A Calcium- and pH-regulated Protein from *Dictyostelium discoideum* That Cross-links Actin Filaments

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ABSTRACT We have purified an actin binding protein from amebas of *Dictyostelium discoideum* which we call 95,000-dalton protein (95K). This protein is rod shaped, ~40 nm long in the electron microscope, contains two subunits measuring 95,000 daltons each, and cross-links actin filaments. Cross-linking activity was demonstrated by using falling-ball viscometry, Ostwald viscometry, and electron microscopy. Cross-linking activity is optimal at 0.1 μM Ca^{++} and pH 6.8, but is progressively inhibited at higher Ca^{++} and pH levels over a physiological range. Half-maximal inhibition occurs at 1.6 μM free Ca^{++} and pH 7.3, respectively. Sedimentation experiments demonstrate that elevated Ca^{++} and pH inhibit the binding of 95K to F-actin which explains the loss of cross-linking activity. Electron microscopy demonstrates that, under optimal conditions for cross-linking, 95K protein bundles actin filaments and that this bundling is inhibited by μM Ca^{++}. Severing of actin filaments by 95K was not observed in any of the various assays under any of the solution conditions used.

Hence, 95K protein is a rod-shaped, dimeric, Ca^{++}- and pH-regulated actin binding protein that cross-links but does not sever actin filaments.

Substantial changes in cytoplasmic consistency have been observed in ameboid cells during locomotion. Numerous studies on cell extracts indicate that these changes involve the interaction of actin with various actin binding proteins (1). We have been interested in identifying those actin binding proteins that account for Ca^{++}- and pH-regulated gelation and solation of actin in cell extracts from amebas of *Dictyostelium discoideum* (2). Studies on these extracts identified numerous proteins that were capable of gelling actin (2–6). In particular, a 95,000-dalton protein (95K), initially identified in actin-enriched fractions from cell extracts (2–4) and purified complexes of actin binding proteins and actin (5, 7), was required for Ca^{++} regulation of gelation. However, the mechanism by which any of these actin binding proteins gelled actin in a Ca^{++}- and pH-dependent manner was not understood.

In this paper we describe the 95K protein from *D. discoideum* and the mechanism by which it gels actin with Ca^{++} and pH dependence.[1]

MATERIALS AND METHODS

The 95K protein was purified from vegetative amebas of *D. discoideum* strain Ax-3. Amebas were grown to a density of 10^7/ml. 6 liters of cells was collected, washed in 0.2% NaCl, and resuspended in 2 vol of ice-cold 0.5 M sucrose, 5 mM dithiothreitol (DTT), 0.5 mM ATP, 10 mM Tris-HCl, 5 mM PIPES, 2 mM EDTA, 2 mM phenylmethyl sulfonylfluoride (PMSF), 0.3 mg/ml soybean trypsin inhibitor, and 0.06 ml/ml trasylol at pH 7.3. All subsequent steps were done on ice.

The cell suspension was homogenized by sonication (twice for 10 s, 70% power, Heat Systems W185D Sonifier, Heat Systems-Ultrasonics, Inc., Plainview, NH) and centrifuged at 100,000 *g* for 1 h. The supernatant was fractionated with powdered ammonium sulfate to make 0–45 and 45–60% cuts. The 45–60% cut was resuspended in 20 mM PIPES, 100 mM KCl, 1 mM EDTA, and 0.5 mM DTT, pH 7.0, and brought to 0.6 M potassium iodide with a 3 M stock. The solution was clarified at 200,000 *g* for 20 min and loaded on a 2.5 × 100-cm column of Bio-Rad A15 M (100–200 mesh, Bio-Rad Laboratories, Richmond, CA) that had been pulsed with 20 ml of 0.6 M KI. The column was equilibrated and eluted with the above buffer.

Gelation activity of column fractions was measured as described previously (7). The second peak of gelation activity from the column was pooled, desalted on a 4 × 18-cm column of Sephadex G25 in 5 mM potassium phosphate, 0.5 mM DTT, 0.5 mM EDTA, pH 7.5, and pumped onto a 2.5 × 10-cm column of Whatman DE52 (Whatman, Inc., Clifton, NJ) at 50 ml/h. The column was washed with one column volume of the above buffer, and protein was eluted with a 400-ml gradient from 0 to 0.45 M NaCl.

