A 27,000-D Core of the *Dictyostelium* 34,000-D Protein Retains Ca^{2+}-regulated Actin Cross-linking But Lacks Bundling Activity

Marcus Fechheimer and Ruth Furukawa

Department of Zoology, University of Georgia, Athens, Georgia 30602

Abstract. Actin cross-linking proteins are important for formation of isotropic F-actin networks and anisotropic bundles of filaments in the cytoplasm of eucaryotic cells. A 34,000-D protein from the cellular slime mold *Dictyostelium discoideum* mediates formation of actin bundles in vitro, and is specifically incorporated into filopodia. The actin cross-linking activity of this protein is inhibited by the presence of micromolar calcium. A 27,000-D fragment obtained by digestion with α-chymotrypsin lacks the amino-terminal six amino acids and the carboxyl-terminal 7,000 D of the intact polypeptide. The 27,000-D fragment retains F-actin binding activity assessed by co-sedimentation assays and by ^{125}I-[F-actin] blot overlay technique, F-actin cross-linking activity as assessed by viscometry, and calcium binding activity. Ultrastructural analyses indicate that the 27,000-D fragment is deficient in the bundling activity characteristic of the intact 34,000-D protein. Actin filaments are aggregated into microdomains but not bundled in the presence of the 27,000-D fragment. A polarized light scattering assay was used to demonstrate that the 34,000-D protein increases the orientational correlation among F-actin filaments. The 27,000-D fragment does not increase the orientation of the actin filaments as assessed by this technique. A terminal segment(s) of the 34,000-D protein, lacking in the 27,000-D fragment, contributes significantly to the ability to cross-link actin filaments into bundles.

CHANGES in the distribution and assembly of cytoplasmic actin are believed to contribute to changes in cell shape and generation of the force for movements of eucaryotic cells. Actin-binding proteins may play a key role in the regulation of the assembly and supramolecular organization of actin in the cytoplasm (Korn, 1982; Pollard and Cooper, 1986; Stossel et al., 1985). Actin cross-linking proteins are potentially important for formation of isotropic cross-linked F-actin networks and anisotropic bundles of filaments that have been implicated in the viscoelastic properties of cytoplasm (Hartwig and Kwiatkowski, 1991; Matsudaira, 1991; Taylor and Condeelis, 1979).

A 30-kD protein from the cellular slime mold *Dictyostelium discoideum* mediates formation of actin bundles in vitro, and is present in the cortical cytoskeleton (Fechheimer, 1987; Fechheimer and Taylor, 1984; Johns et al., 1988). Participation of the 30-kD protein in phagocytosis is indicated by prominent localization of the protein in the region of the phagocytic cup, and statistically significant inhibition of phagocytosis in live cells loaded with antibody specific to this protein (Furukawa et al., 1992). Antigenic homologs have been identified in a variety of vertebrate and invertebrate cells (Furukawa and Fechheimer, 1990; Johns et al., 1988). The F-actin cross-linking activity of this protein is inhibited in the presence of micromolar calcium. Therefore, the 30-kD protein is expected to possess two or more binding sites for actin and at least one binding site for divalent cation. Indeed, from the cDNA sequence, there are two EF-type hands that are potential calcium-binding sites, but sequences with similarity to other previously identified actin-binding motifs are not apparent (Fechheimer et al., 1991).

We initiated a study of structure/function relationship of the 30-kD protein by preparing a 27-kD protein by digestion with α-chymotrypsin. The results indicate that the 27-kD protein is a core that retains the ability to bind and cross-link actin filaments. However, ultrastructural observations indicate that the 27-kD protein is deficient in the bundling activity characteristic of the intact 30-kD protein. We also describe and use a polarized light scattering assay adapted from liquid crystal physics. We have used the polarized components of the scattered light intensity to study the orientational correlation among the F-actin filaments to assess the ability of the intact molecule and chymotryptic fragment to bundle the actin filaments. These polarized light scattering studies provide quantitative evidence that the 27-kD core lacks the ability to induce a detectable increase in the orientational correlation among F-actin filaments. The results support the conclusion that a terminal segment(s) of the 30-kD protein, lacking in the 27-kD fragment, contributes significantly to the ability to cross-link actin filaments into highly aligned bundles. Preliminary reports of portions of this work have

been previously described (Furukawa, R., and M. Fechheimer. 1986. *J. Cell Biol.* 103:108a; Fechheimer, M., and R. Furukawa. 1989. *J. Cell Biol.* 109:237a).

Materials and Methods

Cells

D. discoideum strain AX-3 were grown in suspension in HL-5 broth as previously described (Loomis, 1971).

Purification of the Dictyostelium 30-kD Protein

The 30-kD protein was purified as described previously (Fechheimer and Furukawa, 1991). The purified protein was dialyzed in 2.0 mM Tris (Research Organics, Cleveland, OH), 50 mM KCl, 0.2 mM DTT (U.S. Biochemical Corp., Cleveland, OH), and 0.02% NaN_3 (Sigma Chemical Co., St. Louis, MO), pH 7.0.

Preparation of the 27-kD Protein

The purified 30-kD protein was mixed with α-chymotrypsin (Sigma Chemical Co.) in the final dialysis buffer described above supplemented with 20 mM Tris, pH 7.8. The digest contained a ratio of α-chymotrypsin to the 30-kD protein of 5% (wt/wt), and was held at 37°C for 3 min. Proteolysis was stopped by addition of PMSF (Sigma Chemical Co.) to a final concentration of 2.5 mM and chilling to 0°C. The solution was diluted with 10 vol of hydroxylapatite buffer (10 mM Pipes [Sigma Chemical Co.]; 0.1 mM DTT, 0.1 mM EDTA, and 0.02% NaN_3, pH 6.5) and applied to a column of hydroxylapatite (1.2 × 8 cm) (Calbiochem Biochemicals, La Jolla, CA) previously equilibrated in hydroxylapatite buffer, and eluted with a linear gradient of 400 ml from 0 to 0.1 M NaH_2PO_4. Fractions containing the 27-kD protein were identified by SDS-PAGE, and concentrated by binding to a column of hydroxylapatite (0.5 cm × 4 cm) and elution with 0.2 M NaH_2PO_4 in hydroxylapatite buffer. The concentrated protein was dialyzed versus 2.0 mM Tris, 50 mM KCl, 0.2 mM DTT, and 0.02% NaN_3, pH 7.0.

