A 40,000-Dalton Protein from *Dictyostelium discoideum* Affects Assembly Properties of Actin in a Ca^{2+}-dependent Manner

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ABSTRACT A 40,000-dalton protein that affects the assembly properties of actin in a Ca^{2+}-dependent manner has been purified from *Dictyostelium discoideum*. Gel filtration chromatography indicates that the native form of this protein is a monomer. A major effect of this protein is to reduce the sedimentability of F-actin in a stoichiometric fashion. Nearly complete loss of sedimentability is observed at ratios of the 40,000-dalton protein to actin of greater than 1:10. At low stoichiometries, this protein can accelerate the rate of actin assembly under certain experimental conditions. These effects of the 40,000-dalton protein on the actin assembly properties described above require calcium ion. The 40,000-dalton protein does not exert its effects by proteolyzing actin. Furthermore, peptide maps demonstrate that this protein is not a proteolytic fragment of actin.

It has been clear for a number of years that the actin in nonmuscle cells is constantly being reorganized in response to functional demands such as endocytosis, locomotion, and cytokinesis (4, 8). It seems likely that these reorganizations occur via modulations of the assembly state of actin. Accordingly, many laboratories have embarked on a search for proteins that can be demonstrated to have an effect on the assembly properties of actin (3, 6, 7, 10–15, 22, 23). A number of such proteins have been found, including a category of Ca^{2+}-activated factors (17). We have been searching for such factors in amoebae of *Dictyostelium discoideum*, an organism which has a number of advantages for biochemical, developmental, and genetic studies on motility (19). Here we report on the purification and preliminary characterization of a factor from *Dictyostelium* which exerts several effects on actin in a Ca^{2+}-dependent manner.

MATERIALS AND METHODS

Purification of Actin

Actin was purified from *Dictyostelium discoideum* by the method of Uyemura et al. (21). G-actin was prepared by resuspending a pellet of F-actin in a buffer containing 10 mM triethanolamine, pH 7.5, 0.2 mM DTT, and 0.2 mM ATP, then sonicating twice for 15 s (Kontes sonicator, setting 7; Kontes Co., Vineland, NJ) and incubating for 1 h on ice. F-actin was prepared by assembling G-actin for 3 h at 22°C in 25 mM triethanolamine, pH 7.5, 0.2 mM DTT, 0.2 mM ATP, 50 mM KCl, 2 mM $MgCl_2$.

Assays

"Polymerization" was followed by sedimentation at various times after bringing 200 μg/ml radioactive *Dictyostelium* G-actin (^{35}S-labeled actin, purified from *Dictyostelium* metabolically labeled with [^{35}S]-methionine [18]) to the following final buffer conditions: 25 mM triethanolamine, pH 7.5, 0.2 mM DTT, 0.2 mM ATP, 50 mM KCl, 2 mM $MgCl_2$, and 0.1 mM $CaCl_2$ or 0.5 mM EGTA. Sedimentation was carried out for 10 min in an Airfuge (Beckman Instruments, Inc., Fullerton, CA) operated at 30 psi. These conditions have been shown to efficiently sediment all oligomers larger than ~ decamers[1].

"Disassembly"[2] was followed in the same buffer by sedimentation of 200 μg/ml of ^{35}S-labeled *Dictyostelium* F-actin at various times after addition of 40,000-dalton protein.

"Activity" of the 40,000-dalton protein is defined as its ability to interfere with the incorporation of radioactive G-actin into the sedimentable F-actin pool under steady-state conditions. The assay was based on the report of Simpson and

[1] Cross-linking of actin with *p*-phenylene-*N,N'-bis* (maleimide) yielded actin dimers, trimers, and higher-order aggregates. Sedimentation of this cross-linked population of oligomers under the conditions used here showed that oligomers greater than ~decamers in length were efficiently sedimented (Yamamoto and Spudich, manuscript in preparation.).

