with ^{125}I using chloramine T (21), and had a specific activity of 4×10^6 cpm/ μg . The paper was washed with 8 ml of 5% BSA in 0.15 M NaCl for 15 min and rinsed in 8 ml of the same solution. This wash/rinse procedure was repeated three times. The paper was allowed to dry and

was exposed to Kodak X-Omat AR film. Strips of the nitrocellulose paper were excised using the x-ray film to identify appropriate regions, and were counted in a Packard gamma counter (Packard Instrument Co., Inc., United Technologies, Downers Grove, IL). For each experiment, a standard curve was obtained by measuring the quantity of antibody bound to the paper after polyacrylamide gel electrophoresis, and the transfer of known quantities of the purified 95,000-dalton protein. The standard curve was used to determine the quantity of 95,000-dalton protein present in samples of the soluble extract.

Densitometry: Polypeptides were resolved by SDS PAGE and stained with Coomassie Brilliant Blue R-250 (Bethesda Research Laboratories, Gaithersburg, MD). Gels were scanned with a soft laser scanning densitometer (Biomed Instruments, Inc. Fullerton, CA). The quantity of Coomassie Blue bound in the entire lane and in individual polypeptide bands was determined using a computer program written and kindly provided by R. Enriken and W. McClure (Carnegie-Mellon University, Pittsburgh, PA).

RESULTS

Characterization of Anti-95,000-dalton IgG

Specific reaction of the immune IgG with the 95,000-dalton protein was demonstrated using immunodiffusion, immunoelectrophoresis, and the Western Transfer technique. A precipitin arc in a pattern of identity was observed when immune IgG was tested, using immunodiffusion, for reactivity with the purified 95,000-dalton protein and the soluble extract from *D. discoideum* amoebae (Fig. 1a). No precipitin lines were observed when preimmune IgG was tested with the same concentrations of purified antigen and whole extract (Fig. 1b). Precipitin arcs of identical electrophoretic mobility were observed when anti-95,000-dalton IgG was tested, using immunoelectrophoresis, for reactivity with purified 95,000-dalton protein and extract (Fig. 1c). In addition, immune IgG was reactive with a single polypeptide with an apparent molecular weight of 95,000 in a western blot of both cell homogenates and soluble extracts (Fig. 2).

Quantity of 95,000-dalton Protein in the Soluble Extract

The abundance of the 95,000-dalton actin-binding protein relative to other cytoplasmic proteins in *D. discoideum* amoebae was determined in two ways. First, quantitative densitometry (see Materials and Methods) was employed to demonstrate that 2.7% of the Coomassie Blue bound by the polypeptides of a soluble extract resolved, using electrophoresis, in a polyacrylamide gel in the presence of SDS was associated with a protein(s) having an apparent molecular mass of 95,000 daltons (data not shown). Actin comprised 8% of the protein in the extract as determined using the same method. Second, the Western Transfer technique was employed to devise a radioimmunoassay for the 95,000-dalton protein as described in Materials and Methods. The specificity for the 95,000-dalton protein of the IgG fraction used, and a typical reaction against dilutions of the 95,000-dalton protein on a western blot is shown in Fig. 2. The 95,000-dalton protein was $1.2 \pm 0.2\%$ ($n = 5$) of the protein in the soluble extract as assessed using this technique.

Contribution of the 95,000-dalton Protein to the Gelation of the Soluble Extract

The apparent viscosity of a soluble extract of *D. discoideum* amoebae supplemented with 0.8 mg/ml of rabbit muscle F-actin was measured as a function of the concentration of extract protein, as described in Materials and Methods. In addition, we calculated the apparent viscosities of mixtures of