Fractions with gelation activity were pooled and pumped directly onto a 1 × 7-cm column of Bio-Rad HTP hydroxylapatite (Bio-Rad Laboratories) equilibrated in 10 mM PIPES, 50 mM KCl, 0.5 mM EDTA, 0.25 mM DTT, pH 7.0. The column was washed with one column volume of buffer, and protein was eluted with a 100-ml gradient from 0–0.15 M potassium phosphate pH 7.0. Fractions containing the 95K protein were pooled, dialyzed against 50 vol of buffer, and pumped onto a 1 × 1.5-cm column of hydroxylapatite. Bound 95K protein was eluted at a concentration of 1–2 mg/ml by a 7-ml pulse of 100 mM potassium phosphate, pH 7.0.

[1] This work was presented at the Twenty-first Annual Meeting of the American Society for Cell Biology, November 1981 (8).

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Electrophoresis was performed according to Laemmli (9). Covalent cross-linking with dimethyl suberimidate was done according to Davies and Stark (10). The falling-ball low-shear assay was performed according to MacLean-Fletcher and Pollard (11), and the number of seconds necessary for the ball to pass a measured distance in centimeters was expressed as seconds divided by centimeters equals inverse velocity (Vel^{-1}). Negative staining was done using 2% aqueous uranyl acetate, pH 4.1, on carbon-coated Formvar. Rotary shadowing was done according to Tyler and Branton (12). Measurements of the length of 95K protein were made with a calibrated ocular from prints at a magnification of 80,000. Rabbit muscle actin used in these experiments was prepared according to Spudich and Watt (13) and was chromatographed on a 1.5 × 70-cm column of Sephacryl S200 in 5 mM Tris-HCl, 0.2 mM ATP, 0.5 mM DTT, 0.1 mM $CaCl_2$, pH 7.8, before use. Free Ca^{++} concentrations were calculated with the aid of a computer program developed by Dr. Michael Sanderson (Dept. of Anatomy, Albert Einstein College of Medicine). The apparent dissociation constants used for EGTA-Ca^{++} varied with pH. For example, the range used varied from 5.5×10^6 M^{-1} at pH 6.8 to 3×10^5 M^{-1} at pH 7.8 in the presence of 2 mM $MgSO_4$ and 2 mM EGTA.

RESULTS

Purification of 95K is summarized in Fig. 1. Cross-linking experiments with dimethyl suberimidate demonstrate that this protein consists of two 95,000-dalton subunits, suggesting that the native molecular weight is 190,000 (Fig. 2).

The interaction of 95K with actin is studied most conveniently by falling-ball low-shear viscometry. The effect of Ca^{++} concentration on the ability of 95K to increase the resistance to flow of actin solutions (95K activity) is shown in Fig. 3. At either pH 6.8 or 7.6 the Ca^{++} concentration for optimum activity is 0.1 μM. As the Ca^{++} concentration is increased above this level, the activity of 95K is abolished with half-maximal inhibition at 1.6 μM Ca^{++}.

Similar experiments to study the effect of pH on 95K activity are shown in Fig. 4. Here the pH optimum for activity is 6.8 with half-maximal inhibition at pH 7.3.

As shown in Figs. 3 and 4, changes in pH or Ca^{++} over the ranges studied did not induce 95K to reduce the Vel^{-1} of actin below that of purified actin alone. These results suggest that 95K, although a Ca^{++}-regulated actin binding protein, is not similar to villin (14, 15) or gelsolin (16), which are capable of reducing the viscosity of actin in the presence of micromolar Ca^{++} by severing actin filaments. To study this further, we used Ostwald viscometry as shown in Fig. 5. The presence of 95K resulted in a final viscosity slightly higher than that of actin alone and promoted slight nucleation of actin filament growth. The final steady-state viscosity achieved in mixtures of

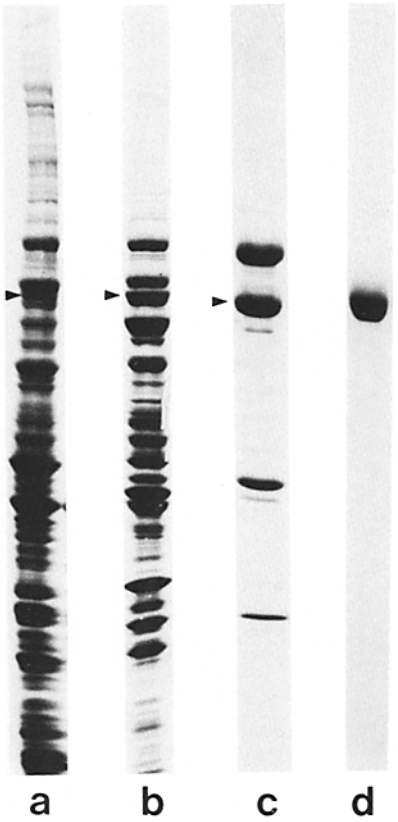

FIGURE 1 SDS PAGE of pooled fractions with gelation activity from (*a*) cell extract, (*b*) A15 M column, (*c*) DE52 column, (*d*) pooled 95K from the hydroxylapatite column. Arrowhead marks the position of 95K.