[2] In this report, "disassembly" refers to loss of sedimentability of F-actin in the presence of the 40,000-mol wt protein. Other methods of analysis demonstrate that this protein causes fragmentation of actin filaments with concomitant increases in the concentration of G-actin (Yamamoto, Pardee, Reidler, Stryer, and Spudich. Manuscript submitted for publication.).

Spudich (18) of the exchange between G- and F-actin pools which can be observed in this manner. The mechanism of exchange as well as the mechanism whereby 40,000-dalton protein interferes with it is still being investigated (see Discussion). The incorporation of radioactivity into the actin filaments was monitored as follows: 80 μl of 250 μg/ml F-actin was mixed with 10 μl of 1 mM $CaCl_2$ or 5 mM EGTA, and 10 μl of various concentrations of 40,000-dalton protein in the same buffer and incubated for 0.5 h. ^{35}S-labeled G-actin, 5 μl of a 200 μg/ml solution, was added and the mixture was incubated for another 0.5 h. The mixture was then centrifuged for 10 min in an Airfuge (Beckman Instruments, Inc.) operated at 30 psi. An 80-μl aliquot of the supernate was counted to determine how much of the radioactivity had become incorporated into the F-actin pellet. For routine monitoring of fractions during the purification, the above 0.5-h incubations were shortened to 10 min, the amount of ^{35}S-labeled G-actin added was increased from 10 to 20 μg/ml, and 500 μg/ml rabbit muscle F-actin (20) was substituted for the 200 μg/ml *Dictyostelium* F-actin. In the absence of 40,000-dalton protein, ~70% of the radioactive G-actin exchanges into the F-actin pool under the above conditions. This amount of exchange is taken as 0% inhibition, and then the amount of inhibition of incorporation of radioactivity into F-actin in the presence of various concentrations of 40,000-dalton protein is determined. We obtained linearity between 25% and 75% inhibition, whether the crude lysate or the final purified material was used. One unit of activity is defined as that required to give 50% inhibition.

Purification of the 40,000-Dalton Protein

The temperature was 4°C, unless otherwise noted. A high-speed supernate (S2) of lysed *Dictyostelium* amoebae was prepared as described for the preparation of actin (21). Nearly 100% of the units of activity (see above) present in the crude lysate were recovered in this fraction.

The buffer concentration of the S2 was brought to 50 mM triethanolamine, pH 8, and solid ammonium sulfate was added to 80% saturation (0.561 g added per ml starting solution). The mixture was stirred for 1 h, then centrifuged for 10 min at 15,000 rpm in an SS34 rotor. Virtually all of the protein and all of the units of activity were precipitated by this procedure. The pellet was resuspended in 40% ammonium sulfate (4 parts saturated ammonium sulfate at 4°C, pH 7.6 plus 6 parts 2 mM triethanolamine, pH 7.5, 0.2 mM DTT) using a volume in ml equal to the starting number of grams of cells used. After stirring for 0.5 h the solution was centrifuged as described above. ~10% of the protein and one-third of the units of activity were recovered in the 40% supernate. The activity in this fraction was much more Ca^{2+}-dependent than the rest of the activity recovered in other ammonium sulfate fractions, or that in the original S2. In a typical preparation, the fold-activation by Ca^{2+} was ≥30 for the 40% supernatant; when the 40% pellet was resuspended in 30% ammonium sulfate and centrifuged, the 30% supernatant showed a fourfold activation and the 30% pellet a 14-fold activation by Ca^{2+}. This suggests that multiple activities, some of which are possibly Ca^{2+}-independent, have been fractionated by this step. If we assume that all of a single activity is recovered in the 40% supernate, then a tenfold purification has been achieved by ammonium sulfate fractionation.

The 40% supernate was dialysed extensively vs. 2 mM triethanolamine, pH 7.5, 0.2 mM DTT, until the conductance was <500 μSiemens. It was then loaded onto DEAE cellulose (Whatman DE52; Whatman Inc., Clifton, NJ) (column equal in volume to the starting number of grams of cells) pre-equilibrated with the same buffer (Fig. 1). The activity did not adhere to DEAE and was recovered in the flow-through, which contained 5–10% of the loaded protein. The flow-through activity was completely Ca^{2+}-dependent (no activity was seen in the presence of EGTA). A second peak of inhibitory activity which bound to the DEAE cellulose was often observed, but this activity was Ca^{2+}-independent. The total recovery of activity on the DEAE cellulose column was ~ 70%. This step gives about a tenfold purification.