Purification of Rabbit Skeletal Muscle Actin

Actin was purified from an acetone powder of rabbit skeletal muscle essentially as described by Spudich and Watt (1971) with two cycles of assembly and sedimentation through 0.8 M KCl, and then fractionated on Sephadex G-150 (Sigma Chemical Co.) in 2 mM Tris, 0.2 mM ATP (U.S. Biochemical Corp.), 0.2 mM $CaCl_2$, 0.2 mM DTT, and 0.02% NaN_3, pH 8.0 (MacLean-Fletcher and Pollard, 1980*a*). Actin was prepared for use in viscometry, co-sedimentation, EM, and light scattering assays by dialysis overnight versus 2 mM Tris, 0.2 mM ATP, 50 μM $MgCl_2$ (1.0 M solution; Fisher Scientific, Orangeburg, NJ), 0.2 mM DTT, and 0.02% NaN_3, pH 7.0.

Protein Determination

Protein concentration was measured using the bicinchoninic acid (Pierce Chemical Co., Rockford, IL) method (Smith et al., 1985) using BSA (Sigma Chemical Co.) as the standard. The concentration of rabbit skeletal muscle G-actin was determined using an extinction coefficient of 0.62 $mg^{-1}ml\ cm^{-1}$ at 290 nm.

Polyacrylamide Gel Electrophoresis

Polypeptides were resolved by electrophoresis in standard size (10 × 15 × 0.075 cm) or mini (9 × 5 × 0.075 cm) slab gels containing 10% acrylamide (Beckman Instruments, Inc., Fullerton, CA) in the presence of SDS (Boehringer Mannheim Biochemicals, Indianapolis, IN) as described previously (Laemmli, 1970).

Interaction of the 30,000- and 27,000-D Proteins with Actin

Falling ball viscometry was performed as described previously (Fechheimer, 1987; MacLean-Fletcher and Pollard, 1980*b*) in solutions containing 20 mM Pipes, 50 mM KCl (4.0 M solution; Fisher Scientific), 1 mM ATP, 0.2 mM DTT, 0.02% NaN_3, 5 mM EGTA (Sigma Chemical Co.), pH 7.0, and either 50 μM or 0.5 mM $MgCl_2$. F-actin at a final concentration of 4.7 μM was mixed with the indicated concentrations of the 30- and 27-kD proteins. Results are the average of triplicate determinations.

Co-sedimentation assays were performed as previously described (Fechheimer, 1987). The solutions contained 29 μM actin with 2 μM of either the 27- or 30-kD proteins in 20 mM Pipes, 5 mM EGTA, 50 μM $MgCl_2$, 1 mM ATP, 50 mM KCl, 0.2 mM DTT, and 0.02% NaN_3, pH 7.0. In some experiments, the solutions also contained either 2 mM $MgCl_2$ or 4.5 mM $CaCl_2$ (0.1 M solution; Orion Research, Inc., Cambridge, MA). Standardized stock solutions of $MgCl_2$, $CaCl_2$, and KCl were used to make more accurate concentrations of these salts.

The presence of an actin-binding site active in the intact protein and 27-kD protein was assessed by an [^{125}I]-F-actin blot overlay assay performed as described previously (Chia et al., 1991).

Calcium Binding

Binding of calcium to the purified 30- and 27-kD proteins was assessed using the method of Maruyama and co-workers (1984). Methods for electrophoretic blotting were as previously described (Fechheimer, 1987*b*). Samples containing 27.5, 55, 110, and 220 pmoles of the 27- and 30-kD proteins, and 45 pmoles of apoaequorin were analyzed. Nitrocellulose paper (BA-85; Schleicher and Schuell, Keene, NH) to which electrophoretically resolved samples had been previously transferred was washed in 60 mM KCl, 5 mM $MgCl_2$, and 10 mM imidazole (Sigma Chemical Co.), pH 6.8, stained with the same solution containing 1 μCi/ml [^{45}Ca]-$CaCl_2$ (ICN Biomedicals, Inc., Costa Mesa, CA) for 10 min, rinsed with distilled water for 5 min at room temperature, dried, exposed to Kodak X-OMAT film (Eastman Kodak Co., Rochester, NY), and developed. The autoradiographs were analyzed using a Molecular Dynamics 300A Computing Densitometer (Sunnyvale, CA).

Electron Microscopy

Mixtures of 29 μM actin with 2 μM of either the 27- or 30-kD proteins were prepared in 20 mM Pipes, 5 mM EGTA, 50 μM $MgCl_2$, 1 mM ATP, 50 mM KCl, 0.2 mM DTT, and 0.02% NaN_3, pH 7.0, held at room temperature for 2 h, diluted tenfold with buffer, and applied to formvar and carbon coated grids for 1 min. The grids were washed, stained for 2 min in 2% aqueous uranyl acetate, and examined using a Philips 400 transmission electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ). Photomicrographs were taken at a magnification of 13,000.

Light Scattering

Physicists have described anisotropic light scattering from liquid crystalline solutions and polymeric films and described the use of this approach for assessing the orientational correlation among the molecules (de Gennes, 1974; Flygare and Gierke, 1974; Chandrasekhar, 1977). As the nematic liquid crystalline phase is approached (i.e., the long axis of the molecules are arranged in a parallel array), the intensity of the anisotropic light scattering component increases dramatically (Chatelain, 1948; Chatelain, 1951). This is measured as a ratio of light scattered from samples with polarizers in the incident and scattered beams in crossed versus parallel orientations. The increase in anisotropic light scattering occurs in conjunction with other phenomena such as the transmission of light through crossed polarizers observed by eye or by polarization microscopy. Since the method has been widely used in studies of the orientational correlation of other polymeric macromolecules, its application to analyses of the bundling of actin filaments seems quite promising.

Light scattering (LS)[1] measurements were performed in a Perkin-Elmer LS-5 spectrofluorometer (Perkin Elmer, Norwich, CT) with polarizing optics in the incident and scattered light paths. Conventional LS terminology is that the first letter (capitalized) is the polarization of the scattered light and the second letter (lower case) is the polarization of the incident light. The abbreviations V and H (upper or lower case as appropriate) are used to indicate vertical and horizontal orientation of the polarizer, respectively. The Vv and Hv components of the scattered light intensity were measured. The 90° (h) polarizer setting (i.e., the polarizer of the incident beam) was adjusted to a minimum scattering signal from 38-nm polystyrene spheres with the analyzer polarizer removed, then rotated to 0° (v). The analyzer

1. *Abbreviations used in this paper*: FRAP, fluorescence recovery after photobleaching; LS, light scattering.

signal (i.e., the polarizer of the scattered light beam) was adjusted to provide a minimum scattering intensity from spheres at the 90° (H) setting. This procedure is necessary to ensure that the polarizers are in the correct plane of polarization. The excitation and emission slits were 10 nm and a wavelength of 450 nm was set for the excitation and emission monochomaters. Latex beads 2.74 μm in diameter (Polysciences, Inc., Warrington, PA) were used to standardize the instrument response to remove gross instrument variations between experiments.