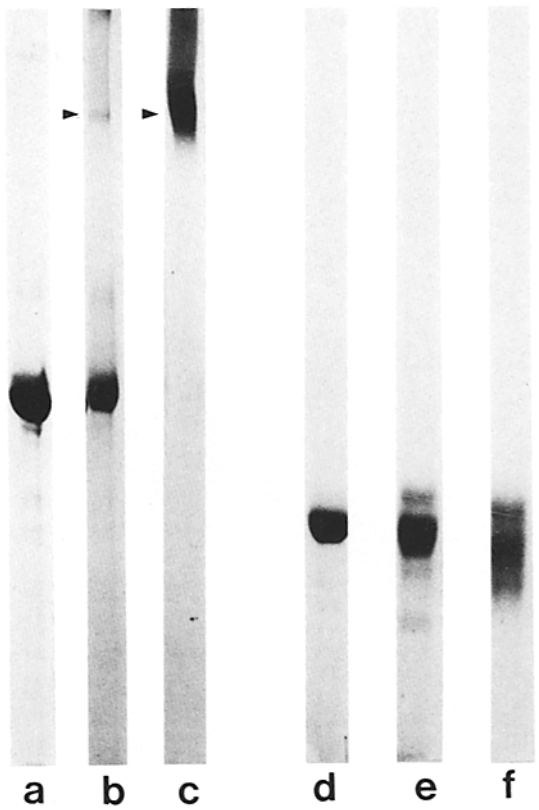

FIGURE 2 SDS PAGE of dimethyl suberimidate (DMSI) cross-linking of 95K. Conditions were 0.2 mg/ml protein in 0.2 M triethanolamine, pH 8.5, and 1 mg/ml DMSI. Reaction was allowed to proceed for 0 (*a*, *d*), 1 (*b*, *e*), or 12 h (*c*, *f*) at 0°C. *a–c* 95K, (*d–f*) bovine serum albumin (BSA). The 95K is cross-linked to a species measuring 190,000 daltons (arrow) while BSA, a monomeric protein, retains its monomeric molecular weight.

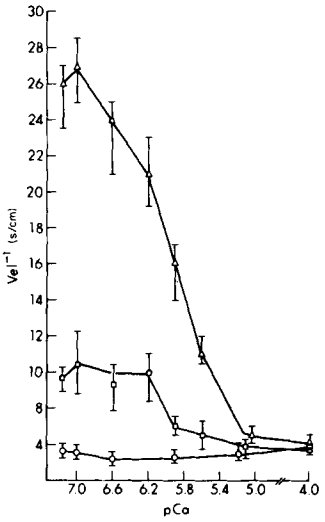

FIGURE 3 Effect of Ca^{++} on 95K activity as measured by the falling-ball assay. Rabbit muscle actin at 19.8 μM and 95K at 0.5 μM (calculations throughout based on molecular weights of 42,000 and 190,000, respectively) were incubated for 18 min at 22°C in 20 mM KCl, 2 mM $MgSO_4$, 5 mM PIPES, 2 mM EGTA, and sufficient $CaCl_2$ to achieve the concentrations shown. Measurements were made at an angle of 45°. Actin plus 95K at pH 7.0 (△) or pH 7.6 (□); actin alone at pH 7.0 (○). Vel^{-1} (S/cm), inverse velocity.

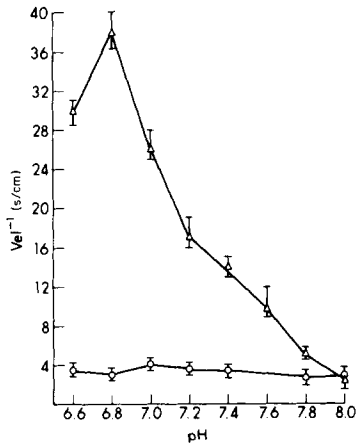

FIGURE 4 Effect of pH on 95K activity as measured by the falling-ball assay. Conditions were the same as in Fig. 3 except Ca^{++} concentration was held at 0.1 μM. Actin plus 95K (Δ), actin alone (○). Vel^{-1}, inverse velocity.

