

The *Dictyostelium discoideum* 30,000-Dalton Protein Is an Actin Filament–bundling Protein That Is Selectively Present in Filopodia

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Abstract. The interaction with actin and intracellular localization of the 30,000-D actin-binding protein from the cellular slime mold *Dictyostelium discoideum* have been investigated to analyze the potential contributions of this protein to cell structure and movement. The formation of anisotropic cross-linked filament networks (bundles) containing actin and the 30,000-D protein has been observed by electron microscopy, light scattering, viscometry, and polarization microscopy. Co-sedimentation experiments indicate that a maximum of one molecule of the 30,000-D protein can bind to 10 actin monomers in filaments with an apparent association constant of 1×10^7 liters/mol. Inhibition of the interaction of the 30,000-D protein with actin by either magnesium or calcium was observed by viscometry, light scattering, polarization microscopy, and direct binding assays. However, the concentration of magnesium required to diminish the interaction is >100 times greater than that of calcium. The association constant of the 30,000-D protein for actin is 4.2×10^6 liters/mol, or $<1 \times 10^5$ liters/mol in the presence of increased concentrations of either Mg^{2+} or Ca^{2+} , respectively. Enzyme-linked immunoassays indicate that the 30,000-D protein comprises 0.04% of the pro-

tein in *D. discoideum*. Extensive interaction of the 30,000-D protein with actin in cytoplasm is predicted from these measurements of the concentration of this protein and its affinity for actin.

The distribution of the 30,000-D protein was analyzed by immunofluorescence microscopy using monospecific affinity-purified polyclonal antibody. The 30,000-D protein exhibits a diffuse distribution in cytoplasm, is excluded from prominent organelles, and is quite prominent in fine extensions protruding from the cell surface. The number, length, and distribution of these extensions containing the 30,000-D protein are similar to those of filopodia observed by scanning electron microscopy. To analyze the effects of cell thickness and the distribution of organelles on the immunofluorescence localization, fluorescein-labeled BSA was incorporated into the cytoplasm of living cells before fixation and staining using a sonication loading technique. The results indicate that the 30,000-D protein is selectively incorporated into filopodia. These results provide a clear distinction between the multiple actin–cross-linking proteins present in *D. discoideum*, and suggest that the 30,000-D protein contributes to organization of bundles of actin filaments in filopodia.

SINGLE cells perform coordinated movements in order to eat, crawl, and divide. The behavior and ultrastructural features of moving cells have been described by a number of methods for light and electron microscopy that continue to define these processes at ever-increasing levels of resolution (Allen, 1985; Heuser and Kirschner, 1980). Concurrently, biochemical approaches have been utilized for isolation of cellular molecules that may contribute to motility (Korn, 1982; Dustin, 1984; Steinert and Parry, 1985). Present investigations of the structures and potential functions of actin, tubulin, intermediate filaments, and their associated proteins seek molecular descriptions of these complex and dynamic processes.

Actin cross-linking proteins have been isolated from a variety of eukaryotic cells, and are hypothesized to contribute

to the formation of isotropic gelled networks of actin filaments and to anisotropic arrays of filament bundles (Taylor and Condeelis, 1979; Stossel et al., 1985). Actin cross-linking proteins having subunit masses of 120,000 D (Condeelis et al., 1982), 95,000 D (Fechheimer et al., 1982; Condeelis and Vahey, 1982), and two distinct proteins of 30,000 D (Fechheimer and Taylor, 1984; Brown, 1985) have been purified from *Dictyostelium discoideum*. Discrimination between the activities and functions of these proteins is a major challenge at the present time.

Immunofluorescence microscopy has been employed to determine whether individual proteins reside in distinct subcellular locations that may suggest specific biological functions of these polypeptides. For example, bundles of actin filaments and specific actin-bundling proteins have been

localized in specialized regions such as attachment plaques, microvilli, and filopodia of a number of cell types (Geiger, 1983; Mooseker, 1985). Attempts to establish the localization of proteins in specific cellular structures of ameboid cells such as *Chaos carolinensis* and amebas of the cellular slime mold *D. discoideum* have been less fruitful, perhaps because these cells exhibit frequent changes in shape and high rates of cytoplasmic streaming and locomotion. Moreover, variations in cell thickness and organelle distribution may account for variations in fluorescence intensity that have been observed, because methods to determine the effects of these variables have generally not been employed (Taylor et al., 1984; Brier et al., 1983; Taylor and Fehheimer, 1982).

In the present work, both biochemical methods and immunofluorescence microscopy have been utilized to investigate the actin-binding properties and intracellular localization of the 30,000-D protein. Results from viscometry, polarization microscopy, light scattering, and direct binding assays indicate that the 30,000-D protein is a divalent cation-sensitive, actin-bundling protein. Normalized immunofluorescence microscopy and scanning electron microscopy indicate that the 30,000-D protein is selectively incorporated into filopodia. It is suggested that the 30,000-D protein contributes to formation of actin filament bundles in the filopodia of *D. discoideum* amebas. Preliminary reports of this work were presented at the 25th and 26th meetings of the American Society for Cell Biology (Fehheimer, 1985; Furukawa and Fehheimer, 1986).

Materials and Methods

Cells

Dictyostelium discoideum strain AX-3 were grown in HL-5 culture broth, and provided large quantities of cells required for isolation of the 30,000-D protein. *Dictyostelium discoideum* strain NC-4 were cultured on 2% agar in petri dishes in the presence of *E. coli* B/r, and were used for immunofluorescence microscopy and scanning electron microscopy. Strain NC-4 amebas were used for microscopy because locomoting cells are both more numerous and more highly polarized than are cells of the axenic strain. Methods for culture of the AX-3 and NC-4 strains have been described previously (Fehheimer and Taylor, 1984; Brier et al., 1983).

Purification of Proteins

Purification of actin from rabbit skeletal muscle acetone powder and the 30,000-D actin-binding protein from vegetative *D. discoideum* amebas (strain AX-3) was performed as previously described (Fehheimer and Taylor, 1984). Samples for protein determination were first precipitated with TCA (Bensadoun and Weinstein, 1976), and then analyzed by the Folin procedure using BSA as a standard (Lowry et al., 1951).

Rabbit antiserum reactive with the 30,000-D protein was elicited by immunization of a young adult female white rabbit with 0.4 mg of the purified protein emulsified in complete Freund's adjuvant, and boosted 11 wk later with an additional 0.4 mg in incomplete Freund's adjuvant. Serum was prepared from blood collected 2–11 wk after the second immunization. An IgG-containing fraction was prepared by precipitation with ammonium sulfate and chromatography on DEAE-cellulose as previously described (Brier et al., 1983). The antibody was affinity purified by binding to and elution from an electrophoretic blot essentially as described (Talian et al., 1982). Isolation of the 30,000-D protein was performed through the DEAE-Sepharcel ion-exchange chromatography step (Fehheimer and Taylor, 1984). The sample was concentrated to a volume of 1.6 ml using a PM-10 membrane (Amicon Corp., Danvers, MA) and the polypeptides were resolved by electrophoresis in an 8.75% polyacrylamide slab gel (separating gel is 10 × 15 cm and 1.5 mm thick) with no comb in the stacking gel (Laemmli, 1970), and transferred to nitrocellulose paper (BA 83, Schleicher & Schuell, Inc., Keene, NH) in the presence of 20% methanol, 25 mM Tris, 192 mM glycine

(Towbin et al., 1979) in a Transblot Cell (BioRad Laboratories, Richmond, CA) by application of 60 V for 6 h (field strength 7.5 V/cm). The nitrocellulose was treated with 5% BSA (Sigma Chemical Co., St. Louis, MO) for 1 h at room temperature, and then washed with 0.15 M NaCl. The paper was then immersed in 30 ml of phosphate-buffered saline (PBS) containing 30 mg of IgG reactive with the 30,000-D protein, and shaken at 4°C overnight. Elution of affinity-purified antibody was performed as described (Talian et al., 1982). The antibody was concentrated by vacuum dialysis. The yield in two separate experiments was 275 and 590 µg of affinity-purified antibody.

The specificity of the antibody was examined by electrophoretic blotting. Various fractions obtained during purification of the 30,000-D protein were resolved by SDS-PAGE, and transferred to nitrocellulose paper as described above. The nitrocellulose paper was then shaken for 2 h in 100 ml of a solution of 5% dried milk, 0.01% anti-foam A (Sigma Chemical Co.) in PBS (blocking solution) as previously described (Johnson et al., 1984), washed, and stained for 2 h in 5 ml of blocking solution containing 3 µg/ml of the affinity-purified rabbit IgG reactive with the 30,000-D protein. The paper was washed three times for 10 min in 20 ml of blocking solution, immersed in blocking solution containing a 1:2,000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase (Cappel Laboratories, West Chester, PA), washed three times in PBS, and stained with a solution prepared by addition of 50 mg of diaminobenzidine and 10 µl of H₂O₂ (30%) to 100 ml of PBS. The blots were photographed with Kodak Technical Pan film (Eastman Kodak Co., Rochester, NY).