Mixtures of 12 μM actin and either the 27- or 30-kD proteins were polymerized in the cuvettes with 20 mM Pipes, 1 mM ATP, 50 μM $MgCl_2$, 50 mM KCl, 5 mM EGTA, 0.2 mM DTT, and 0.02% NaN_3, pH 7.0. The cuvettes, pipette tips, and glassware were rinsed exhaustively previously with water filtered through a 0.22-μm filter (Millipore Continental Water Systems, Bedford, MA) in a laminar flow hood to remove dust. All buffers and proteins were filtered through a 0.22-μm filter into dust free glassware. The solutions were degassed immediately after mixing, capped, and held at room temperature for 24 h before measurement of Vv and Hv light scattering at a wavelength of 450 nm. Dust free samples of latex beads, BSA, and F-actin were also prepared and measured. Results of mixtures of 27- or 30-kD proteins with F-actin were compared to those of F-actin alone.

Results

Digestion of the 30-kD protein with α-chymotrypsin results in a decrease in intensity of the polypeptide with M_r 34,000, and concomitant appearance of an M_r 27,000 fragment (Fig. 1). The 27-kD fragment is fairly stable, generated in good yield, and is present in maximal concentration after ~2 to 5 min of digestion. The 27-kD protein is routinely purified from digests held at 37°C for 3 min, and then purified to homogeneity as described in Materials and Methods. The yield of the 27-kD protein after digestion, chromatography on hydroxylapatite, and concentration is ~30% of the maximal theoretical yield. The NH_2-terminal amino acid sequence of the 27-kD protein has been reported previously showing that it begins at amino acid seven (alanine), and lacks approximately the COOH-terminal 7 kD of the intact protein (Fechheimer et al., 1991).

The F-actin cross-linking activity of the 27-kD protein was compared to that of the 30-kD protein by falling ball viscometry. In the presence of 50 μM $MgCl_2$ and no added calcium, the viscosity of mixtures of F-actin with both the 27- and 30-kD proteins increases as cross-linked structures form in the presence of low concentrations of the cross-linking proteins, and decreases in the presence of higher concentrations of the cross-linking proteins (Fig. 2 *a*). The viscosity of mixtures of the 27-kD protein and F-actin increases at lower molar concentrations of cross-linking protein than do matched samples containing the intact 30-kD protein (Fig. 2 *a*). The decrease in viscosity is observed in the presence of higher concentrations of the 27-kD protein than of the intact 30-kD protein.

In the presence of 0.5 mM $MgCl_2$, a similar difference in the activities of the 30- and 27-kD proteins is observed (Fig. 2 *b*). Lower molar concentrations of the 27-kD protein than of the 30-kD protein are required to cause an increase in viscosity of a solution of actin filaments. A higher concentration of the 27-kD protein than of the 30-kD protein is required to induce a decrease in the apparent viscosity of the actin solutions. The results also indicate a slight reduction of the activity of the 30-kD protein as the concentration of $MgCl_2$ is increased as previously reported (Fechheimer, 1987).

An actin-binding site detected by the binding of [^{125}I]-F-actin to the 30-kD protein immobilized on nitrocellulose has

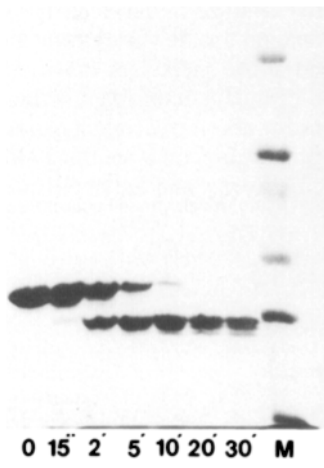

Figure 1. Digestion of the 30,000-D protein with α-chymotrypsin. The 30-kD protein was mixed with α-chymotrypsin (5% wt/wt) in the presence of 22 mM Tris, 50 mM NaCl, 0.2 mM DTT, and 0.02% NaN_3, pH 7.8, and held at 37°C. At the times indicated, samples were placed on ice and PMSF was added to a final concentration of 2.5 mM. Samples were resolved by electrophoresis in a gel of 10% polyacrylamide in the presence of SDS (Laemmli, 1970). Molecular weight standard proteins (*M*) (Diversified Biotech, Newton Center, MA) from top to bottom are: 95,500 (phosphorylase B), 55,000 (glutamate dehydrogenase), 43,000 (ovalbumin), 36,000 (lactate dehydrogenase), 29,000 (carbonic anhydrase), and 18,400 (lactoglobulin). The 27-kD protein is generated in good yield between 2 and 5 min of digestion. The purity of the 27-kD fragment isolated from these digests by chromatography on hydroxylapatite is shown in Fig. 3.

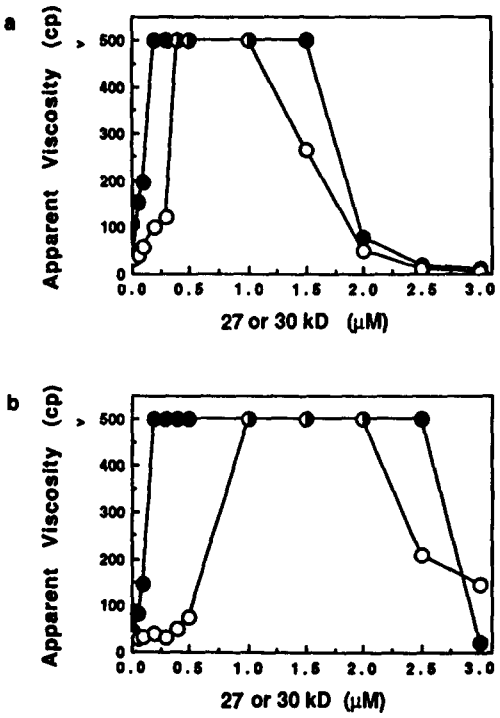

Figure 2. Comparison of the activities of the 30,000- and 27,000-D proteins by falling ball viscometry. Falling ball viscometry was performed as described in Materials and Methods using solutions containing 20 mM Pipes, 50 mM KCl, 1 mM ATP, 0.2 mM DTT, 0.02% NaN_3, 5 mM EGTA, pH 7.0, and either 50 μM (*a*) or 0.5 mM $MgCl_2$ (*b*). F-actin at a final concentration of 4.7 μM was mixed with the indicated concentrations of the 30- (○) and 27-kD (●) proteins. Results are the average of triplicate determinations from one experiment. Similar results were obtained with independent preparations of the proteins.