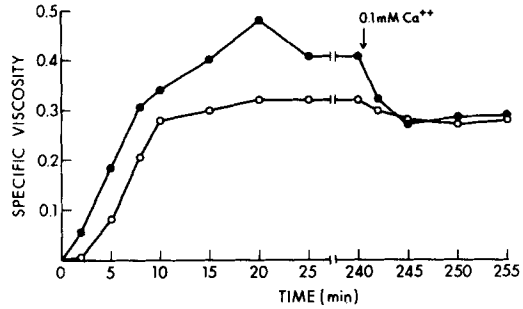

FIGURE 5 Effect of 95K on viscosity of actin measured by Ostwald viscometry. Actin at 11 μM was mixed with 0.5 μM 95K in 5 mM PIPES, 2 mM EGTA, 0.3 mM $MgSO_4$, and 0.1 mM $CaCl_2$, pH 6.8, and immediately loaded into a Cannon-Manning semi-microviscometer. Specific viscosity was measured at 25°C. At the time indicated 2.0 mM $CaCl_2$ was added at pH 6.8 to achieve a free Ca^{++} concentration of ~0.1 mM. Actin (○), actin plus 95K (●).

95K and actin depended on both the amount of shearing to which the sample was subjected and the Ca^{++} concentration. Samples subjected to multiple measurements in the Ostwald viscometer exhibited sequentially lower viscosities, presumably due to shearing of 95K-actin complexes in the capillary during flow. Addition of Ca^{++} to concentrations >10 μM resulted in a reduction of the viscosity of 95K-actin mixtures. However, this reduction was never below the viscosity of F-actin alone, confirming that 95K does not sever actin filaments.

The ability of 95K to increase the viscosity of actin suggests that 95K cross-links filaments. Inhibition of cross-linking activity by Ca^{++} and pH could, therefore, result from either the loss of binding of 95K to actin or the breaking of 95K cross-links without loss of binding to actin. To distinguish between these two possibilities the binding of 95K to F-actin was investigated with a sedimentation assay as shown in Fig. 6. At 0.1 μM Ca^{++} and pH 6.8, conditions for optimal 95K activity, 95K pellets with F-actin. However, if the Ca^{++} concentration is raised to 10 μM at pH 6.8 or the pH is raised to 7.8 at 0.1 μM Ca^{++}, 95K does not pellet with F-actin but is recovered in the supernatant. This indicates that the ability of 95K to bind to F-actin is inhibited at elevated Ca^{++} or pH. Furthermore, the same amount of F-actin is pelleted in the presence of 10 μM Ca^{++} as with 0.1 μM Ca^{++}, indicating once again that 95K does not sever actin filaments at elevated Ca^{++} concentrations.

The results reported so far can be interpreted to mean that 95K cross-links actin filaments. To visualize possible cross-linking activity directly we used the negative staining technique as shown in Fig. 7. Under the conditions of these experiments, actin, in the absence of 95K, formed unaggregated filaments of indefinite length (Fig. 7*a*) in the presence of both 0.1 and 10 μM Ca^{++}. However, when 95K was added, filaments became aligned to form bundles in 0.1 μM Ca^{++} (Fig. 7*b* and *d*) but not in 10 μM Ca^{++} (Fig. 7*c*). The size of these bundles was determined by the molar ratio of 95K to actin. At low ratios the bundles were small, usually containing 3–10 filaments in side-to-side association (Fig. 7*b*) whereas at high ratios macroscopic bundles formed containing hundreds of filaments (Fig. 7*d*).

These results demonstrate that 95K cross-links actin filaments. However, the 95K containing cross-link could not be unequivocally identified in preparations like those shown in Fig. 7. Therefore, to identify the 95K protein both negative staining and rotary shadowing were carried out with the purified protein in the absence of actin. Fig. 8 shows a field of 95K after negative staining in uranyl acetate. Under these conditions, the 95K protein appears as a rod with an average length of 38.0 nm. One of these rods is shown at higher magnification in Fig. 9*b*. The actual distribution of lengths measured in fields like the one shown in Fig. 8*a* is presented as a histogram in Fig. 10.

The 95K protein after rotary shadowing is shown in Fig. 9. Under these conditions, the 95K protein appears as a rod with an average length of 39.8 nm. The distribution of lengths measured after rotary shadowing is shown in Fig. 11.