Immunofluorescence Microscopy

Amebas (NC-4) were allowed to spread on glass slides in 17 mM phosphate buffer, pH 6.1, containing 1 mM CaCl₂ for 10–30 min. In some experiments, 10 mg/ml of glucose and 0.03 M NaCl were also present in the solution. The cells were then fixed for 20 min with 3.7% formaldehyde in 17 mM phosphate, 1 mM CaCl₂, pH 7.1, rinsed in the same solution without formaldehyde, extracted with acetone at –20°C for 2 min, air-dried, treated with 17 mM phosphate, 150 mM NaCl, pH 7.1, containing 10 mg/ml BSA, stained with 10 µg/ml affinity-purified anti-30K IgG or normal rabbit IgG for 45 min, washed with PBS, stained with rhodamine-labeled goat IgG reactive with rabbit IgG (Cappel Laboratories; 1:500 dilution of stock), and washed. Light microscopy was performed using either a photomicroscope (Carl Zeiss, Inc., Thornwood, NY) as previously described (Brier et al., 1983) for Fig. 2, or a Zeiss inverted microscope equipped with a 100-W mercury arc lamp, and ×40 (N.A. 0.9) and ×100 (N.A. 1.3) objectives for Figs. 3 and 5. Images were recorded on Eastman Kodak Co. Tri-X film (ASA 400).

Normalized Immunofluorescence Microscopy

The effects of cell thickness and organelle distribution on the immunofluorescence images were estimated by recording the distribution of FITC-labeled BSA (FITC-BSA) present in the cytoplasm of the cell. FITC-BSA was prepared by mixing 1 g of BSA with 80 mg of FITC (Sigma Chemical Co.) in 50 mM sodium carbonate, pH 9.0, at room temperature for 1 h, desalting on Sephadex G-25, and dialysis against 17 mM phosphate, 1 mM CaCl₂, 10 mg/ml glucose, pH 6.1. The FITC-BSA conjugate was loaded into the cytoplasm of *D. discoideum* amebas by controlled sonication. Cells loaded by this method appear normal in morphology, locomotion, chemotaxis, phagocytosis, and development (Fehheimer et al., 1986). 10 million *D. discoideum* amebas (strain NC-4) were suspended in 42 mg/ml of FITC-BSA (dye/protein ratio of 4), sonicated at power 1 for 2 s using a model 200 sonicator (Branson Sonic Power Co., Danbury, CT), diluted into 10 ml of ice-cold buffer to prevent pinocytosis, washed four times in buffer, and plated onto glass slides. Approximately 40% of the cells were recovered, and 2% were loaded with the FITC-BSA. Cells were fixed and stained with antibody reactive with the 30,000-D protein as described above.

Scanning Electron Microscopy

Amebas were allowed to attach and locomote on circular glass coverslips (1.2-cm diam) for 15 min in 17 mM phosphate, 1 mM CaCl₂, 10 mg/ml glucose, pH 6.1. The cells were then fixed on ice in either (a) one part saturated mercuric chloride, three parts 4% osmium tetroxide, and three parts water for 2 min (Parducz, 1966); or (b) 2% glutaraldehyde in 0.1 M cacodylate, pH 7.2, for 1 h, and washed twice for 10 min in 0.1 M cacodylate, 5% sucrose, pH 7.2. After fixation, cells were dehydrated in a graded ethanol series, and subjected to critical-point drying using a Samdri-780A (Tousimis

Research Corp., Rockville, MD). The cells were coated with a layer of gold palladium $\sim 200\text{\AA}$ thick, and examined in a Philips 505T scanning electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ) operated at an accelerating voltage of 15 kV. Images were recorded on Polaroid 665 film (Polaroid Corp., Cambridge, MA).

Buffer Solutions and Calculation of Free Metal Ion Concentrations

The interaction of actin and the 30,000-D protein was investigated in solutions containing 20 mM Pipes, 50 mM KCl, 5 mM EGTA, 1 mM ATP, and 50 μM MgCl_2 , pH 7.0 (buffer B), or the above solution containing 1.05 mM MgCl_2 (buffer B plus Mg^{2+}), or the above solution containing 50 μM MgCl_2 and 5 mM CaCl_2 (buffer B plus Ca^{2+}). The concentrations of free calcium ion and of free magnesium ion present in these three solutions were calculated using the program of Perrin and Sayce (1967). Stability constants of the metal ion complexes used in the calculations are from Sillen and Martell (1964, 1971). These calculations include the complexes of metals both with EGTA and with ATP. The pH of all solutions was maintained at 7.0. Buffer B contains 3.8 μM free magnesium and 2.2 pM free calcium. Buffer B plus Mg^{2+} contains 0.24 mM free magnesium and 0.47 nM free calcium. Buffer B plus Ca^{2+} contains 4.2 μM free magnesium and 12.4 μM free calcium. Some error in the actual free calcium ion concentration is expected in buffer B plus Ca^{2+} , because EGTA is not a good buffer at high ratios of calcium to EGTA, and because the concentration of calcium was not independently investigated by atomic absorption analysis.

Transmission Electron Microscopy of Negatively Stained Proteins

Actin and the 30,000-D protein were mixed at final concentrations of 5 and 2.5 μM , respectively, dialyzed against buffer B for 2 d, and negatively stained essentially as described previously (Valentine et al., 1968). Samples were allowed to adhere for 30 s to carbon films that had been deposited on freshly cleaved mica chips. The mica chips were used to transport the carbon film from sample to buffer B to a staining solution containing 2% aqueous uranyl acetate. The samples were then lifted from the stain onto copper grids, blotted with filter paper, and examined using a Philips 400 transmission electron microscope operated at 80 kV.

Falling Ball Viscometry

The apparent viscosities of mixtures of actin and the 30,000-D protein were measured by falling ball viscometry as previously described (Fechheimer and Taylor, 1984). Samples contained 19 μM rabbit skeletal muscle actin and varying concentrations of the 30,000-D protein in buffer B, buffer B plus Mg^{2+} , or buffer B plus Ca^{2+} .

Light Scattering

Light scattering at a wavelength of 350 nm was measured as an indication of the interaction of the 30,000-D protein with actin. This assay has been used previously to monitor the formation of cross-linked actin structures (Hellewell and Taylor, 1979). Samples of rabbit skeletal muscle actin (19 μM) and varying concentrations of the 30,000-D protein were mixed in semi-micro cuvettes in buffer B or buffer B plus Mg^{2+} . The absorbance at 350 nm was monitored intermittently for 1 h at 28°C using a Gilford 260 recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH).

Polarization Microscopy

Samples for polarization microscopy were sealed between the slide and coverslip immediately after addition of all components, and were held for 2 h in a humid atmosphere at room temperature before examination. Samples contained 19 μM actin and 0, 1, or 2 μM of the 30,000-D protein in either buffer B or buffer B plus Mg^{2+} . A rectified polarization objective ($\times 40$, N.A. 0.65, Nikon Inc., Garden City, NY) and 16 mm condenser (N.A. 0.52) were employed on a Zeiss Ultraphot II using a 200-W mercury arc illuminator. Images were recorded on Kodak Tri-X film.

Binding of the 30,000-D Protein to Actin

Binding of the 30,000-D protein to actin was assessed by co-sedimentation as previously described (Fechheimer and Taylor, 1984). The proteins were

mixed in either buffer B, buffer B plus Mg^{2+} , or buffer B plus Ca^{2+} , held at room temperature for 2 h, sedimented at 23 psi for 30 min in an air-driven ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). The concentrations of the 30,000-D protein and actin were varied from 0.83 to 3.5 μM and 9.5 to 24 μM , respectively. Supernatant and pellet fractions were analyzed by electrophoresis in gels of 10% polyacrylamide in the presence of SDS (Laemmli, 1970), and stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Rockville Center, NY). The quantity of the 30,000-D protein present in individual fractions was determined by scanning the gels with a Quick Scan densitometer (Helena Laboratories, Beaumont, TX). Standard samples of the 30,000-D protein were examined to relate the densitometric scans to the quantity of the protein present and to demonstrate linearity with protein concentration. Results were analyzed by the method of Scatchard (1949).