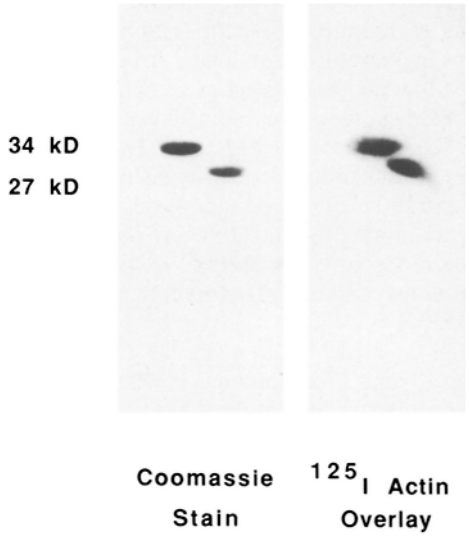

Figure 3. An actin binding site detected by F-actin overlay assays. The 27- and 30-kD proteins were resolved by electrophoresis, and either stained with Coomassie blue, or transferred to nitrocellulose, and allowed to bind [^{125}I]-F-actin before autoradiography. The actin binding site is present in both the 30-kD protein and the 27-kD fragment.

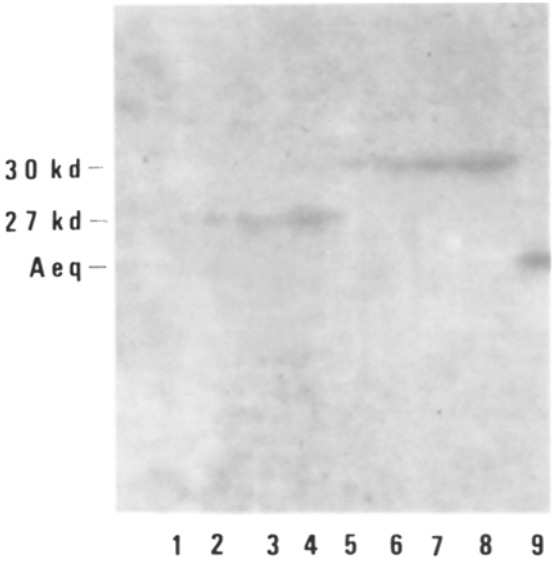

Figure 4. Binding of calcium to the 27,000- and 30,000-D proteins. Samples were resolved by electrophoresis in a gel of polyacrylamide in the presence of SDS, transferred to nitrocellulose, allowed to bind [^{45}Ca]$CaCl_2$ in the presence of 5 mM $MgCl_2$, dried, and autoradiography performed as described in Materials and Methods. Lanes *1–4* contained 27.5, 55, 110, and 220 pmoles of the 27-kD protein; lanes *5–8* contained 27.5, 55, 110, and 220 pmoles of the 30-kD protein; lane *9* contained 45 pmoles of apoaequorin. Densitometry indicates that the 27-kD protein binds 82% as much calcium as the 30-kD protein.

been reported previously (Chia et al., 1991). This actin-binding site is retained in the 27-kD fragment (Fig. 3). The binding of the 27- and 30-kD proteins to F-actin was also measured by their ability to co-sediment with actin filaments in the presence of either a low or elevated concentration of magnesium or calcium. Virtually all of the 27- and 30-kD proteins were bound to F-actin in the presence of low concentrations of divalent cations (Table I). A slight decrease in the F-actin binding was observed in the presence of 2 mM $MgCl_2$ in agreement with previous results (Fechheimer, 1987*b*), but no significant difference in the binding of the 30- and 27-kD proteins to F-actin was apparent. A major decrease in the binding of both the 30- and 27-kD proteins was observed in the presence of 4.5 mM calcium (~10 μM free calcium ion). Thus, no significant difference in the binding of the intact protein and the 27-kD protein to F-actin was apparent (Table I).

Measurements of the binding of calcium to the 30- and 27-kD proteins were performed by the electrophoretic blotting method of Maruyama and co-workers (1984). The binding of calcium to the 27- and 30-kD proteins was quite similar (Fig. 4). Densitometry indicates that binding of calcium to the 27-kD protein is 82% of that binding to the 30-kD protein.

Negatively stained mixtures of actin filaments with either the 27- or 30-kD proteins were examined in the electron microscope to obtain some explanation for the viscometric and F-actin binding data described above. Mixtures of F-actin and the 30-kD protein formed abundant actin filament bundles in the presence of low concentrations of divalent cations (Fig. 5 *b*). By contrast, mixtures of F-actin with the 27-kD protein formed more isotropic networks, and did not display

Table I. Divalent Cation Sensitivity of the Binding of the 27,000 and 30,000-D Polypeptides to Actin

Condition‡	Protein	Percent bound*		
		Experiment I	Experiment II	Experiment III
50 μM $MgCl_2$	30,000	98	98	ND
	27,000	94	100	ND
2 mM $MgCl_2$	30,000	88	94	95
	27,000	87	93	94
50 μM $MgCl_2$, 4.5 mM $CaCl_2$	30,000	20	46	44
	27,000	16	44	26

* The binding of the 27,000- and 30,000-D proteins to actin was assessed by a co-sedimentation assay as described in Materials and Methods. The percent bound was calculated as: $(p/[p + s]) \times 100$ in which p and s indicate the quantities of the polypeptides in the pellet and supernatant fractions, respectively.
‡ The solutions contained 20 mM Pipes, 50 mM KCl, 1 mM ATP, 5 mM EGTA, 29 μM of rabbit skeletal muscle actin, 2 μM of the 30,000- or 27,000-D proteins, and the indicated concentrations of divalent cations.