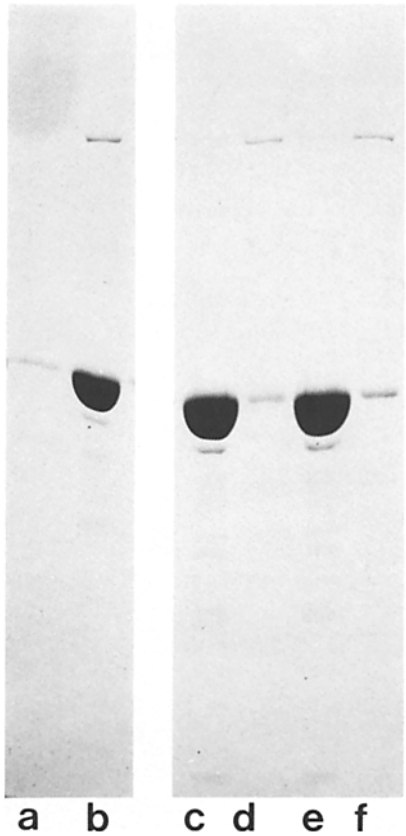

FIGURE 6 Sedimentation of 95K and F-actin. Actin at 9.5 μM and 95K at 0.24 μM were incubated for 30 min at 25°C in the buffer in Fig. 3 at pH 6.8 or 7.8 and sufficient $CaCl_2$ to achieve free Ca^{++} concentrations of 0.1 or 10 μM. F-actin was pelleted at 100,000 g for 30 min in a Beckman airfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) at 25°C. (*a*, *b*) pH 6.8, 0.1 μM Ca^{++}, (*a*) supernatant, (*b*) pellet. (*c*, *d*) pH 6.8, 10 μM Ca^{++}, (*c*) pellet, (*d*) supernatant. (*e*, *f*) pH 7.8, 0.1 μM Ca^{++}, (*e*) pellet, (*f*) supernatant.