Determination of the Quantity of the 30,000-D Protein in AX-3 Amebas

Duplicate samples of either a homogenate of *D. discoideum* amebas (strain AX-3) or the purified 30,000-D protein were applied to 0.45- μm nitrocellulose paper (Schleicher & Schuell, Inc.) using a microfiltration manifold (Bio-Rad Laboratories) essentially according to the manufacturer's instructions. The homogenate was prepared by boiling 10^8 cells for 2 min in 1 ml of 20 mM Tris, 500 mM NaCl, 0.1% SDS, 1 mM dithiothreitol (DTT), 1 mM EDTA, 4% Trasylol, 10 $\mu\text{g/ml}$ leupeptin, and 1 $\mu\text{g/ml}$ pepstatin, pH 7.5. The homogenate was diluted before use to contain between 2 and 15 μg of protein per 0.1-ml sample in 0.005% SDS. Standard samples contained 0, 0.05, 0.5, 5, 50, or 500 ng of the 30,000-D protein per 0.1-ml sample diluted in the same buffer containing 20 $\mu\text{g/ml}$ of BSA and 0.005% SDS. These sample solutions all contained 20 mM Tris, 500 mM NaCl, pH 7.5, and were supplemented with 0.05% Tween-20, and with 1% BSA for washing and blocking, respectively. Blocked samples were stained with 5 $\mu\text{g/ml}$ of affinity-purified rabbit antibody reactive with the 30,000-D protein, a 1:4,000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad Laboratories), and stained with a solution prepared by mixing 100 ml of PBS, 20 ml of a 3 mg/ml solution of 4-chloro-1-naphthol (Sigma Chemical Co.) in methanol, and 5 μl of 30% hydrogen peroxide. Stained blots were photographed with Kodak Technical Pan film.

Results

Selective Localization of the 30,000-D Protein in Filopodia

Antibody specific for the *D. discoideum* 30,000-D actin-binding protein was elicited in rabbits and affinity-purified as described in Materials and Methods. The specificity of the antibody was examined by electrophoretic blotting. Numerous polypeptides are observed in a Coomassie Blue-stained polyacrylamide gel in which the soluble extract of *D. discoideum* amebas and fractions obtained during purification of the 30,000-D protein have been resolved (Fig. 1 A). Staining an electrophoretic blot of an identical gel with the affinity-purified antibody reactive with the 30,000-D protein revealed a single polypeptide that migrated with an apparent molecular mass of 30,000 D (Fig. 1 B). It is interesting to note that the 30,000-D protein is nearly quantitatively incorporated into the fraction of proteins sedimented after gelation and contraction of the soluble extract (Fig. 1 B, lane c). The quantity of the 30,000-D protein in the fraction remaining soluble after gelation and contraction is extremely small (Fig. 1 B, lane b).

Differential interference-contrast and fluorescence images of cells stained with antibodies reactive with the 30,000-D protein are shown in Fig. 2. Fluorescence is detectable in both the central and cortical regions of most cells. Regions of low fluorescence intensity in the cell interior suggest that this protein is excluded from prominent organelles. Bright

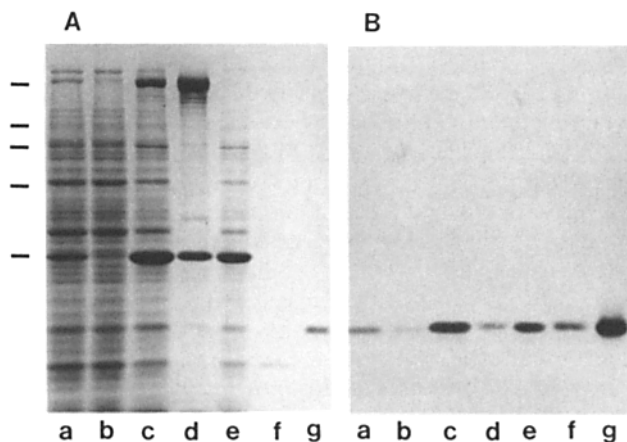


Figure 1. The specificity of affinity-purified antibody reactive with the 30,000-D protein assessed by electrophoretic blotting. Samples were first resolved in a gel of 10% polyacrylamide and then either (A) stained with Coomassie Blue, or (B) transferred to nitrocellulose and stained with antibody reactive with the 30,000-D protein as described in Materials and Methods. The following samples obtained during purification of the 30,000-D protein as described by Fechtmeier and Taylor (1984) were analyzed: (a) 25 μ g of soluble extract of *D. discoideum* (strain AX-3); (b) 25 μ g of contraction supernatant; (c) 25 μ g of contracted gel pellet; (d) 10 μ g of actomyosin; (e) 10 μ g of DEAE-Sephacel load; (f) 1.5 μ g of hydroxylapatite load; (g) 1.5 μ g of 30,000-D protein. Molecular mass markers illustrated at the left are from top to bottom: myosin (200 kD), β -galactosidase (116 kD), phosphorylase b (92 kD), BSA (68 kD), and ovalbumin (45 kD).

fluorescence is frequently observed in the tails and pseudopods of these cells. In this respect, the distributions of the 30,000- and 95,000-D proteins appear quite similar (Brier et al., 1983). However, fluorescence is also detected in numerous fine extensions present on a large fraction of the cells stained with antibody reactive with the 30,000-D protein (Figs. 2, b and d, and 3, a and e). The largest extensions are detectable by Nomarski differential interference-contrast microscopy (Fig. 2, a and c). However, most extensions observed in cells stained with antibody reactive with the 30,000-D protein are not detected by Nomarski, presumably owing to their small diameter. The specificity of immunofluorescence localization of the 30,000-D protein is demonstrated by lack of staining in the absence of primary antibody (Fig. 3 b), or in the presence of nonimmune rabbit IgG (Fig. 3 c). In addition, the staining is almost completely eliminated by mixing the 30,000-D protein with the affinity-purified antibody before staining (Fig. 3 d).

The shape, motility, and morphology of *D. discoideum* amoebas may be influenced by the strain, the state of differentiation, the ionic conditions in the media, and the substrate (Eckert et al., 1977; Gingell and Vince, 1982a, b). Therefore, the cells were observed by scanning electron microscopy to examine in detail the ultrastructure of the fine extensions present at the surface and the variations of cell shape and thickness. The cell preparations and experimental conditions were identical to those employed for fluorescence microscopy up to the time of fixation. Filopodia are present on virtually all cells, although there is considerable heterogeneity in the number, length, and distribution of these structures (Fig. 4). Some cells are extensively coated with filopodia,

whereas others display a rather smooth contour. Filopodia, when present, may be at the front, back, sides, or upper surfaces of cells. These extensions are usually long and straight, and occasionally branched. The filopodia range in length from the shortest detectable structures up to 8 μ m, or approximately one cell diameter in length. The majority of the filopodia are in the range of 2–3 μ m long and 0.1–0.2 μ m in diameter, and are not significantly tapered. Large pseudopodial lamellae are detected on some cells. Rounded cells often exhibited a circumferential ring of filopodia. These observations are consistent with the interpretation that the structures observed by immunofluorescence microscopy using antibody reactive with the 30,000-D protein are filopodia.

Large variations in cell thickness are also evident in the scanning electron micrographs. The thickest portion of a cell may be present either in the center or at one end. Thus, the images also illustrate the fact that methods to estimate variations in cytoplasmic volume are required for interpretation of immunofluorescence images of these cells.

To determine the effects of cell thickness and organelle distribution on the apparent localization of the 30,000-D protein, the distribution of fluorescence intensity of the 30,000-D protein was compared with that of FITC-BSA incorporated into the living cells by controlled sonication as described in Materials and Methods. The distribution of the FITC-BSA exhibits local variation in the cytoplasm, presumably reflecting the variation in cell thickness and organelle distribution of each individual cell. This spatial variation in fluorescence intensity of a soluble molecule is also evident