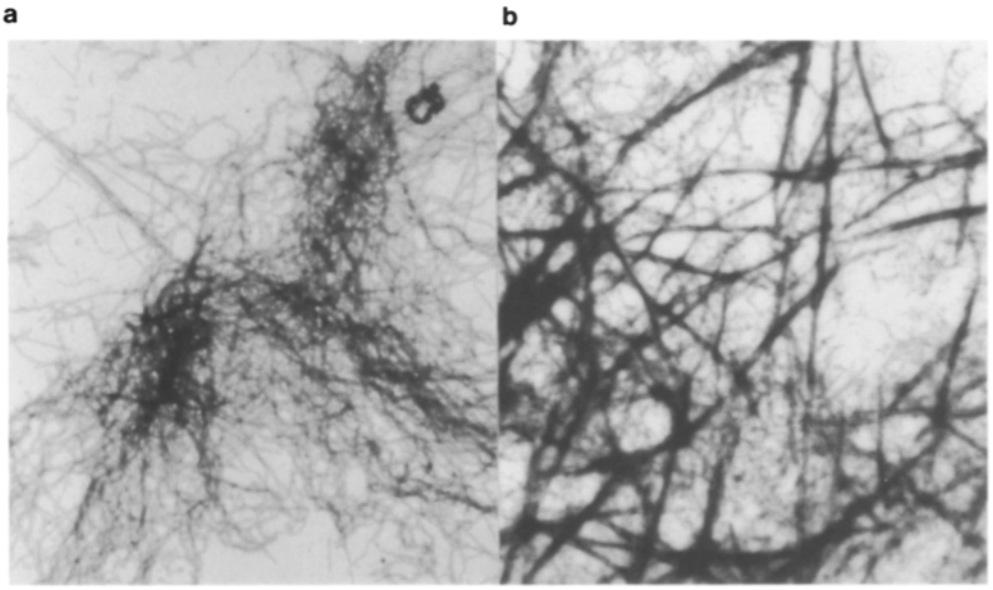

Figure 5. Morphology of mixtures of actin with the 27,000- and 30,000-D proteins. Mixtures of 29 μM actin with 2 μM of either the 27- (*a*) and 30-kD (*b*) proteins were prepared in 20 mM Pipes, 5 mM EGTA, 50 μM $MgCl_2$, 1 mM ATP, and 50 mM KCl, and held at room temperature for 2 h. Negative staining and EM were performed as described in Materials and Methods. Actin filaments in the presence of the 30-kD protein are contained in bundles (*b*), while filaments in mixtures of actin with the 27-kD protein are highly concentrated in microdomains, but are not bundled (*a*). Bar, 0.5 μm.

extensive formation of filament bundles (Fig. 5 *a*). Actin filaments cross-linked by the 27-kD protein were collected into microdomains. Extensive formation of bundles was not observed in mixtures of actin with the 27-kD protein even after extending the time of incubation from 2 h to 24 h (data not shown).

A polarized light scattering assay has been used to verify the observation that the 27-kD protein cross-links but does not bundle actin filaments. We have used the polarized components of the scattered light intensity to study the orientational correlation among the F-actin filaments to assess the ability of the 30-kD protein and 27-kD fragment to bundle the actin filaments.

The Vv and Hv components of the scattered light and Hv/Vv ratio have been measured for 38-nm-diam polystyrene spheres, BSA, and F-actin. The ratio of Hv/Vv for beads and BSA is 0.023 ± 0.002 and 0.025 ± 0.006, respectively, over a 40-fold range of bead concentration, and a 100-fold range in concentration of BSA from 0.8 to 80 mg/ml. The Hv/Vv ratio for 12 μM F-actin is 0.029 ± 0.01. These measurements on samples known to lack anisotropically oriented structures serve to validate the method using our experimental technique and apparatus.

Both the 30- and 27-kD proteins cross-link F-actin to form supramolecular aggregates as detected by an increase in the viscosity of the solutions (Fig. 2), by EM (Fig. 5), and by the increase in the Vv component of the scattered light intensity (data not shown). As the concentration of either actin-binding protein increases, the aggregation size is increasing. As the concentration of 30-kD actin–binding protein increases, the ratio of Hv/Vv scattered light intensity increases and approaches an asymptotic value even though the molecular mass is still increasing (Fig. 6). This implies that ordering among the F-actin filaments approaches a limit such as would be achieved in a bundle with more filaments able to enter the bundle but the order among the filaments is already at a maximum. In mixtures of actin and the 27-kD protein, the Vv component increases as the concentration of the cross-linking protein increases, and the Hv component increases proportionally (Fig. 6). The ratio of the Hv/Vv scattered light intensity normalized to that of actin alone is one within experimental error with increasing concentration of the 27-kD protein. These results indicate that the 30-kD protein induces an increase in the orientational correlation among the F-actin filaments, but that the 27-kD fragment lacks bundling activity.

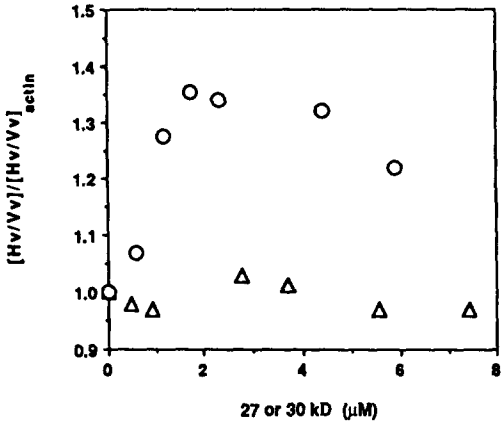

Figure 6. Polarized light scattering from mixtures of actin with the 27,000- and 30,000-D proteins. The concentration dependence of the ratio Hv/Vv for mixtures of the 30- (○) or 27-kD (△) proteins with F-actin is normalized to that of F-actin alone (0.029 ± 0.01). Results shown are representative of six experiments with different preparations of the 30-kD protein, and four different preparations of the 27-kD protein. G-actin (12 μM) was polymerized in the fluorescence cuvettes in the presence of 20 mM Pipes, 1 mM ATP, 50 mM KCl, 5 mM EGTA, 50 μM $MgCl_2$, 0.2 mM DTT, and 0.02% NaN_3, pH 7.0, and the indicated concentration of cross-linking protein. The Hv and Vv light scattering were determined 24 h after mixing with excitation and emission monochrometers set to 450 nm. The relative error averaged over the entire data set is 7%. An increase in the orientational correlation among the actin filaments is observed in mixtures of F-actin with the 30- but not the 27-kD protein.

Discussion

Actin cross-linking proteins can cross-link actin filaments into isotropic (gel) or anisotropic (bundle) aggregates. These structures have fundamentally different physical and mechanical properties with significant consequence to the cell or region of cytoplasm in which they are found. For instance, intestinal microvilli (Mooseker, 1985), stereocilia of hair cells in the ear (Tilney et al., 1980), echinoderm sperm and eggs (DeRosier et al., 1977), and filopodia of cultured cells contain F-actin bundles. Pseudopods of amoeboid cells and cortical cytoplasm contain more isotropic F-actin networks (Hartwig and Shevlin, 1986; Small, 1981). *D. discoideum* amoebae contain a minimum of seven actin cross-linking proteins (Luna and Condeelis, 1990), and at least some redundancy of function among this group has been inferred from the results of initial genetic experiments (Brink et al., 1990; Cox et al., 1992; Noegel and Witke, 1988; Witke et al., 1992). Yet, the molecular parameters which dictate the subcellular localization, and the formation of either isotropic or anisotropic cross-linked F-actin structures remain incompletely characterized. These questions have motivated development of a quantitative assay that can discriminate gelled and bundled cross-linked actin structures, our studies of structure/function relationships in a 30-kD actin bundling protein from *Dictyostelium*, and our interest in the mechanisms of actin filament cross-linking and bundling.