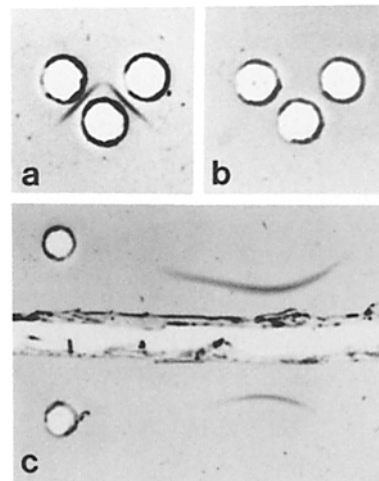


FIGURE 1 Characterization of anti-95,000-dalton IgG by immunodiffusion and immunoelectrophoresis. The purified 95,000-dalton protein (0.07 mg/ml, top left well in a and b) and the soluble extract of *D. discoideum* (20 mg/ml, top right well in a and b) were tested, using immunodiffusion, for reactivity with 8 mg/ml of either anti-95,000-dalton IgG (lower well in a) or preimmune IgG (lower well in b). The 95,000-dalton protein (0.28 mg/ml, top well in c)

and the soluble extract (20 mg/ml, lower well in c) were tested, using immunoelectrophoresis, for reactivity with anti-95,000-dalton IgG (8 mg/ml, trough in c).

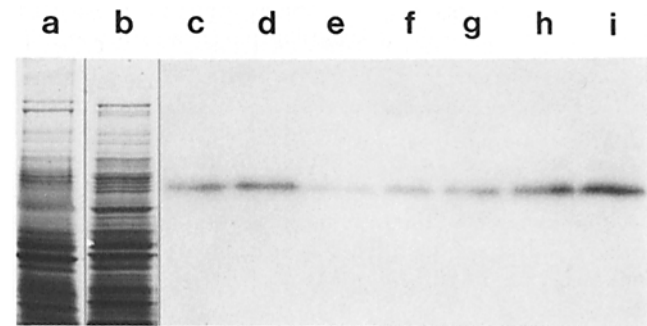


FIGURE 2 Reactivity of anti-95,000-dalton IgG with the 95,000-dalton protein assessed by the Western Transfer technique. Polypeptides were resolved in a minigel composed of a gradient of 5–10% polyacrylamide in the presence of SDS, and stained with Coomassie Blue (a and b), or transferred to nitrocellulose paper, exposed to [125 I] anti-95,000-dalton IgG, and visualized by autoradiography (c–i), as described in Materials and Methods. Samples analyzed were 25 μ g of a homogenate of *D. discoideum* amoebae (a and c); 26 μ g of a soluble extract of *D. discoideum* amoebae (b and d); 55, 165, 275, 385, and 495 ng of the purified 95,000-dalton protein (e, f, g, h, and i). The quantity of 95,000-dalton protein in the soluble extract was determined as described in Materials and Methods. In the experiment shown, the 26- μ g extract sample contained 370 ng of the 95,000-dalton protein.

0.8 mg/ml of actin with the soluble extract that are expected from the contribution of the 95,000-dalton protein in the extract. The quantity of 95,000-dalton protein in an extract sample was calculated from its abundance (95,000-dalton protein comprised 1.2% of the protein in the extract; Fig. 2), and the apparent viscosities of mixtures of 0.8 mg/ml actin with various concentrations of the purified 95,000-dalton protein have been determined previously (15). The actual apparent viscosities were compared with the apparent viscosities predicted from the expected activity of the 95,000-dalton protein (Fig. 3). A solution of 5 mg/ml of extract protein supplemented with 0.8 mg/ml of actin contained 60 μ g/ml of the 95,000-dalton protein and had an apparent viscosity of 10 cp. The apparent viscosity of 60 μ g/ml of the purified 95,000-dalton protein with 0.8 mg/ml actin was >500 cp. Thus, increases in the apparent viscosity of the extract were less than predicted from the expected contribution of the 95,000-dalton protein alone.