The DEAE flow-through was loaded onto a hydroxyapatite column (Fig. 2) (Bio-Gel HTP; [Bio-Rad Laboratories, Inc., Richmond, CA] one-tenth the volume of the DEAE column; pretreated with 0.1 mg/ml BSA to reduce losses of activity on the column, then washed with 300 mM potassium phosphate followed by 10 mM potassium phosphate, pH 6.5). This resulted in the appearance of a pink band at the top of the column. The column was washed with 0.1 M KCl in 10 mM potassium phosphate, pH 6.5, until the pink band passed through the column. Then a 0.1 M to 0.6 M KCl gradient (20 times column volume) in 10 mM potassium phosphate was run, and the active fractions (2 times column volume) were pooled. Total recovery of protein on this column was ~70%, 10–20% of which was in the pooled fractions. An approximate fivefold purification was obtained at this step, giving a hydroxyapatite (HAP) pool estimated to be 80% pure by scanning of acrylamide gels.

The pool was concentrated tenfold by vacuum dialysis in a collodion bag with a 25,000-dalton cutoff, which resulted in further purification by elimination of low molecular weight contaminants; 90% of the protein was recovered at this step. This final fraction was estimated to be ~90% pure by gel scanning.

Other Methods

SDS PAGE was carried out by the method of Laemmli (16). Protein concentration was determined by the method of Bradford (2). Actin concentration was also determined optically, using an absorbance at 290 nm of 0.62 for 1 mg/ml actin and a 1 cm path length (9).

RESULTS

The scheme used to purify the 40,000-dalton protein involved ammonium sulfate fractionation of a high-speed supernate followed by DEAE and HAP chromatography and concentration by vacuum dialysis. The details of each step are presented in Materials and Methods. The proteins present at the various stages of purification are displayed by SDS PAGE in Fig. 3. The final preparation of 40,000-dalton protein is ~90% pure.

We conclude that the 40,000-dalton protein is responsible

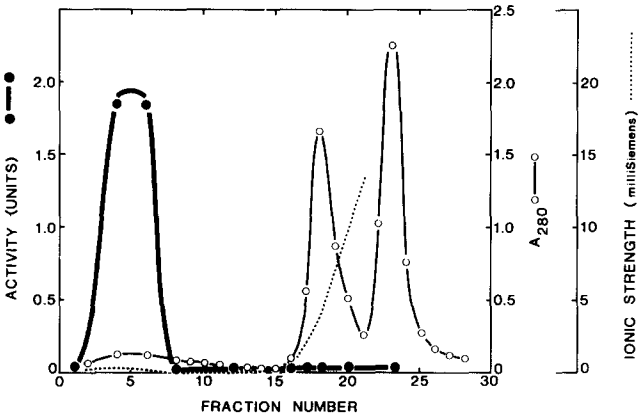

FIGURE 1 DEAE chromatography of the 40% ammonium sulfate fraction. The 40% ammonium sulfate supernate (40 ml) in 2 mM triethanolamine, pH 7.5, 0.2 mM DTT was loaded onto a 35-ml DE 52 (Whatman) column (2.5 cm × 6.3 cm) preequilibrated with 400 ml of the same buffer. 12.5 ml was collected per fraction, and fractions 4–6 (equal to the flow-through volume of the column) were pooled. After a wash (fractions 8–14), a gradient of 0–1 M KCl was applied. Activity (see Materials and Methods) was assayed across the column.