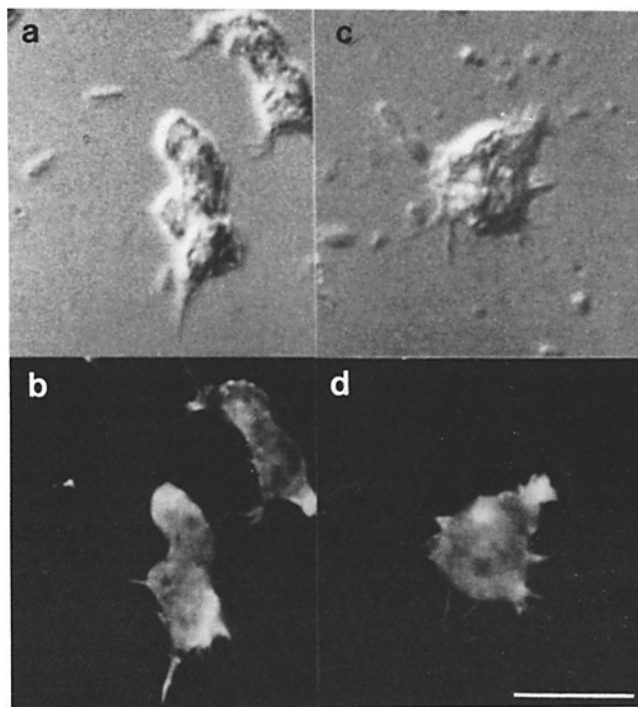
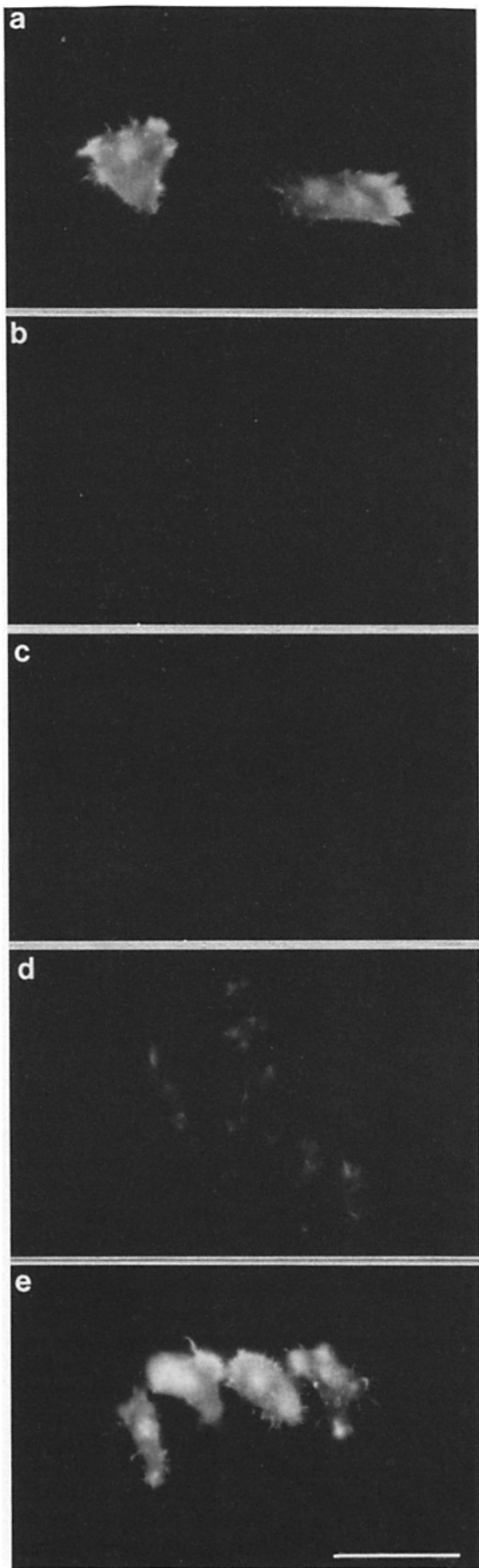


Figure 2. Localization of the 30,000-D protein in *D. discoideum* amoebas using immunofluorescence microscopy. Cells (strain NC-4) were fixed and stained with affinity-purified antibody reactive with the 30,000-D protein and rhodamine-labeled goat antibody reactive with rabbit antibody as described in Materials and Methods. (a, c) Differential interference contrast and (b, d) fluorescence images of stained cells are illustrated. Bar, 10 μ m.



in living *D. discoideum* amoebae (Brier et al., 1983; Fechheimer et al., 1986). The fine extensions are not visible in the images of this fluorescein-labeled (Fl) soluble molecule, suggesting that the volume in these processes is quite small (Fig. 5 a). Thus, the quantity of Fl-BSA in these extensions is too low to detect. The low fluorescence intensity from Fl-BSA observed in the filopodia is not due to extraction during preparation for immunofluorescence microscopy, in that fluorescence is not observed in filopodia in living cells loaded with Fl-dextran or Fl-BSA (Fechheimer et al., 1986; Brier et al., 1983). The 30,000-D protein is readily detected in numerous filopodia present on the same cell (Fig. 5 b). These results indicate that the 30,000-D protein is selectively incorporated into filopodia.

Divalent Cation-sensitive, Actin Filament-bundling Activity of the 30,000-D Protein

Studies of the interaction of the 30,000-D protein with actin were performed in the presence of solutions designed to regulate the concentrations of both free calcium and free magnesium at the desired levels. Buffer B maintains a relatively low concentration of both calcium and magnesium ions, whereas buffer B plus Mg^{2+} and buffer B plus Ca^{2+} contain elevated concentrations of free Mg^{2+} and free Ca^{2+} , respectively (see Materials and Methods). In buffer B plus Mg^{2+} (1.05 mM total Mg^{2+} and 240 μ M free magnesium), the apparent viscosities of mixtures of the 30,000-D protein and actin increase exponentially after the critical concentration of the 30,000-D protein has been exceeded as previously described (Fechheimer and Taylor, 1984) (Fig. 6 B). There are two differences in the apparent viscosities of these mixtures of actin and the 30,000-D protein in buffer B as compared with those in buffer B plus Mg^{2+} . First, the concentration of the 30,000-D protein required to induce an increase in the viscosity of a solution of actin filaments is lower in buffer B. Second, the viscosities decrease dramatically in the presence of increasing concentrations of the 30,000-D protein in buffer B (Fig. 6 A).

The decrease in viscosity in the presence of the highest concentrations of the 30,000-D protein could be explained either by a decrease in the quantity or length of single actin filaments. A decrease in the quantity of single actin filaments can occur either by depolymerization of filaments, by a decrease in the number of single filaments coupled with an increase in the filament lengths, or by the formation of aggregate structures induced by the addition of the 30,000-D

Figure 3. Specificity of the localization of the 30,000-D protein in *D. discoideum* amoebae using immunofluorescence microscopy. Cells (strain NC-4) were fixed and stained with rabbit IgG and rhodamine-labeled goat anti-rabbit IgG as described below: (a, e) cells stained with 10 μ g/ml of affinity-purified rabbit antibody reactive with the 30,000-D protein; (b) cells stained with omission of rabbit IgG; (c) cells stained with 10 μ g/ml of normal rabbit IgG; (d) cells stained with 10 μ g/ml of affinity-purified rabbit antibody reactive with the 30,000-D protein in the presence of 0.15 mg/ml of purified 30,000-D protein. All of the slides were stained with rhodamine-labeled goat anti-rabbit IgG. Experimental conditions, microscopy, photography, and darkroom procedures were carefully controlled to allow direct comparison of the images shown. Bar, 10 μ m.

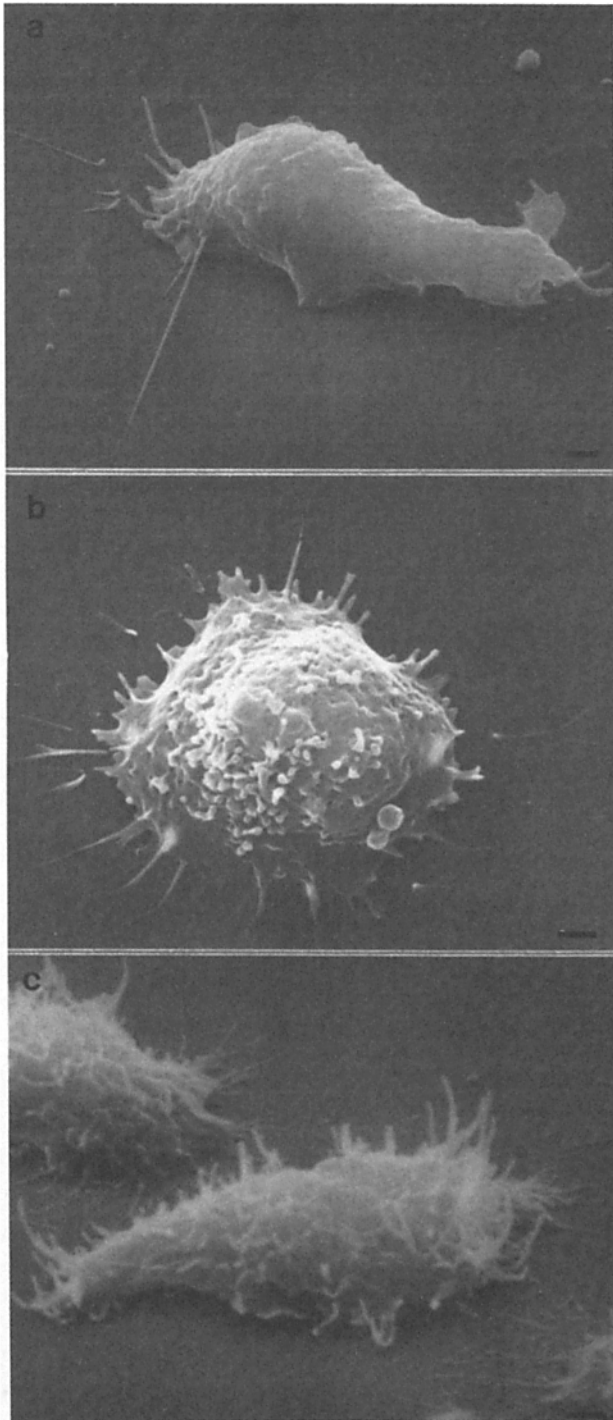


Figure 4. Filopodia of *Dictyostelium discoideum* amebas (strain NC-4) viewed by scanning electron microscope. Considerable heterogeneity in cell morphology, surface contour, and in the number, length, and distribution of filopodia is observed. The filopodia observed by scanning electron microscope are quite similar to the fine projections observed by immunofluorescence microscopy in amebas stained with antibody reactive with the 30,000-D protein. Vegetative amebas (strain NC-4) were fixed on glass coverslips (*a*, *b*) using mercuric chloride/osmium tetroxide, or (*c*) glutaraldehyde as described in Materials and Methods. Bar, 1 μ m.