A Quantitative Assay to Discriminate Gelation and Bundling

A number of techniques have been used to investigate the structure of cross-linked actin networks. Aggregate structures formed upon interaction of actin cross-linking proteins with actin have been characterized by sedimentation, viscometry, light and electron microscopy, and rheology (Pollard and Cooper, 1982). While all of the assays can detect cross-linking, the microscopic assays have been used most frequently to yield information that is sensitive to the arrangement of the actin filaments in the network. Fluorescence recovery after photobleaching (FRAP) has been used to characterize directly the molecular dynamics in F-actin networks (Loftus, D. J., M. Sato, D. Wachsstock, J. A. Cooper, C. Frieden, and T. D. Pollard. 1987. *J. Cell Biol.* 105:114a; Simon et al., 1988). However, this method is not designed to reveal orientation among actin filaments.

Orientation in actin filament solutions has been assessed by optical methods using linearly polarized light including flow birefringence (reviewed by Cooper and Pollard, 1982), and polarization microscopy (Hou et al., 1990). A most innovative study reported development of a fluorescence polarization, fluorescence photobleaching, rheometer (Cortese and Frieden, 1988) that was used to observe formation of birefringent microdomains under shear in F-actin and in mixtures of F-actin with filamin. A key advantage of these optical methods is that the orientation may be assessed with no shear or disruption of the network. The orientation dependent absorbance of actin filaments and microtubules has been exploited in recent studies of the formation of liquid crystal domains in these polymers (Hitt et al., 1990; Suzuki et al., 1991). An isotropic to nematic phase transition has also been observed in solutions of F-actin and microtubules using rheology and polarization microscopy (Buxbaum et al., 1987; Kerst et al., 1990). Application of optical methods for characterization of F-actin and F-actin networks seems extremely promising at the present time, and may complement the results obtained from rheological approaches.

Physicists have described anisotropic light scattering from liquid crystalline materials, both small organic (Chatelain, 1948; Chatelain, 1951) as well as polymeric molecules (Miller et al., 1978), and polymeric films (Flygare and Gierke, 1974) and described the use of these parameters for assessing the orientational correlation among the molecules (de Gennes, 1968, 1974; Chandrasekhar, 1977). We have used the anisotropic light scattering properties of actin for characterization of the orientation of actin filaments in networks by light scattering. The Vv and Hv components of the scattered light and Hv/Vv ratio have been measured for polystyrene spheres, BSA, and F-actin. Our measurements over a broad concentration range of spheres and BSA exhibit isotropic light scattering, consistent with the known light scattering properties of these species. The Hv/Vv ratio for F-actin shows a transition that depends on filament length and actin concentration demonstrating spontaneous ordering of actin filaments in the absence of applied shear (Furukawa, R., R. Kundra, and M. Fechheimer, unpublished results). Orientation is not observed at the actin concentrations used in the present investigation. These studies support the conclusion that this assay can be used as a measure of filament orientation in actin solutions in the absence of exogenous shear forces.

The 27,000-D Core Can Cross-link but Not Bundle Actin Filaments

A calcium-regulated actin cross-linking protein such as the *Dictyostelium* 30-kD protein is expected to contain at least two binding sites for actin and at least one binding site for divalent cation. The present proteolytic dissection experiments were initiated with the goal of identifying the binding sites for actin and divalent cations within the primary sequence of the protein. A 27-kD fragment of the protein was characterized by direct Edman degradation, and found to begin at amino acid seven of the intact protein (Fechheimer et al., 1991). Thus, the 7-kD difference in molecular mass between the intact protein and the fragment is due primarily to a truncation from the carboxyl terminus of the molecule.

The 27-kD fragment retains F-actin–binding, F-actin cross–linking, and calcium-binding activities. These results and the relative stability of the 27-kD protein in the presence of α-chymotrypsin support the conclusion that this fragment is a core of the intact protein.

Studies of the cross-linking of actin filaments with the 30-kD protein and the 27-kD core were performed using viscometry, EM, and polarized light scattering. These techniques are complementary and combine to characterize and discriminate between the macromolecular structures which are formed. The results from EM indicate qualitatively that the 27-kD protein lacks the bundling activity present in the intact protein (Fig. 5). The decrease in viscosity of actin solutions observed in the presence of high concentrations of the 30- and 27-kD proteins are due to concentration of most of the filaments from the solution in bundles and microdomains, respectively. This interpretation is supported by EM. A decrease in viscosity accompanying bundling of actin by

actin binding proteins has been reported previously (Fechheimer, 1987; Griffith and Pollard, 1982; Hartwig et al., 1992). It is noteworthy that mixtures of F-actin with elevated concentrations of the 27-kD protein exhibit a decrease in viscosity even though formation of actin filament bundles is not observed. Thus, such a decrease in viscosity is not necessarily an indication of bundle formation, and results simply from a decrease in the concentration of F-actin in the bulk solution as most of the polymer is collected into dense aggregates (i.e., microdomains). The concentration of 27-kD protein required to increase the viscosity of actin is much greater than that of filamin on a molar basis. This could reflect the fact that the 27-kD protein is not a purely isotropic cross-linker. An alternative explanation is that the 27-kD protein is an isotropic cross-linker (Fig. 6), but is less efficient than filamin at forming productive cross-links between filaments.

The 30- and 27-kD proteins both induce formation of large aggregates of actin filaments as indicated by the increase in Vv light scattering intensity as additional cross-linking molecules are added to the solution. The Hv/Vv ratio for mixtures of F-actin and the 30-kD protein increases from 0 to 2 μm 30-kD protein, and subsequently reaches a plateau value, indicative of the formation of optically oriented structures (i.e., bundles). This observation correlates well with previous data that birefringence is observed using polarization microscopy with mixtures of F-actin and 1 μm 30-kD protein and increases with 2 μm 30-kD protein (Fechheimer, 1987). By contrast, the 27-kD core forms networks without any increase in the orientational correlation among the actin filaments, in agreement with the results from EM.

Factors Affecting Formation of Isotropic and Anisotropic Cross-Linked F-Actin Structures

These results are significant to discussion of the differences between the structure of cross-linking proteins that induce formation of either networks or bundles of actin filaments (Matsudaira, 1991; Pollard and Cooper, 1986; Stossel et al., 1985). Cross-linking proteins that form isotropic F-actin gels such as filamin, spectrin, and the *Dictyostelium* 120-kD protein have a flexible extended conformation. By contrast, bundling proteins such as fascin and the *Dictyostelium* 30-kD protein are commonly described as short rigid molecules lacking the flexibility required for formation of an isotropic F-actin gel. This generalization is too simplistic to account for our observations regarding the 30-kD protein, or for a number of observations in the literature regarding other cross-linking proteins.