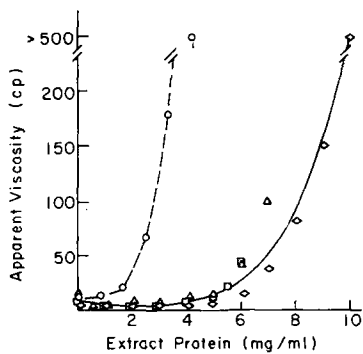


FIGURE 3 Contribution of the 95,000-dalton protein to gelation of the soluble extract. A soluble extract of *D. discoideum* was prepared and eluted from a column of Sephadex G-25 as described in Materials and Methods. The apparent viscosity was measured using falling ball viscometry in the presence of 0.8 mg/ml rabbit skeletal muscle actin, 20 mM PIPES, 50 mM KCl, 5 mM EGTA, 0.25 mM CaCl_2 , 1 mM MgCl_2 , and 1 mM ATP, pH 6.8. Results obtained with three preparations of the soluble extract are shown (\square , Δ , \diamond). The concentration of the 95,000-dalton protein present in each sample of the extract has been calculated assuming that this protein is 1.2% of the protein in the soluble extract. The apparent viscosity of mixtures of the purified 95,000-dalton protein with 0.8 mg/ml of muscle actin has been determined in the same salt and buffer conditions (Fig. 9 in reference 15). The apparent viscosities of mixtures of the soluble extract with muscle actin that are expected from the interaction of the 95,000-dalton protein with muscle actin are illustrated (\circ).

Distribution of the 95,000-dalton Actin-binding Protein in Cells

Nomarski and fluorescence images of cells stained first with immune IgG, preimmune IgG, or immune IgG mixed with the purified 95,000-dalton protein, followed by Rh-GAR IgG, are shown in Fig. 4. A cell stained with anti-95,000-dalton IgG is depicted in Fig. 4*b*. Controls stained with preimmune IgG (Fig. 4*d*) or immune IgG mixed with antigen at a molar ratio of 20 IgG:1 dimeric 95,000-dalton protein (Fig. 4*f*), and stained with Rh-GAR IgG were extremely dim when recorded under the conditions used for Fig. 4*b*. Similar results were obtained for cells stained with Rh-GAR IgG alone or cells not treated with any antibody (data not shown). These results indicate that the fluorescence observed in amoebae stained with anti-95,000-dalton IgG was specific for the *D. discoideum* 95,000-dalton protein.

Vegetative amoebae having a variety of shapes and sizes are illustrated in Figs. 4*b* and 5, which show Nomarski and fluorescence images of cells stained with anti-95,000-dalton IgG. Distinct cortical fluorescence with less bright staining in the central region was observed in the immunofluorescence image of the 95,000-dalton protein in a rounded cell (Fig. 5*b*). The same cell photographed with Nomarski optics appeared round with an edge of hyaline cytoplasm corresponding to the bright fluorescence.

Bright staining was observed in the pseudopods of motile amoebae (Figs. 4*b* and 5, *d* and *f*). Areas of intense fluorescence in pseudopods corresponded to hyaline cytoplasm, which excludes organelles (Figs. 4*a* and 5, *c* and *e*). The central regions of cells were moderately fluorescent and contained domains that were nonfluorescent (Figs. 4*b* and 5, *d* and *f*). The Nomarski images of these cells demonstrated that the nonfluorescent domains contained numerous organelles (Figs. 4*a* and 5, *c* and *e*). The tails of motile cells were sometimes fluorescent (4*b* and 5, *d* and *f*). Cells with less prominent pseudopods exhibited more uniform fluorescence except where organelles displaced the cytoplasm (Fig. 5, *g* and *h*). A filopod at the lower right of this cell was also stained.

Developing cells oriented in gradients of cAMP were elongated, monopodial, and had tapered tails (Fig. 6). The intense fluorescence at the tips of pseudopods of cells stained with anti-95,000-dalton IgG was present in hyaline cytoplasm (Fig. 6). The intensity of fluorescence in the tails of oriented cells was variable. A fluorescence image of cells linked in succession during chemotaxis in response to cAMP is shown in Fig. 6*g*. Bright fluorescence was seen at regions of cell-cell contact.

Distribution of FTC-Ovalbumin in Living Cells

Cells microinjected with FTC-ovalbumin exhibited a non-uniform distribution of fluorescence. The pattern of fluorescence was affected by cell shape, organelle distribution and motility. The relatively organelle-free hyaline regions of extending pseudopods showed the brightest fluorescence. The organelle-rich central cytoplasm had the weakest fluorescence (Fig. 6, *a* and *b*).