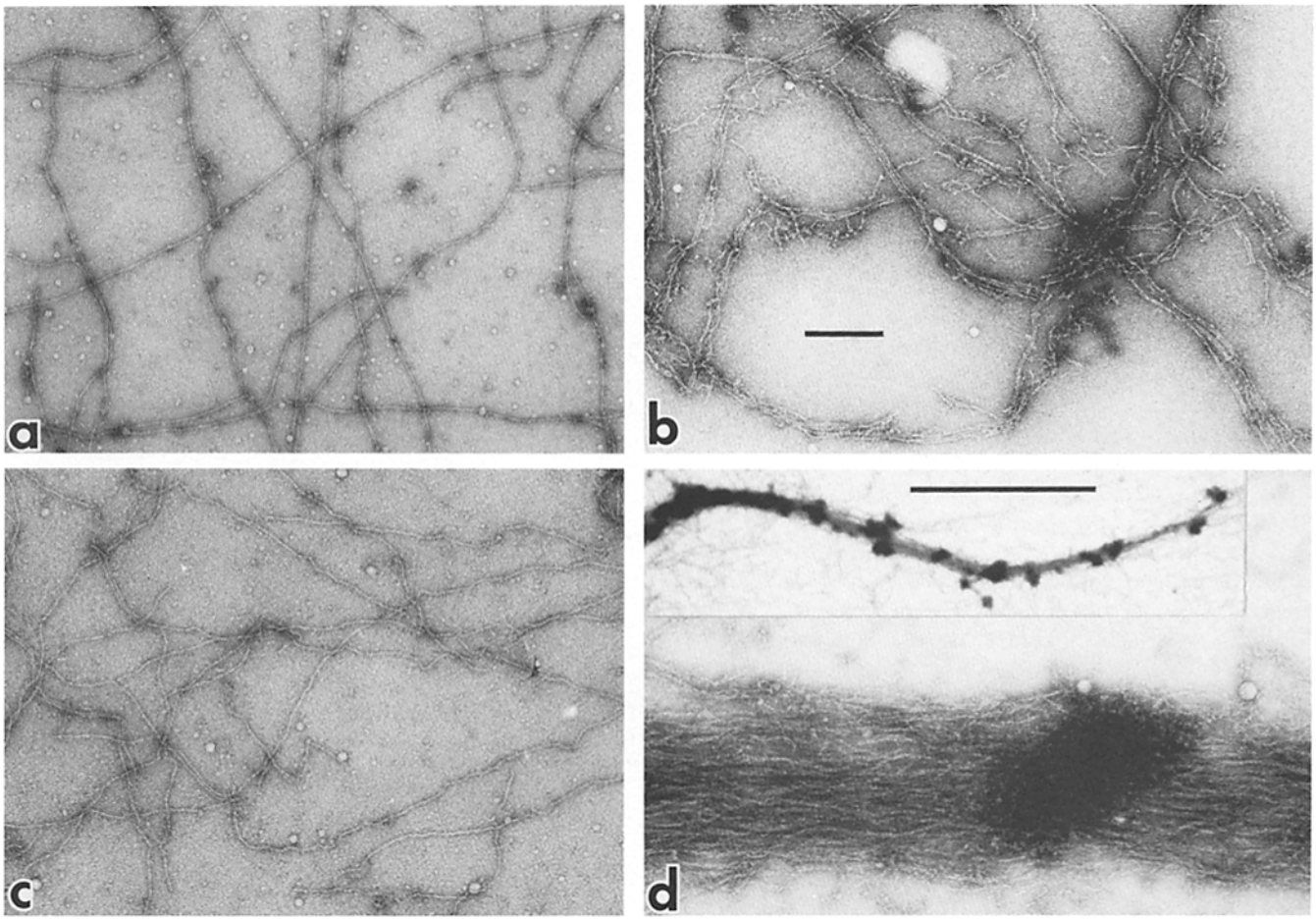

FIGURE 7 Cross-linking of actin filaments by 95K. Actin was polymerized for 15 min at 25°C in the presence or absence of 95K in 20 mM KCl, 2 mM $MgSO_4$, 2.5 mM PIPES, 2 mM EGTA pH 6.8, and sufficient $CaCl_2$ to achieve the Ca^{++} concentration indicated. (*a*) 1.5 μM actin in 0.1 μM Ca^{++}, (*b*) 1.5 μM actin plus 0.1 μM 95K in 0.1 μM Ca^{++}, (*c*) same as *b* but with 10 μM Ca^{++}, (*d*) 1.5 μM actin plus 0.5 μM 95K in 0.1 μM Ca^{++}. *Inset* in *d* shows low magnification view of filament bundle in *d*. *a–d* are all the same magnification. Bar, 0.2 μm. *Inset* bar, 5.0 μm. (*a–d*) × 50,000. (*Inset*) × 5,000.

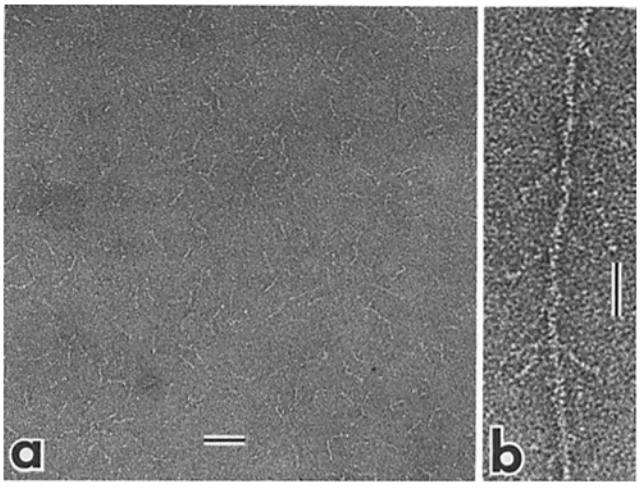

FIGURE 8 Structure of 95K protein after negative staining in uranyl acetate. (*a*) Field of 95K protein stained from a solution containing 0.5 μM protein in 0.1 μM Ca^{++}. (*b*) F-actin at 0.15 μM labeled with 0.1 μM 95K protein in 0.1 μM Ca^{++}. Proteins were incubated in the buffer described in Fig. 7. Bars: (*a*) 80 nm, (*b*) 40 nm. (*a*) × 62,500. (*b*) × 175,000.

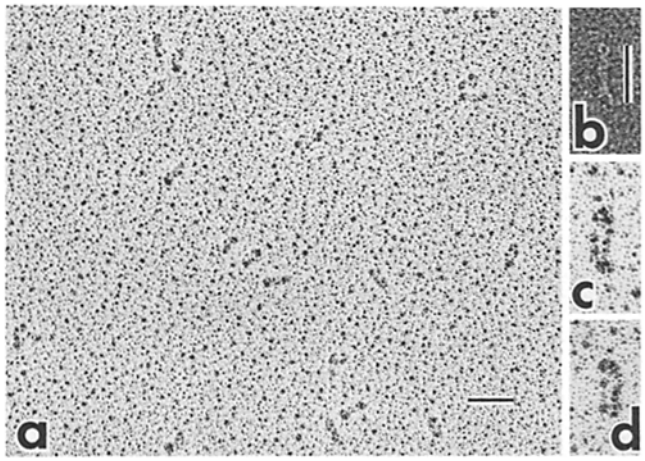

FIGURE 9 Structure of 95K protein after rotary shadowing. (*a*) Field of 95K protein dried on mica from a solution containing 0.016 μM 95K in 5 mM Tris-HCl, 0.1 mM $CaCl_2$, 0.2 mM ATP, and 0.5 mM DTT pH 7.8. Higher magnification of 95K protein (*b*) negatively stained as in Fig. 8, (*c*, *d*) rotary shadowed. Bars: (*a*) 80 nm, (*b–d*) 40 nm. (*a*) × 75,000. (*b–d*) × 187,500.