Certainly, the cross-linking proteins such as filamin and spectrin are long, flexible macromolecules, while bundling proteins are shorter and presumably less flexible. This distinction is one major factor that must be taken into account. For example, a comparison of α-actinins indicates that the bundling activity of α-actinins correlates with molecular length emphasizing the importance of this parameter (Meyer and Aebi, 1990). The effect of the molar ratio of cross-linking protein to actin has received some attention with the conclusion that formation of anisotropic structures is promoted in the presence of higher concentrations of cross-linking protein (Hou et al., 1990; Rockwell et al., 1984). Application of controlled shear also induces formation of bundles in F-actin filamin mixtures (Cortese and Frieden, 1988). Filamin was reported to induce formation of perpendicularly branched actin networks if added to G-actin before polymerization, and bundles if added directly to F-actin (Hartwig and Stossel, 1981; Hartwig et al., 1980; Niederman et al., 1983). This difference may arise from alignment induced by shear during mixing of filamin with F-actin. In addition, kinetic studies using polarization microscopy indicate that cross-linked networks of F-actin and chicken gizzard filamin develop anisotropy as bundles form with time (Hou et al., 1990; Rockwell et al., 1984). Thus, the data indicate that these flexible cross-linking molecules can form either isotropic or anisotropic F-actin structures. The slow transition to a highly oriented configuration suggests that the bundle is the lowest free energy state for these mixtures, with isotropic networks predominating at early times in the absence of shear. A recent study of bundle formation in mixtures of actin and fascin indicates that the size and degree of order of the bundles increases over a period of days after the initial mixing (Stokes and DeRosier, 1991). Quantitative studies of other parameters such as polymer concentration and filament length on the formation of isotropic versus anisotropic structures have not received careful attention due at least in part to lack of a quantitative solution assay that can discriminate between formation of these two types of structures. A recent study indicates that a decrease in filament length imposed by addition of actophorin favors the formation of bundled structures in mixtures of F-actin and *Acanthamoeba* α-actinin (Maciver et al., 1991).

Our data imply that a terminal domain(s) of the 30-kD protein is important for formation of actin filament bundles. This domain could function as a third actin-binding site in addition to the two required for actin filament cross-linking by the 27-kD protein. Alternatively, the terminal domain could function to orient the two actin-binding sites in the core to promote bundle formation. In this case, the 30- and 27-kD proteins could both be fairly short rigid molecules with the distinction between formation of isotropic and anisotropic structures dictated by the orientation of the actin-binding sites with respect to each other and with respect to actin. Further studies of structure/function relationships in the 30-kD protein may add to our knowledge of the nature of the binding sites for actin and divalent cations, and the molecular mechanisms for actin filament cross-linking and bundling.

We thank the laboratory of Dr. M. J. Cormier (Department of Biochemistry, University of Georgia, Athens, GA) for the gift of recombinant apoaequorin and for the use of the spectrofluorometer.

This work was supported by National Science Foundation DCB 8903336 and NSF 9105087.

Received for publication 17 July 1992 and in revised form 20 October 1992.

References

Brink, M., G. Gerisch, G. Isenberg, A. A. Noegel, J. E. Segall, E. Wallraff, and M. Schleicher. 1990. A *Dictyostelium* mutant lacking an F-actin cross-linking protein, the 120-kD gelation factor. *J. Cell Biol.* 111:1477–1489.

Buxbaum, R. E., T. Dennerll, S. Weiss, and S. R. Heidemann. 1987. F-actin and microtubule suspensions as indeterminate fluids. *Science (Wash. DC).* 235:1511–1514.

Chandrasekhar, S. 1977. Liquid Crystals. Cambridge University Press, Cambridge. 1–342.

Chatelain, P. 1948. Sur la diffusion, par les cristaux liquides du type nématique,