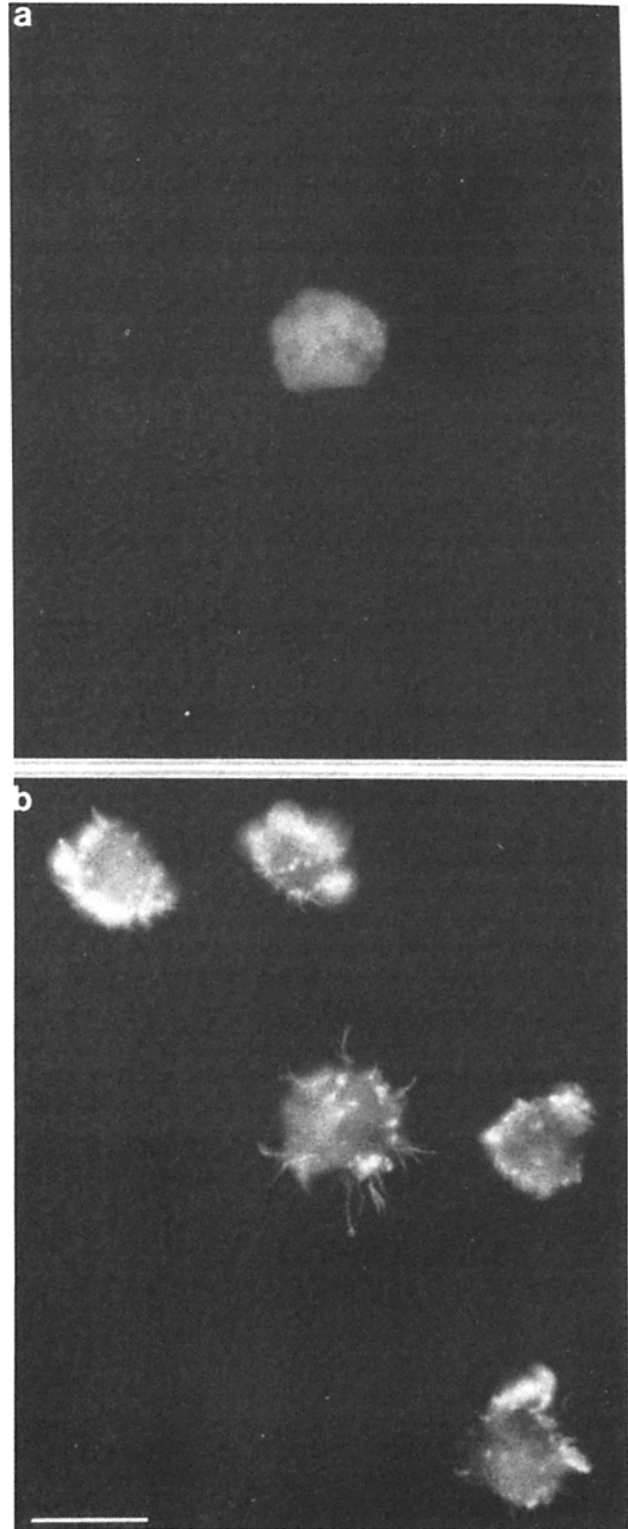


Figure 5. Normalized immunofluorescence microscopy. (*a*) Fluorescein-labeled BSA was introduced into living cells (strain NC-4) by the sonication loading technique, and fixed during preparation of the cells for immunofluorescence microscopy. (*b*) Rhodamine-labeled 30,000-D protein was observed in the same cells by immunofluorescence microscopy. One cell has been loaded with fluorescein-labeled BSA. All five of the cells contain the 30,000-D protein. The results indicate that the 30,000-D protein is selectively incorporated into filopodia. Bar, 10 μ m.

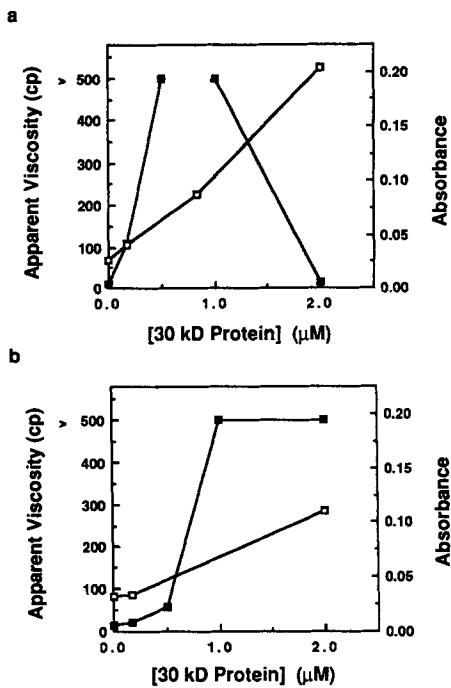


Figure 6. Apparent viscosity and light scattering of mixtures of actin and the 30,000-D protein. Actin (19 μM) and the 30,000-D protein were mixed in the presence of (a) 20 mM Pipes, 50 mM KCl, 5 mM EGTA, 1 mM ATP, and 50 μM MgCl_2 (buffer B), or (b) the same solution containing 1.05 mM MgCl_2 . The apparent viscosity (solid squares) and light scattering at a wavelength of 350 nm (empty squares) were measured as described in Materials and Methods. Inhibition of the interaction of the 30,000-D protein and actin by magnesium is indicated by the relatively high critical concentration of the 30,000-D protein required to increase the apparent viscosity and the relatively low light scattering observed in b.

protein. Light scattering, polarization microscopy, viscometry, sedimentation, and electron microscopy were employed to distinguish between the various possibilities.

The 30,000-D protein does not appear to depolymerize actin filaments, because no change in the quantity of sedimentable actin can be detected (Fechheimer and Taylor, 1984). In addition, the 30,000-D protein does not sever actin filaments, in that no inhibition of the gelation of actin by filamin can be detected (Fechheimer and Taylor, 1984). The linear increase in light scattering observed as the concentration of the 30,000-D protein is increased also indicates that the decrease in viscosity in the presence of high concentrations of the 30,000-D protein cannot be explained either by depolymerization or by severing of the actin filaments, and indicates the formation of aggregate structures (Fig. 6).

The aggregate structures can be either isotropic with a random orientation of the actin filaments with respect to each other or anisotropic with parallel alignments of the actin filaments. Formation of anisotropic filament aggregates was examined directly by polarization microscopy. Anisotropic structures detectable by polarization microscopy are present in F-actin solutions containing 1 μM of the 30,000-D protein, and are more prominent in F-actin solutions having very low viscosity that contain 2 μM of the 30,000-D protein (Fig. 7). These results from polarization microscopy are consistent with the existence of bundles. The possibility that the bun-

dles were induced by flow is considered unlikely for three reasons. First, the low viscosity of these preparations offers independent evidence for the presence of bundles. Second, the anisotropic structures exhibit a range of orientations in the field. Third, negatively stained images of mixtures of actin and the 30,000-D protein in buffer B also demonstrate that bundle formation does occur efficiently under these ionic conditions (Fig. 8). The small size of the bundles observed by electron microscopy may be due to the lower concentration of actin employed for these studies. A detailed investigation of the effects of protein concentration on the morphology of the bundles may clarify this point.

The lower concentration of the 30,000-D protein required to cause an increase in the viscosity of a solution of actin filaments in buffer B as compared with that in buffer B plus Mg^{2+} could be explained by postulating that the activity of the 30,000-D protein is inhibited by magnesium. This hypothesis was tested by light scattering, polarization microscopy, viscometry, and by direct measurements of the binding of the 30,000-D protein to actin. Light scattering from mixtures of 19 μM actin and 2 μM 30,000-D protein is approximately half as great in the presence of buffer B plus Mg^{2+} as in buffer B (Fig. 6). The formation of anisotropic aggregates detectable by polarization microscopy is significantly greater in the presence of buffer B (Fig. 7, d-f) than in buffer B plus Mg^{2+} (Fig. 7, a-c) in the presence of either 1 or 2 μM of the 30,000-D protein. In addition, free magnesium ion reduces the apparent viscosity of mixtures of the 30,000-D protein and actin. The apparent viscosities of mixtures of actin and the 30,000-D protein are reduced as the total concentration of magnesium is increased from 50 μM to 1.05 mM (Fig. 9), with half-maximal viscosity observed at $\sim 250 \mu\text{M}$ magnesium (23 μM free Mg^{2+}). The apparent viscosity of mixtures of the 30,000-D protein and actin is half-maximally inhibited in the presence of $<0.1 \mu\text{M}$ free calcium in the presence of 50 μM total magnesium (Fig. 9). The level of free calcium required for half-maximal inhibition was previously reported to be $1 \times 10^{-8} \text{ M}$ (Fechheimer and Taylor, 1984). Thus, the interaction of the 30,000-D protein and actin appears to be sensitive to calcium at concentrations more than 1,000 times less than that of magnesium as assessed by falling ball viscometry.