When the 95K protein is rotary shadowed, many of the rods appear to open in the center to expose two strands that terminate in globular domains at each end of the rod. This is shown at higher magnification in Fig. 9 *c* and *d*. It is not certain what these strands represent, but since covalent cross-linking demonstrates that 95K protein contains two subunits (Fig. 2) these

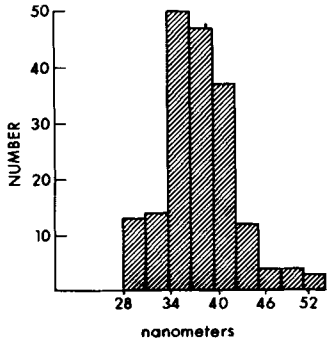

FIGURE 10 A histogram of lengths of 95K protein after negative staining in uranyl acetate as in Fig. 8 *a*.

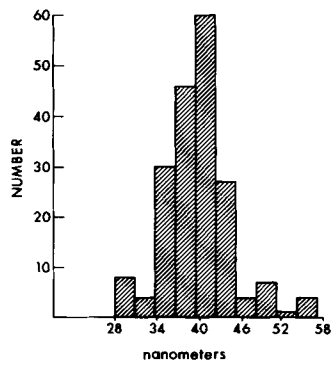

FIGURE 11 A histogram of lengths of 95K protein after rotary shadowing as in Fig. 9 *a*.

strands might be the two 95K subunits of the native protein in side-to-side association.

To visualize the binding of 95K to F-actin, the concentration of F-actin was diluted to decrease the probability of 95K-induced cross-links between filaments, and 95K was then added to the filaments (Fig. 8*b*). Under these conditions aggregates are not readily formed. After negative staining with uranyl acetate, free F-actin filaments are found that are associated with 95K protein. The end of the 95K rod attaches to F-actin to form an angle that varies from 42° to 55°, suggesting that 95K forms a cross-link between actin filaments by binding at each of its ends to an actin filament.

DISCUSSION

The 95K protein increases the consistency of actin solutions by cross-linking actin filaments. Cross-linking activity is demonstrated by low and high shear viscometry and most directly by negative staining electron microscopy. Cross-linking activity is inhibited by increasing Ca^{++} or pH over a physiological range (0.1–1.6 μM Ca^{++} or pH 6.8–7.3, optimal activity to half-maximal inhibition), suggesting that the activity could be regulated by Ca^{++} or pH in vivo. Our sedimentation experiments indicate that inhibition of cross-linking results from the inhibition of binding of 95K to F-actin at elevated Ca^{++} or pH, rather than breaking of cross-bridges between actin filaments without loss of 95K-actin binding.

The 95K protein is a rod-shaped, dimeric actin binding protein. Our viscosity, sedimentation, and electron microscope experiments demonstrate that 95K does not sever actin filaments at elevated Ca^{++} concentrations, indicating that 95K is not a villin (14, 15) or gelsolin (16) like protein. Hence, 95K protein appears to belong in a class of rod-shaped actin binding proteins including α-actinin (17, 18), actinogelin (19), and *Acanthamoeba* gelation protein (20) as proposed by Burridge and Feramisco (18). Further work on the physical and chemical properties of 95K protein is in progress to determine whether it is an α actininlike protein.[2]

We have demonstrated elsewhere that 95K protein copurifies with 120,000-dalton protein (120K), a very potent actin gelation protein in *Dictyostelium* amebas (7, 21). During purification, mixtures of 120K, 95K, and actin demonstrate gelation activity that is inhibited by micromolar Ca^{++}. However, when the 120K and 95K proteins are separated from each other, gelation activity recovered with the 120K protein is no longer regulated by Ca^{++} (7, 21). These results suggested to us that the 95K protein was, by an unknown mechanism, endowing the 120K-actin mixture with Ca^{++} regulation (7). From the results presented in this paper we believe that the mechanism by which 95K "regulates" gelation of 120K-actin mixtures can now be explained as follows. In a mixture of 120K, 95K, and actin, the critical cross-linker concentration (22) required to gel actin is the sum of the activity of both the 120K and 95K proteins, because both proteins are capable of cross-linking actin filaments. When the calcium concentration is raised above 1 μM, the ability of 95K protein to cross-link actin filaments is inhibited, whereas the ability of 120K protein to cross-link filaments is not. Hence, gelation would be inhibited in mixtures where the critical cross-linker concentration necessary for gelation was greater than the concentration of 120K present. This interpretation is consistent with the properties of mixtures of the three proteins (7), the 120K protein (21), and 95K protein as described here.