DISCUSSION

Quantity of the 95,000-dalton Protein

The 95,000-dalton actin-binding protein comprises 1.2% of the protein in a soluble extract of vegetative *D. discoideum*

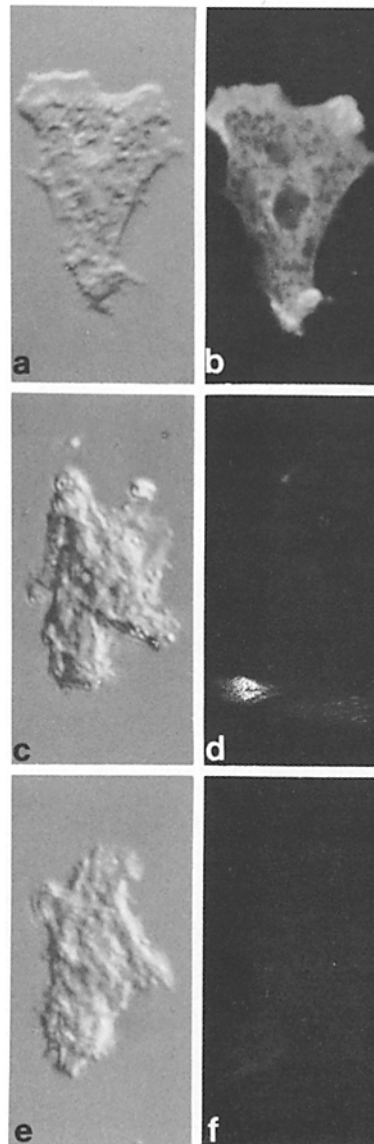


FIGURE 4 Specificity of localization of the 95,000-dalton protein in *D. discoideum* amoebae by immunofluorescence microscopy. Cells were placed on poly-L-lysine-coated glass slides, fixed, and stained with rabbit IgG and Rh-GAR IgG as described in Materials and Methods. Nomarski and fluorescence images of cells stained with anti-95,000-dalton IgG (a and b), preimmune IgG (c and d), and anti-95,000-dalton IgG mixed with the purified 95,000-dalton protein using a molar ratio of 20 IgG:1 dimeric 95,000-dalton protein molecule (e and f) are shown.

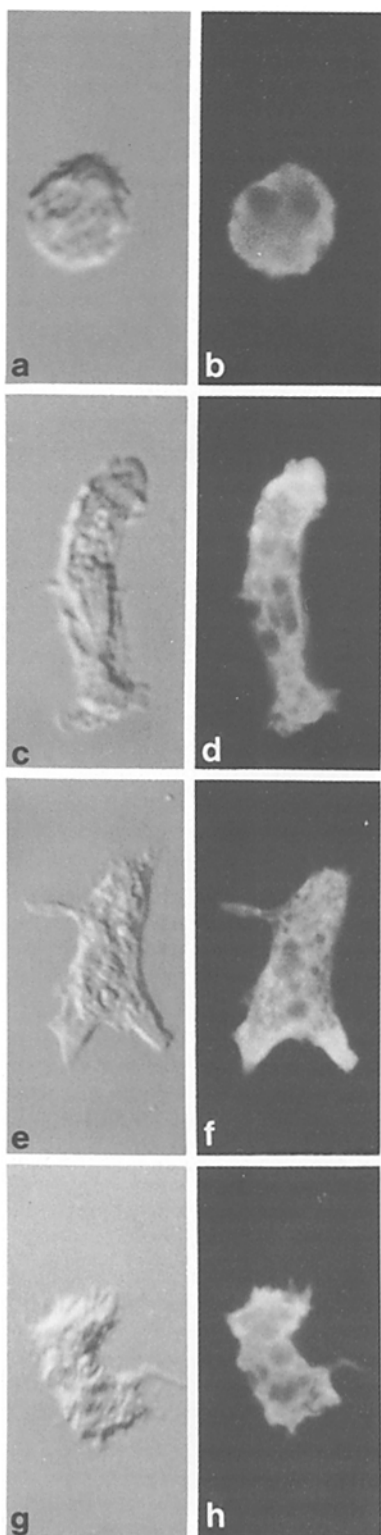


FIGURE 5 Distribution of the 95,000-dalton protein in vegetative *D. discoideum* amoebae. Cells were placed on poly-L-lysine-coated glass slides, fixed, and stained with anti-95,000-dalton IgG and Rh-GAR IgG as described in Materials and Methods. Nomarski (a, c, e, and g) and fluorescence (b, d, f, and h) images are shown.