The effects of calcium and magnesium on the binding of the 30,000-D protein to actin were investigated by measurement of the co-sedimentation of the 30,000-D protein with actin filaments. The affinity of the 30,000-D protein for actin in buffer B plus Mg^{2+} is significantly less than that in buffer B. In buffer B plus Ca^{2+} (12.4 μM free Ca^{2+}), the interaction decreases to a level that is difficult to quantify by the co-sedimentation assay (Fig. 10). Binding of the 30,000-D protein to actin is saturated at a level of 1 molecule of the 30,000-D protein to 9.5 molecules of actin in buffer B, and 1 molecule of the 30,000-D protein to 10.3 molecules of actin in buffer B plus Mg^{2+} (averages from experiments with three different preparations of the 30,000-D protein). The apparent association constants were 1×10^7 , and 4.2×10^6 liters/mol in buffer B and buffer B plus Mg^{2+} , respectively (averages from experiments with three different preparations of the 30,000-D protein) (Fig. 10). Assuming that the interaction also saturates at a level of 1 molecule of the 30,000-D protein to 10 actin monomers in filaments in buffer B plus

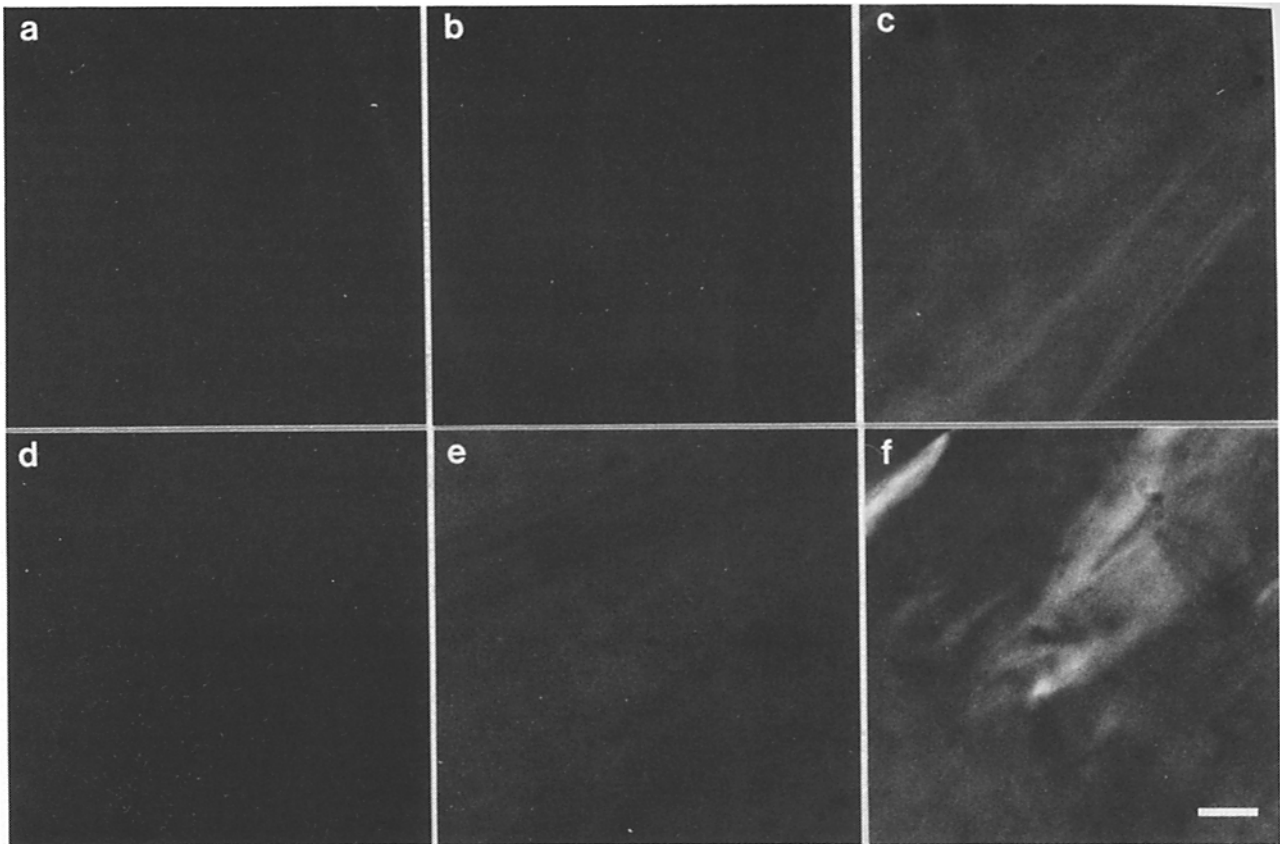


Figure 7. Examination by polarization microscopy of the structures formed in mixtures of actin and the 30,000-D protein. Mixtures contained 19 μM actin, and (a, d) 0, (b, e) 1.0, or (c, f) 2.0 μM of the 30,000-D protein in the presence of (d-f) buffer B or (a-c) buffer B plus Mg^{2+} . No anisotropic structures are observed in the absence of the 30,000-D protein. Formation of anisotropic structures detectable by polarization microscopy is significantly inhibited in the presence of the higher concentration of magnesium. Bar, 10 μm .

Ca^{2+} , the apparent association constant is $<1 \times 10^5 \text{ L/M}$ in this solution.

The binding of the 30,000-D protein to actin was also examined using the co-sedimentation assay in the presence and absence of both Mg^{2+} and Ca^{2+} at a single concentration of actin and the 30,000-D protein. The fraction of the 30,000-D protein co-sedimenting with actin was 99, 73, 29, and 14% in the presence of buffer B, buffer B plus Mg^{2+} , buffer B plus Ca^{2+} , and buffer B plus Ca^{2+} and Mg^{2+} , respectively. Thus, the effects of both calcium and magnesium are inhibitory, and are roughly additive when the two cations are present simultaneously.

The effect of ATP on the interaction of the 30,000-D protein with actin was analyzed by the co-sedimentation assay in buffer B. No significant differences in the quantity of the 30,000-D protein co-sedimenting with actin were detected in the presence of 0.08, 1.08, and 5.08 mM ATP (data not shown).

The quantity of the 30,000-D protein in AX-3 amoebas was determined by a quantitative dot blot procedure as described in Materials and Methods (Fig. 11). The affinity-purified antibody used for these determinations appears specific for the 30,000-D protein as assessed by electrophoretic blotting (Fig. 1). The results indicate that the 30,000-D protein constitutes 0.04% of the total protein in these cells (Fig. 11).

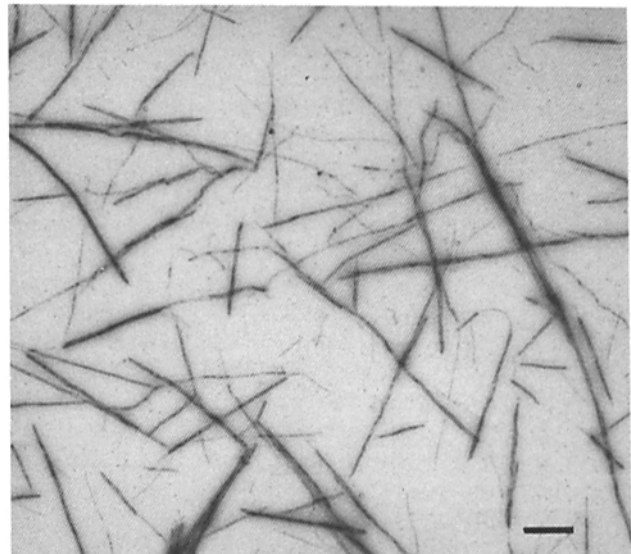


Figure 8. Negatively stained bundles of actin and the 30,000-D protein. Mixtures of actin and the 30,000-D protein in buffer B were negatively stained and observed in the transmission electron microscope as described in Materials and Methods. Filament bundles are formed under these ionic conditions. Bar, 0.5 μm .