de la lumière polarisée. *Acta Crystallographica.* 1:315–323.
Chatelain, P. 1951. Étude théorique de la diffusion de la lumière par un fluide présentant un seul axe d'isotropie: application aux cristaux liquides du type nématique. *Acta Crystallographica.* 4:453–457.
Chia, C. P., A. L. Hitt, and E. J. Luna. 1991. Direct binding of F-actin to ponticulin, an integral plasma membrane glycoprotein. *Cell Motil. Cytoskeleton.* 18:164–179.
Cortese, J. D., and C. Frieden. 1988. Microheterogeneity of actin gels formed under controlled linear shear. *J. Cell Biol.* 107:1477–1487.
Cox, D., J. Condeelis, D. Wessels, D. Soll, H. Kern, and D. A. Knecht. 1992. Targeted disruption of the ABP-120 gene leads to cells with altered motility. *J. Cell Biol.* 116:943–955.
de Gennes, P.-G. 1968. Fluctuations d'orientation et diffusion Rayleigh dans un cristal nematique. *C. R. Acad. Sc.* B 266:15–17.
de Gennes, P.-G. 1974. The Physics of Liquid Crystals. Clarendon Press, Oxford. pp. 333.
DeRosier, D. J., E. Mandelkow, A. Sillman, L. Tilney, and R. Kane. 1977. Structure of actin-containing filaments from two types of non-muscle cells. *J. Mol. Biol.* 113:679–695.
Fechheimer, M. 1987. The *Dictyostelium discoideum* 30,000-dalton protein is an actin filament-bundling protein that is selectively present in filopodia. *J. Cell Biol.* 104:1539–1551.
Fechheimer, M., and R. Furukawa. 1991. Purification of the 30,000 dalton actin binding protein from *Dictyostelium discoideum. Methods Enzymol.* 196:84–91.
Fechheimer, M., D. Murdock, M. Carney, and C. V. C. Glover. 1991. Isolation and sequencing of cDNA clones encoding the Dictyostelium discoideum 30,000 dalton actin bundling protein. *J. Biol. Chem.* 266:2883–2889.
Fechheimer, M., and D. L. Taylor. 1984. Isolation and characterization of a 30,000-dalton calcium-sensitive actin crosslinking protein from *Dictyostelium discoideum. J. Biol. Chem.* 259:4514–4520.
Flygare, W., and T. D. Gierke. 1974. Light scattering in noncrystalline solids and liquid crystals. *Ann. Rev. Mat. Sci.* 4:255–285.
Furukawa, R., S. Butz, E. Fleischmann, and M. Fechheimer. 1992. The *Dictyostelium discoideum* 30,000 dalton protein contributes to phagocytosis. *Protoplasma.* 169:18–27.
Furukawa, R., and M. Fechheimer. 1990. Localization, expression, evolutionary conservation, and structure of the 30,000 dalton actin bundling protein of *Dictyostelium discoideum. Dev. Gen.* 11:362–368.
Griffith, L. M., and T. D. Pollard. 1982. Cross-linking of actin filament networks by self-association and actin-bonding macromolecules. *J. Biol. Chem.* 257:9135–9142.
Hartwig, J. H., and D. J. Kwiatkowski. 1991. Actin-binding proteins. *Curr. Opin. Cell Biol.* 3:87–97.
Hartwig, J. H., and P. Shevlin. 1986. The architecture of actin filaments and the ultrastructural location of actin-binding protein in the periphery of lung macrophages. *J. Cell Biol.* 103:1007–1020.
Hartwig, J. H., and T. P. Stossel. 1981. Structure of macrophage actin-binding protein molecules in solution and interacting with actin filaments. *J. Mol. Biol.* 145:563–581.
Hartwig, J. H., M. Thelen, A. Rosen, P. A. Janmey, A. C. Nairn, and A. Aderem. 1992. MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium-calmodulin. *Nature (Lond.).* 356:618–622.
Hartwig, J. H., J. Tyler, and T. P. Stossel. 1980. Actin-binding protein promotes the bipolar and perpendicular branching of actin filaments. *J. Cell Biol.* 87:841–848.
Hitt, A. L., A. R. Cross, and R. C. Williams. 1990. Microtubule solutions display nematic liquid crystalline structure. *J. Biol. Chem.* 265:1639–1647.
Hou, L., K. Luby-Phelps, and F. Lanni. 1990. Brownian motion of inert tracer macromolecules in polymerized and spontaneously bundled mixtures of actin and filamin. *J. Cell Biol.* 110:1645–1654.
Johns, J. A., A. M. Brock, and J. D. Pardee. 1988. Colocalization of F-actin and 34-kilodalton actin bundling protein in *Dictyostelium* and cultured fibroblasts. *Cell Motil. Cytoskeleton.* 9:205–218.
Kerst, A., C. Chmielewski, C. Livesay, R. E. Buxbaum, and S. R. Heidemann. 1990. Liquid crystal domains and thixotropy of filamentous actin suspensions. *Proc. Natl. Acad. Sci. USA.* 87:4241–4245.
Korn, E. D. 1982. Actin polymerization and its regulation by proteins from nonmuscle cells. *Physiol. Rev.* 62:672–737.
Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
Loomis, W. F. 1971. Sensitivity of *Dictyostelium discoideum* to nucleic acid analogues. *Exp. Cell Res.* 64:484–486.
Luna, E. J., and J. S. Condeelis. 1990. Actin-associated proteins in *Dictyostelium discoideum. Dev. Gen.* 11:328–332.
Maciver, S. K., D. H. Wachsstock, W. H. Schwarz, and T. D. Pollard. 1991. The actin filament severing protein actophorin promotes the formation of rigid bundles of actin filaments crosslinked with alpha-actinin. *J. Cell Biol.* 115:1621–1628.
MacLean-Fletcher, S. D., and T. D. Pollard. 1980*a*. Identification of a factor in conventional muscle actin preparations which inhibits actin filament self-association. *Biochem. Biophys. Res. Commun.* 96:18–27.
MacLean-Fletcher, S. D., and T. D. Pollard. 1980*b*. Viscometric analysis of the gelation of *Acanthamoeba* extracts and purification of two gelation factors. *J. Cell Biol.* 85:414–428.
Maruyama, K., T. Mikawa, and S. Ebashi. 1984. Detection of calcium binding proteins by ^{45}Ca autoradiography on nitrocellulose membrane after sodium dodecyl sulfate gel electrophoresis. *J. Biochem.* 95:511–519.
Matsudaira, P. 1991. Modular organization of actin crosslinking proteins. *Trends Biochem. Soc.* 16:87–92.
Meyer, R. K., and U. Aebi. 1990. Bundling of actin filaments by alpha-actinin depends on its molecular length. *J. Cell Biol.* 110:2013–2024.
Miller, W. G., L. Kou, K. Tohyama, and V. Voltaggio. 1978. Kinetic aspects of the formation of the ordered phase in stiff-chain helical polyamino acids. *J. Polym. Sci. Polym. Symp.* 65:91–106.
Mooseker, M. S. 1985. Organization, chemistry, and assembly of the cytoskeletal apparatus of the intestinal brush border. *Ann. Rev. Cell Biol.* 1:209–241.
Niederman, R., P. C. Amrein, and J. Hartwig. 1983. Three-dimensional structure of actin filaments and of an actin gel made with actin-binding protein. *J. Cell Biol.* 96:1400–1413.
Noegel, A. A., and W. Witke. 1988. Inactivation of the alpha-actinin gene in *Dictyostelium. Dev. Gen.* 9:531–538.
Pollard, T. D., and J. A. Cooper. 1982. Methods to measure actin filament networks. *Methods Enzymol.* 85:211–233.
Pollard, T. D., and J. A. Cooper. 1986. Actin and actin-binding proteins. A critical evaluation of mechanisms and functions. *Ann. Rev. Biochem.* 55: 987–1035.
Rockwell, M. A., M. Fechheimer, and D. L. Taylor. 1984. A comparison of methods used to characterize gelation of actin *in vitro. Cell Motility.* 4:197–213.
Simon, J. R., R. H. Furukawa, B. R. Ware, and D. L. Taylor. 1988. The molecular mobility of alpha-actinin and actin in a reconstituted model of gelation. *Cell Motil. Cytoskeleton.* 11:64–82.
Small, J. V. 1981. Organization of actin in the leading edge of cultured fibroblasts: influence of osmium tetroxide and dehydration on the ultrastructure of actin meshworks. *J. Cell Biol.* 91:695–705.
Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150:76–85.
Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. *J. Biol. Chem.* 246:4866–4871.
Stokes, D. L., and D. J. DeRosier. 1991. Growth conditions control the size and order of actin bundles *in vitro. Biophys. J.* 59:456–465.
Stossel, T. P., C. Chaponnier, R. M. Ezzell, J. H. Hartwig, P. A. Janmey, D. J. Kwiatkowski, S. I. Lind, D. B. Smith, F. S. Southwick, H. L. Yin, and K. S. Zaner. 1985. Nonmuscle actin-binding proteins. *Ann. Rev. Cell Biol.* 1:355–402.
Suzuki, A., T. Maeda, and T. Ito. 1991. Formation of liquid crystalline phase of actin filament solutions and its dependence on filament length as studied by optical birefringence. *Biophys. J.* 59:25–30.
Taylor, D. L., and J. S. Condeelis. 1979. Cytoplasmic structure and contractility in amoeboid cells. *Int. Rev. Cytol.* 56:57–144.
Tilney, L. G., D. J. DeRosier, and M. J. Mulroy. 1980. The organization of actin filaments in the steriocilia of cochlear hair cells. *J. Cell Biol.* 86: 244–259.
Witke, W., M. Schleicher, and A. A. Noegel. 1992. Redundancy in the microfilament system: abnormal development of *Dictyostelium* cells lacking two F-actin cross-linking proteins. *Cell.* 68:53–62.