amoebae. Furthermore, the mRNA coding for this protein is well represented in the total RNA obtained from these cells (unpublished observations). The molar ratio of dimeric 95,000-dalton protein to actin monomer in the extract is 1:30, assuming that 95,000-dalton protein and actin comprise 1.2 and 8.0% of the total soluble protein, respectively. This molar ratio of 95,000-dalton protein to actin in the extract is twice that required to form a gel *in vitro* in the presence of 0.8 mg/ml actin, as assessed using falling ball viscometry (15). There-

fore, the activity of the 95,000-dalton protein in amoebae, which is predicted from its activity *in vitro*, is sufficient to cross-link all of the cellular actin into a network of filaments. However, the cytoplasm of amoebae is not rigidly and uniformly gelled to the extent implied by our *in vitro* experiments (35). We suggest that local variation in the consistency of cytoplasm in cells may be due to variations in (a) the extent of formation and length distribution of actin filaments; (b) the local concentrations of potential regulatory ions such as Ca^{++} and H^+ ; and (c) the distribution of different types of actin-binding proteins.

Quantification of Gelation Activity

Quantitative analyses of gelation activity have been used previously to assess the significance of specific actin-binding proteins to gelation of extracts derived from *Acanthamoeba* (26), macrophages (5), *D. discoideum* (7), and Ehrlich tumour cells (27). We have estimated the relative contribution of the 95,000-dalton protein to gelation of the soluble extract from *D. discoideum* amoebae, since several actin-binding proteins have been identified in these cells (19, 7, 15, 12, 39, 9). Falling ball viscometry has been used as a semiquantitative measure of actin cross linking activity (25, 17), and as a method to determine the relative contributions of other actin-binding proteins from *D. discoideum* (7). We compared the apparent viscosities of mixtures of actin and purified 95,000-dalton protein with apparent viscosities of mixtures of actin and identical concentrations of the 95,000-dalton protein in the soluble extract. Our analysis indicates that the activity of the 95,000-dalton protein is sufficient to account for >100% of the activity. This result would not be substantially affected by a large (twofold) overestimate of the quantity of the 95,000-dalton protein present in the extract. It is difficult to reconcile our results with a previous report that 90% of the activity in the soluble extract from these same cells was due to a combination of the 250,000- and 120,000-dalton proteins (7).

Other studies also indicate that quantitative measurements of gelation activity in crude extracts may be inaccurate. It was reported that four low molecular weight proteins from *Acanthamoeba* accounted for 97% of the gelation activity of the soluble extract (26). Subsequently, a protein that inhibits gelation and an additional potent actin-binding protein were discovered in these cells (20, 29). In addition, the gelation activity present in two partially purified fractions from *D. discoideum* was greater than the gelation activity of the contracted pellet fraction from which they were isolated (19).

Quantitative comparisons of the gelation activity of soluble extracts with that of purified proteins are subject to two major sources of error. First, falling ball viscometry is not a quantitative assay for the formation of actin filament networks (48). Furthermore, no other assay has been described in which the formation of actin filament networks is directly proportional to the concentration of the actin-binding protein. Although it was reported that a sedimentation method did meet this criterion (5), we have observed that measurements with this method are not directly proportional to the concentration of the actin-binding protein (30). Thus, it is difficult to define a unit of gelation activity using these methods. Second, inhibitory, competitive, and/or synergistic interactions of actin-binding proteins may contribute to the total activity of crude extracts, as previously proposed (19). A number of proteins such as gelsolin (47), villin (28), severin (6), *Acanthamoeba*

