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.
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) by modifying the method of Towbin et al. (43), which was recommended by K. Lawley and D. Branton (Harvard University, Cambridge, MA). 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 MgCl2, 1 mM ATP, 5 mM EGTA, 0.25 mM CaCl2, 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 MgCl2 and 50 mM KCl. The extract was mixed with F-actin on ice, drawn into 100-μl glass capillaries, and held at 28°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 viscosity, 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, pH 6.8. This solution maintains a free [Ca++] of ~2−7 × 10−10 M, a free [Mg++] of 50 mM, and a free [ATP] of 1 mM. Actin was polymerized for 1 h at 20°C in the presence of 20 mM PIPES, pH 6.8, 1 mM MgCl2, 1 mM ATP, 0.1% sodium azide, pH 6.8. This solution maintains a free [Ca++] of ~2−7 × 10−10 M, a free [Mg++] of 50 mM, and a free [ATP] of 1 mM.

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°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°C, and removing the precipitate by centrifugation at 12,000 g for 10 min. The pellets were resuspended in 10 mM KH2PO4, 10 mM K2HPO4, 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 as polyacrylamide gel electrophoresis (23) and stored at −20°C.

Analysis of antibody-antigen interaction using immunodiffusion and immunoelectrophoresis was performed as described (46). Glass microtodes were coated with 0.6% agarose-L (LK B 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% formaldehyde in 17 mM phosphate buffer (pH 7.1) for 20 min. Acetone buffer, extracted with acetone for 5 min at −20°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°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°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 XP 1100 film.

Controls for Interpreting Immunofluorescence: Fluorescein-labeled ovalbumin (FITC-ovalbumin) was microinjected into living vegetative D. discoideum amoebae (strain AX-3). The cells were washed in 17 mM phosphate buffer containing 1 mM CaCl2, 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). 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).

Fluorescence Microscopy: 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 electrotransferred to a sheet of nitrocellulose 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 [3H]IgG reactive with the 95,000-dalton protein. The IgG was labeled with [3H]IgG using chloramine T (21), and had a specific activity of 4 × 106 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 excited 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. Entriken 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. 1 a). No precipitin lines were observed when preimmune IgG was tested with the same concentrations of purified antigen and whole extract (Fig. 1 b). 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. 1 c). 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 radiolmmunoassay 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 ± 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 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, 0.07 mg/ml, top 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).
the nonfluorescent domains contained numerous organelles except where organelles displaced the cytoplasm (Fig. 5, g and h). A filopod at the lower right of this cell was also stained.

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.
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). Therefore, 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 μM 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 acumenin (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-
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 Ca²⁺, 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 solution-contraction coupling hypothesis (19, 40, 7, 38).

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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.