Discussion

A number of proteins with the ability to cross-link actin filaments *in vitro* are present both in *D. discoideum*, and in other cell types (Korn, 1982; Stossel et al., 1985). *D. discoideum* is known to contain actin cross-linking proteins with subunit molecular weights of 95,000 D (Fechheimer et al., 1982; Condeelis and Vahey, 1982), 120,000 D (Condeelis et al., 1982), and 30,000 D (p30b) (Brown, 1985) that are distinct from the 30,000-D protein (p30a) (Fechheimer and Taylor, 1984). Evidence that distinguishes the two 30,000-D proteins, p30a and p30b, consists of differences in one-dimensional peptide maps, calcium sensitivity of the actin-binding activity, and reactivity with antibody specific for p30a (Brown, 1985). The presence of multiple actin cross-linking proteins has stimulated both curiosity and inquiry concerning the physiological significance, activities, and potential differences in the cellular functions of these polypeptides.

Nearly all cell movements are directional and have distinct polarity. Therefore, it is hypothesized that some nonuniformity of the molecules contributing to the movement must exist in the cytoplasm of the moving cell. This asymmetry may be due either to a difference in the concentration of one or more of these proteins in the cytoplasm of the cell and/or to a difference in activity of these proteins such as the polymerization, orientation, cross-linking, covalent modification, and attachment to other cellular structures (Taylor and Fechheimer, 1982). Large differences in the concentrations of cytoskeletal proteins between distinct regions of the cytoplasm have not generally been observed in rapidly moving amoeboid cells (Taylor et al., 1980). Most cytoskeletal proteins that have been analyzed in such cells appear either diffuse or generally cortical. Thus, the primary goals of the present investigation were to analyze the interaction of the 30,000-D protein with actin *in vitro*, and to examine its distribution in *D. discoideum* amoebas in order to develop a hypothesis concerning the physiological function of this protein.

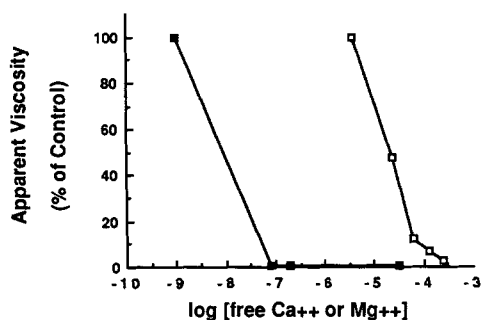


Figure 9. Effect of the concentrations of free calcium and free magnesium on the apparent viscosity of mixtures of actin and the 30,000-D protein. Apparent viscosity in the buffer solution described in Fig. 6 modified to contain the indicated concentrations of (solid squares) free calcium or (empty squares) free magnesium. The solutions contained (solid squares) 19 μ M actin, 0.7 μ M 30,000-D protein, and 50 μ M $MgCl_2$, or (empty squares) 19 μ M actin, 1.0 μ M 30 kD protein, and no added calcium. Half-maximal inhibition is observed in the presence of 0.01 μ M free calcium ion, and 23 μ M free magnesium ion.

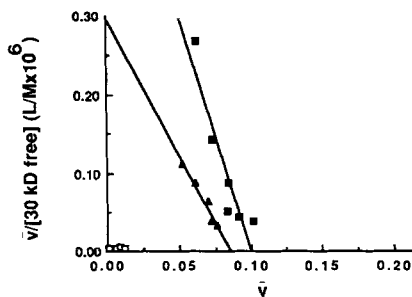


Figure 10. Quantitative analysis of the binding of the 30,000-D protein to actin. Binding of the 30,000-D protein to actin was assessed by the co-sedimentation assay as described in Materials and Methods. Binding was assessed in (solid squares) buffer B, (solid triangles) buffer B plus Mg^{2+} , or (empty squares) buffer B plus Ca^{2+} in solutions containing 9.5, 16.6, or 24 μ M actin, and 0.83–3.5 μ M 30,000-D protein. Results were analyzed by the method of Scatchard (1949).

Selective Incorporation of the 30,000-D Protein into Filopodia

Affinity-purified antibody reactive with the 30,000-D protein was employed in indirect immunofluorescence microscopy. The protein is present in central cytoplasm, in pseudopods and posterior regions of polarized cells, and in filopodia, fine extensions extending from the surface of the cell (Figs. 2, 3, and 5). Filopodia have been observed previously in amoebas of the cellular slime mold *D. discoideum* using darkfield (Rifkin and Speisman, 1976), interference reflection (Gingell and Vince, 1982 a, b), and scanning and transmission electron microscopy (Yumura and Fukui, 1983). These filopodia have a length as long as 30 μ m and a diameter of 0.1–0.2 μ m, and are frequently branched (Rifkin and Speisman, 1976; Rifkin and Isik, 1984). The number and length of filopodia is increased after treatment of amoebas with chemotactic stimuli (Kobilinsky et al., 1976; Rifkin and Isik, 1984).

Localization of the 30,000-D protein in filopodia suggests that this protein contributes to the structure and/or function of these extensions. Cellular roles proposed for filopodia are to promote motility, substrate attachment, and exploration (Albrecht-Buehler, 1976; Gingell and Vince, 1982); to mediate the intercellular interactions of lymphoid (McFarland and Schechter, 1970), neuronal (Bastiani and Goodman, 1984), and slime mold cells (Kobilinsky et al., 1976); to en-

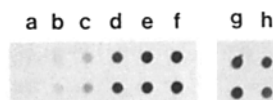


Figure 11. Determination of the quantity of the 30,000-D protein present in a homogenate of *D. discoideum* (AX-3) amoebas. Known quantities of the 30,000-D protein

and of a homogenate of *D. discoideum* amoebas were immobilized on nitrocellulose, and stained first with affinity-purified antibody reactive with the 30,000-D protein, and then with horseradish peroxidase conjugated to goat antibody reactive with rabbit antibody as described in Materials and Methods. Samples were spotted in duplicate and contained: (a) 0, (b) 0.05, (c) 0.5, (d) 5.0, (e) 50.0, (f) 500.0 ng of the 30,000-D, or (g) 12 and (h) 6 μ g of homogenate protein. In the experiment shown, 6 μ g of homogenate contains 5.0 ng of the 30,000-D protein. The 30,000-D protein comprises 0.04% of total cell protein (average of six such determinations).

hance the rates of endocytosis and exocytosis by increasing the cell surface area; to sense chemotactic gradients and maintain oriented cell movement (Rifkin and Isik, 1984); and to transduce mechanical stimuli into electrical signals in sensory cells of the inner ear (Tilney et al., 1980; Hudspeth, 1985).

The presence of actin filaments in filopodia of *D. discoideum* has been demonstrated previously (Eckert and Lazarides, 1978; Yumura and Fukui, 1983; Luna et al., 1984). The presence of the 30,000-D protein in filopodia was particularly striking, because this distribution was not observed in immunofluorescence investigations of calmodulin (Bazari and Clarke, 1982), myosin (Bazari and Clarke, 1982; Yumura et al., 1984; Yumura and Fukui, 1985), 120,000-D actin-binding protein (Condeelis et al., 1981), *Dictyostelium* α -actinin (Brier et al., 1983), and tubulin (White et al., 1983) in these cells.

The observation that the 30,000-D protein is detected in filopodia whereas other cytoskeletal polypeptides were not provokes additional inquiry. Is detection of the 30,000-D protein in filopodia indicative of specific localization in this structure? The relative concentration of a protein in different regions of the cytosol may be assessed by comparison of the distribution of fluorescence intensity with that of a soluble molecule free to diffuse throughout the cytoplasm of the same cell (Taylor et al., 1984). The potential differences in available cytoplasmic volume in distinct regions of a single vegetative amoeba are dramatically demonstrated by scanning electron micrographs of these cells (Fig. 4). The technique of normalized immunofluorescence microscopy has been adapted for use in *D. discoideum* using a sonication loading technique to introduce fluorescein-labeled BSA into amoebas before fixation and staining (Fechheimer et al., 1986). Comparison of the distribution of the 30,000-D protein with that of BSA present in the same cell indicates that this actin-binding protein is selectively incorporated into filopodia (Fig. 5). Thus, the present results indicate that variations in the distributions of individual cytoskeletal proteins in amoeboid cells can contribute to differences in the structure and activity of cytoplasm in local regions of these cells. Application of normalized immunofluorescence microscopy to other cytoskeletal proteins may reveal subtle variation in concentration that is difficult to discern, and will allow objective evaluation of observations of specific subcellular distributions of these proteins.

Application of a layer of agar to mechanically flatten cells has been employed as an alternative method for studies of the distributions of cytoskeletal proteins in *D. discoideum* amoebas (Yumura et al., 1984). The suggestion that variation in cell thickness or organelle distribution cannot affect the distribution of fluorescence intensity observed by this method is not substantiated (Yumura et al., 1984). The effects of variations in cell thickness and organelle distribution on the immunofluorescence images may be especially profound in cells that have been squashed. For example, these path-length effects are quite profound in cultured fibroblasts (McNeil et al., 1984), a cell type that is noted for extensive spreading to a fairly flattened morphology. Use of the normalized fluorescence method in conjunction with the agar technique would facilitate interpretation of the fluorescence images.