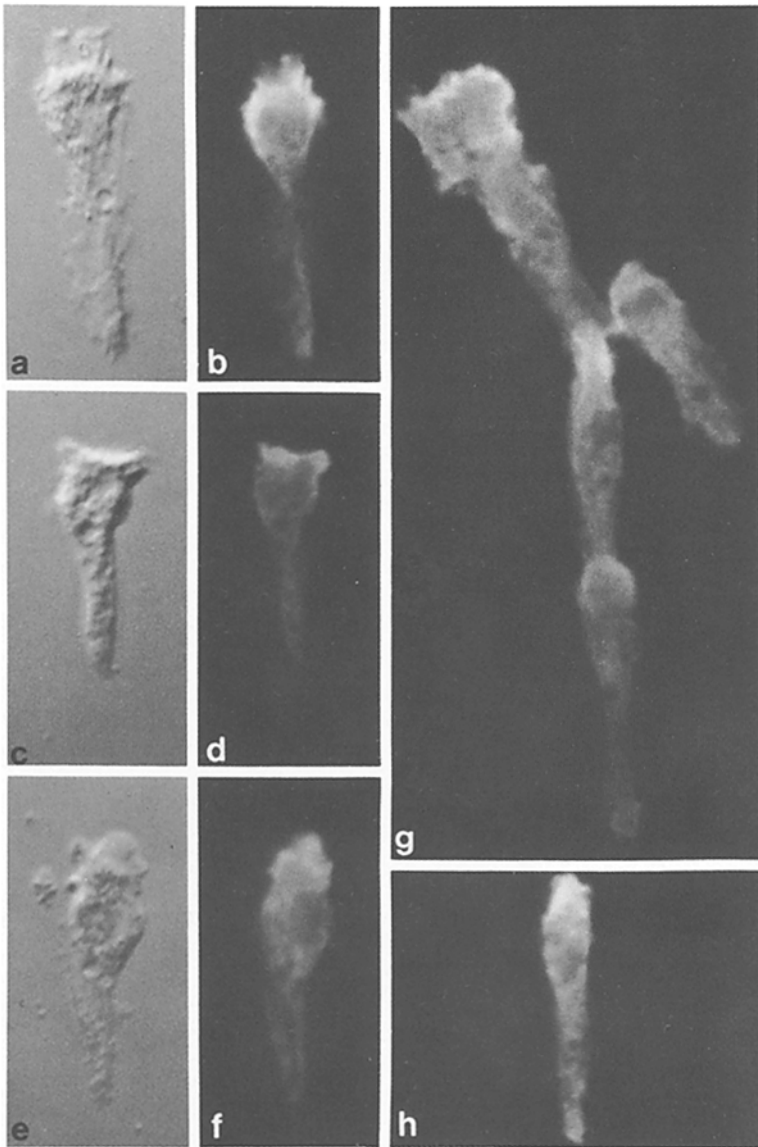


FIGURE 6 Distribution of the 95,000-dalton protein in *D. discoideum* amoebae oriented in a gradient of cAMP. Amoebae were starved to render them sensitive to cAMP as a chemotactic agent, placed in a gradient of 0–2 μ M cAMP on a Zigmond chemotaxis chamber (49), fixed, and stained with anti-95,000-dalton IgG and Rh-GAR IgG, as described in Materials and Methods. Nomarski (a, c, and e) and fluorescence (b, d, f, g, and h) images are shown.

capping protein (20), and acumentin (32) can modulate gelation by affecting the lengths or interactions of actin filaments.

We suggest that the 95,000-dalton protein contributes to gelation of soluble extracts of *D. discoideum* but that a quantitative evaluation awaits development of new approaches.

Distribution of the 95,000-dalton Actin-binding Protein

Molecular hypotheses of cell structure and cell movement must be based on accurate descriptions of the distribution of cytoskeletal and contractile proteins in living cells. Because of its sensitivity and specificity, immunofluorescence microscopy has been used to determine the distribution of specific contractile proteins in a wide variety of fixed and extracted cells (18). The apparent distribution of fluorescence intensity observed using immunofluorescence microscopy must be interpreted with caution, since it may be affected by many factors, including antigen concentration in the living cell, partial extraction of the antigen, cell thickness, and organelle distribution.

It has been stated or inferred in some studies employing

immunofluorescence that the fluorescence intensity is directly related to the concentration of the antigen. Migrating *D. discoideum* amoebae stained with anti-actin antibody exhibited bright fluorescence in both the pseudopods and the tails. It was suggested that actin redistributed into these regions during the transition from rounded feeding cells into the more elongated migrating cells (14). Furthermore, selective localization of the 120,000-dalton actin-binding protein, of calmodulin, and of myosin in the cortex of spread cells has been proposed from interpretations of immunofluorescence images (10, 3).