Finally, the ability of 95K to cross-link actin filaments at submicromolar but not micromolar calcium concentrations is consistent with the solation-contraction coupling hypothesis that we have proposed in detail elsewhere (2–5, 7; reviewed in reference 5). This hypothesis states that actin filaments that have been cross-linked by calcium- or pH-regulated actin binding proteins would not be free to participate in a sliding filament type mechanism of contraction if the concentration of cross-links is high compared with the myosin concentration. A rise in Ca^{++} concentration or pH would remove cross-links and release the inhibition to filament sliding imposed by cross-linking. Hence, the 95K protein from *D. discoideum* may function to regulate both assembly of the actin cytoskeleton and contraction in nonmuscle cells.

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[2] Vahey, M., and J. Condeelis, Manuscript in preparation.

REFERENCES

1. Taylor, D., and J. Condeelis. 1979. Cytoplasmic structure and contractility in amoeboid cells. *Int. Rev. Cytol.* 56:57–144.
2. Condeelis, J., and D. Taylor. 1977. Control of gelation, solation and contraction in extracts from *D. discoideum*. *J. Cell Biol.* 74:901–927.
3. Condeelis, J., and S. Geosits. 1979. Properties of gelation factors from *D. discoideum* and the solation-contraction-coupling hypothesis. *J. Cell Biol.* 83:315a (Abstr.).
4. Hellewell, S., and D. Taylor. 1979. The solation-contraction coupling hypothesis. *J. Cell Biol.* 84:633–648.
5. Condeelis, J. 1981. Reciprocal interactions between the actin lattice and cell membrane. *Neurosci. Res. Program Bull.* 19:83–99.
6. Condeelis, J., and J. Wolosewick. 1980. The actin lattice: composition, structure and

membrane attachment. 38th Annual Proceedings of the Electron Microscopy Society of America. 420–423.
7. Condeelis, J. 1981. Microfilament-membrane interactions in cell shape and surface architecture. International Cell Biology, 1980–1981. 306–320.
8. Condeelis, J. 1981. A new Ca^{++} and pH regulated actin binding protein from *Dictyostelium* amoebae. *J. Cell Biol.* 91:289a.
9. Laemmli, V. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
10. Davies, G., and G. Stark. 1970. Use of dimethyl suberimidate in studying the subunit structure of oligomeric proteins. *Proc. Natl. Acad. Sci. U. S. A.* 66:651–656.
11. MacLean-Fletcher, S., and T. Pollard. 1980. Viscometric analysis of the gelation of *Acanthamoeba* extracts and purification of two gelation factors. *J. Cell Biol.* 85:414–428.
12. Tyler, J., and D. Branton. 1980. Rotary shadowing of extended molecules dried from glycerol. *J. Ultrastruct. Res.* 71:95–102.
13. Spudich, J., and S. Watt. 1971. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866–4871.
14. Mooseker, M., T. Graves, K. Wharton, N. Falco, and C. Howe. 1980. Regulation of microvillus structure. *J. Cell Biol.* 87:809–822.
15. Bretscher, A., and K. Weber. 1980. Villin is a major protein of the microvillus cytoskeleton which binds both G and F actin in a calcium dependent manner. *Cell.* 20:839–847.
16. Yin, H., K. Zaner, and T. Stossel. 1980. Ca^{++} control of actin gelatin. *J. Biol. Chem.* 255:9494–9500.
17. Suzuki, A., D. Goll, I. Singh, R. Allen, R. Robson, and M. Stromer. 1976. Some properties of purified skeletal muscle α-actinin. *J. Biol. Chem.* 251:6860–6870.
18. Burridge, K., and J. Feramisco. 1981. Non-muscle α-actinins are calcium sensitive actin binding proteins. *Nature (Lond.).* 294:565–567.
19. Mimura, N., and A. Asano. 1979. Ca^{++} sensitive gelation of actin filaments by a new protein factor. *Nature (Lond.).* 282:44–48.
20. Pollard, T. 1981. Purification of a calcium sensitive actin gelation protein from *Acanthamoeba*. *J. Biol. Chem.* 256:7666–7670.
21. Condeelis, J., S. Geosits, and M. Vahey. 1982. Isolation of a new actin binding protein from *Dictyostelium discoideum*. *Cold Spring Harbor Conf. Cell Proliferation.* 3(Book A):in press.
22. Flory, P. 1946. Fundamental principles of condensation polymerization. *Chem. Rev.* 39:137–197.