Actin Filament-bundling Activity of the 30,000-D Protein

Examination of the structures formed in mixtures of actin and the 30,000-D protein in vitro by a variety of analytical techniques all support the conclusion that the 30,000-D protein is an actin filament-bundling protein. The biphasic increase and decrease in the apparent viscosity of actin solutions as a function of the concentration of the 30,000-D protein is indicative of an increase in the orientational correlation among the filaments, and is consistent with the formation of filament bundles (Fig. 6). This suggestion is supported by the continuous increase in light scattering in the same solutions as the concentration of the 30,000-D protein is increased (Fig. 6), and the fact that the 30,000-D protein neither depolymerizes nor severs actin filaments (Fechheimer and Taylor, 1984). A decrease in viscosity upon orientation of macromolecular polymers in solution has been demonstrated in studies of other rod-shaped molecules such as poly- γ -benzyl-L-glutamate (Hermans, 1962), and has been observed in previous studies of the formation of actin filament bundles (Griffith and Pollard, 1982; Brown, 1985; Siegel and Branton, 1985). Observations by polarization microscopy (Fig. 7) and electron microscopy (Fig. 8) offer additional evidence that the 30,000-D protein does induce formation of actin filament bundles in solution in vitro. These studies of bundle formation in solution validate the previous suggestion of bundling activity of the 30,000-D protein (Fechheimer and Taylor, 1984).

Comparison of the 30,000-D Protein with Other Actin-bundling Proteins

The actin filament-bundling activity and filopodial distribution of the *Dictyostelium* 30,000-D protein motivate comparison of this protein to fascin from echinoderm eggs (Burgess and Schroeder, 1977; Bryan and Kane, 1978; Spudich and Amos, 1979; DeRosier and Censullo, 1981) and coelomocytes (Otto et al., 1979; DeRosier and Edds, 1980; Otto and Schroeder, 1984), fimbrin of mammalian fibroblasts (Bretscher and Weber, 1980a) and intestinal brush border epithelium (Glenney et al., 1981; Matsudaira et al., 1983), and villin (Bretscher and Weber, 1980b). These proteins also organize bundles of actin filaments in vitro, are monomeric and asymmetric, and are present in filopodia. However, these proteins have molecular weights approximately two or three times that of the 30,000-D protein and exhibit numerous functional differences. For instance, the proteins differ in their sensitivity to divalent cations. Actin filament-bundling proteins may be relatively insensitive to the concentration of free calcium ion such as fascin and *Dictyostelium* p30b (Bryan and Kane, 1978; Brown, 1985), specifically sensitive to calcium such as villin (Bretscher and Weber, 1980b), or sensitive to both calcium and magnesium such as fimbrin and the *D. discoideum* 30,000 dalton protein (Glenney et al., 1981; this paper). The proteins also differ in the level at which their interaction with actin is saturated. Fimbrin, fascin, and the 30,000-D protein exhibit saturable binding at a level of 1 actin-binding protein to 3 (Glenney et al., 1981), 5 (Bryan and Kane, 1978), and 10 (this paper) actin monomers in filaments, respectively. Finally, the proteins also differ in the degree of order present in the actin bundles

whose formation they mediate. Fascin induces the formation of the most highly ordered bundles (DeRosier and Censullo, 1981). Comparison of the structural and functional properties of the 30,000-D protein, fascin, and fimbrin by analyses of primary amino acid sequences, divalent cation-binding sites, and interaction with actin to form filament bundles will help to reveal similarities and differences. Additional studies of the *D. discoideum* 30,000-D protein may be quite valuable in this comparison, in that the structural requirements for formation of filament bundles and for interaction with divalent cations appear to be present in this protein in a most compact form.

Physiological Significance of the 30,000-D Protein

The results presented in the present paper allow evaluation of the potential physiological significance of the interaction of the 30,000-D protein with actin in living cells. The concentration of the 30,000-D protein in *D. discoideum* is 1.33 μM (0.04% of 100 mg/ml total protein; Fig. 11). The average concentration of F-actin in *D. discoideum* is 71 μM (3 mg/ml F-actin, or one half of the total actin \times 100 mg/ml total protein). Using the apparent association constant of 4.2×10^6 liters/mol (Fig. 10) measured in buffer B plus Mg^{2+} , a large fraction of the 30,000-D protein is expected to complex with actin in cells. There is approximately one molecule of the 30,000-D protein per 50 polymerized actin monomers in cells, whereas a maximum of one molecule of the 30,000-D protein can bind to 10 actin monomers in filaments in vitro. Thus, the 30,000-D protein is expected to bind tightly to actin in cells and to leave a large fraction of the actin filaments available for interaction with other macromolecules. The observation that virtually all of the 30,000-D protein is included in the contracted pellet fraction that forms in vitro in a functional cell extract of *D. discoideum* (Fig. 1 B, lane c) is consistent with the prediction that a large fraction of the 30,000-D protein is complexed with actin in cytoplasm.

Calcium and magnesium both inhibit the interaction of the 30,000-D protein with actin as assessed by viscometry, light scattering, polarization microscopy, and direct binding studies (Figs. 6, 7, 9, and 10). The activity of the 30,000-D protein as assessed by falling ball viscometry appears half-maximally inhibited by 2×10^{-8} M free calcium ion (Fig. 9; Fechheimer and Taylor, 1984), and by 23 μM free magnesium (Fig. 9). The observation that the interaction of the 30,000-D protein with actin as assessed by falling ball viscometry is half-maximally inhibited in the presence of 23 μM free Mg^{2+} (Fig. 9) would appear to contradict the suggestion that the 30,000-D protein binds to actin under physiological conditions. However, it is important to note that the absolute values of the concentrations of the divalent cations that inhibit the interaction as assessed by falling ball viscometry are much lower than the quantities required to inhibit binding, because a small change in the number of cross-links can cause a large change in the apparent viscosity measured by the falling ball technique. Consistent with this interpretation is the fact that Ca^{2+} appears 1,000 times more effective than Mg^{2+} as assessed by falling ball viscometry, but only 50 times more effective than magnesium as assessed by direct binding studies (see below). Moreover, an increase in free magnesium from 3.8 to 240 μM causes only a twofold decrease in the apparent association constant of the 30,000-D

protein for actin (from 1×10^7 to 4.2×10^6 L/M; Fig. 10). The results from viscosity (Fig. 6), light scattering (Fig. 6), and polarization microscopy (Fig. 7) are also consistent with a relatively minor (twofold) change in activity induced by magnesium. Thus, the data are all consistent with the proposal that the protein may be active and would complex extensively with actin under physiological conditions as discussed above.

The results also indicate that a change in free Ca^{2+} may regulate this interaction under physiological conditions. Ca^{2+} is 1,000 times more potent than Mg^{2+} in inhibiting the rise in apparent viscosity of mixtures of actin and the 30,000-D protein (Fig. 9). Direct binding studies indicate that the affinity of the 30,000-D protein for actin in the presence of 12.4 μM free calcium is more than 50 times less than that in the presence of 240 μM free magnesium (Fig. 10). Moreover, Mg^{2+} and Ca^{2+} act roughly in an additive fashion with the strong inhibitory effects of calcium predominating when the two cations are present together. Thus, an increase in the free calcium ion concentration could cause a decrease in the interaction of the 30,000-D protein with actin in cells as previously proposed (Fechheimer and Taylor, 1984).

The present results are not due to changes in the concentration of free ATP, because large variations in the nucleotide concentration did not affect the interaction of the 30,000-D protein with actin. However, the possibility that a change in the divalent cation bound to actin also contributes to the results of these experiments must be considered inasmuch as distinct differences in the properties of magnesium actin and calcium actin have been documented (Maruyama, 1981; Bonder et al., 1983; Gershman et al., 1984; Selden et al., 1986). Further investigation of the number, affinity, and selectivity of the divalent cation-binding sites of the 30,000-D protein is required to clarify the molecular basis of the effects of calcium and magnesium on the activity of the 30,000-D protein.

The results described in the present paper support the interpretation that the 30,000-D protein is distinct from other actin cross-linking proteins in *D. discoideum* both in intracellular localization and biochemical activity. The observation that the 30,000-D protein is present in (Figs. 2 and 3) and selectively incorporated into filopodia (Fig. 5), and possesses divalent cation-sensitive, actin-bundling activity suggests that this protein may function physiologically to organize actin filaments in these cellular extensions, and would be required for cellular behaviors in which filopodia are known to participate. A critical examination of the function and interaction of the 30,000-D protein with actin in living cells is required to test this hypothesis.

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