We have studied the distribution of the 95,000-dalton actin-binding protein in *D. discoideum* amoebae that were either rounded or highly polarized. Our methods for fixation, staining, and observation have been selected to carefully compare fluorescence images with the organization of organelles and cytoplasm in the cells. Immunofluorescence images of the 95,000-dalton protein demonstrate that it is distributed throughout the cytoplasm and is largely excluded from the prominent organelles. Cell shape and polarity also influence the apparent distribution of the 95,000-dalton protein observed using immunofluorescence microscopy. The most intense fluorescence is often observed in the relatively organelle-

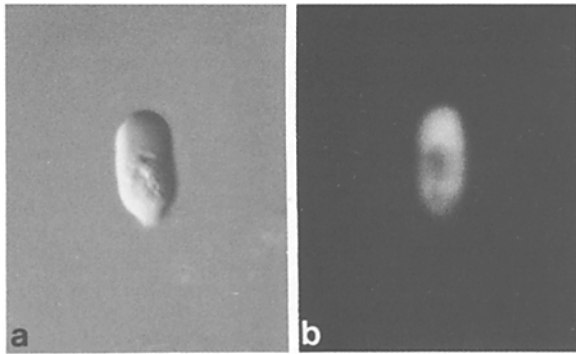


FIGURE 7 *D. discoideum* amoebae injected with fluorescein-labeled ovalbumin. A Nomarski image of a living motile *D. discoideum* amoeba (AX-3) having a prominent hyaline pseudopod is shown (a). Fluorescence image of a similar amoeba microinjected with fluorescein-labeled ovalbumin is shown (b). Bright fluorescence is observed in the hyaline cytoplasm of the pseudopod.

free hyaline cytoplasm in the cortex of rounded cells and in the pseudopods of polarized cells. In contrast, the least intense fluorescence is detected in the central region of cells that contain large numbers of organelles (34).

Fluorescence images of molecules in any cell type cannot be interpreted in terms of the concentration of those molecules without some normalization method for pathlength and accessible volume (41, 44). For example, a cell of uniform thickness and protein distribution may show variations in fluorescence intensity due to the presence of organelles that displace or exclude contractile proteins and thus limit the accessible volume. Therefore, the apparent localization of a cytoplasmic protein, as judged by variations in fluorescence intensity, may not represent an actual variation of concentration in the cytoplasm of the living cell (44, 1). In addition, the possible artifacts due to extraction of antigens or steric hindrance of antibody binding make it even more difficult to interpret immunofluorescence images in terms of local concentrations of protein in the intact cells (1). Control experiments must be performed to determine whether spatial variations in fluorescence intensity are due to uneven distribution or redistribution of specific proteins in cells.

We have evaluated the possible effects of pathlength and accessible volume in *D. discoideum* amoebae by analyzing fluorescence images of living cells injected with FTC-ovalbumin. The fluorescence of labeled ovalbumin in a motile amoeba was brightest in the hyaline cytoplasm of the pseudopod (Fig. 7). The simplest interpretation of this result is that the relatively organelle-free hyaline cytoplasm contains the maximum accessible volume for any soluble protein. Therefore, the bright fluorescence observed in cells stained with anti-95,000-dalton IgG must be interpreted with caution. The apparent localization of the 95,000-dalton protein in the hyaline cytoplasm may be less dramatic than the images suggest and is not necessarily indicative of dramatic variation in the concentration of this protein in the cytoplasm. We are presently developing a general technique that will normalize images from fluorescent analog cytochemistry and immunofluorescence microscopy, in order to analyze quantitatively the distributions of specific proteins in cells.

Functional Implications

The abundance, activity, and distribution of the various classes of actin-binding proteins must be determined before

assessing their relative contribution to the structure and contractility of cytoplasm. The 95,000-dalton actin-binding protein from *D. discoideum* is abundant, is regulated by changes in the concentration of H^+ and Ca^{++} , and is present in areas of cytoplasm not occupied by organelles. Therefore, this protein could play a fundamental role in the regulation of both cytoplasmic structure and contractility as proposed in the solation-contraction coupling hypothesis (19, 40, 7, 8, 37, 38).

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