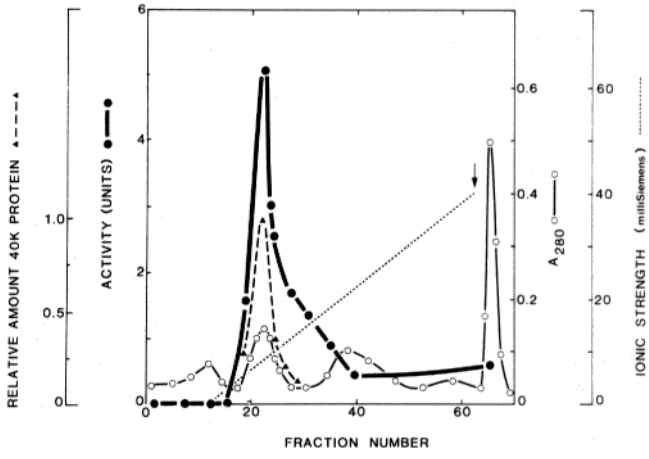

FIGURE 2 Hydroxyapatite chromatography of the DEAE pool. The DEAE pool was loaded onto a 2.5-ml hydroxyapatite column (0.8 cm × 4.5 cm) which had been preequilibrated with 10 mM potassium phosphate, pH 6.5. A 0–1 M KCl gradient in 10 mM potassium phosphate, pH 6.5 (40 ml total) was then applied. Beginning with fraction number 62 (arrow), the column was washed with 0.3 M potassium phosphate, pH 6.5. Activity (see Materials and Methods) was assayed across the column, and fractions 19–29 (0.5 ml each) were pooled.

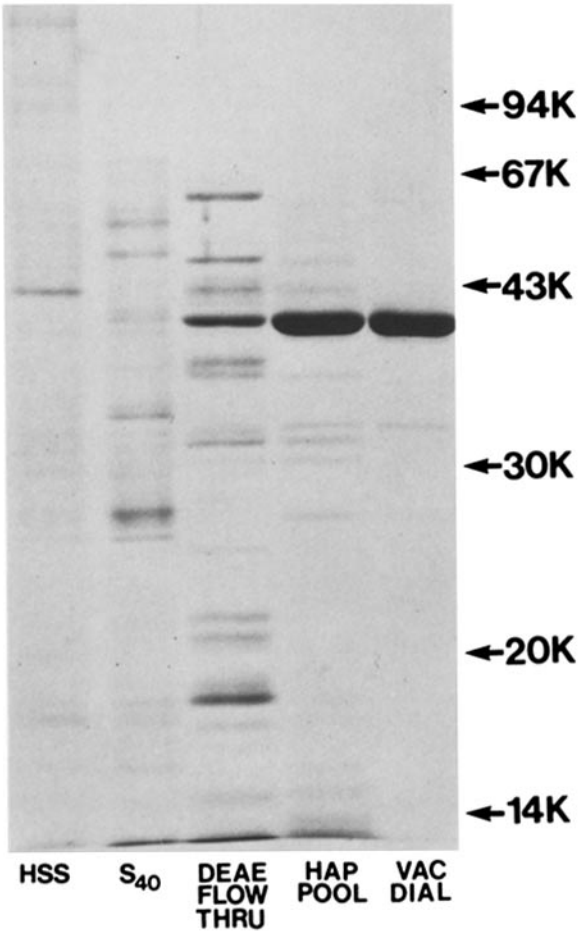

FIGURE 3 SDS PAGE of the various fractions during purification of the 40,000-dalton protein. All lanes were loaded with 3.5 μg of protein. The first lane is the high-speed supernate of a *Dictyostelium* lysate; the second, the 40% ammonium sulfate supernate; the third, the pooled DEAE flow-through; the fourth, the hydroxyapatite pool; and the fifth, the final vacuum dialysis concentrate. A set of molecular weight standards (Pharmacia Fine Chemicals) was also run on this gel; their positions are indicated. The gel was 12% acrylamide, with a 5% stacking gel.

for the activity, because there was excellent agreement between elution profiles of the 40,000-dalton protein and activity with hydroxyapatite chromatography using either a phosphate or a KCl gradient (these give very different separations; see reference 1), with carboxylmethyl cellulose chromatography, and with Bio-Gel P-100 chromatography (Fig. 4; Bio-Rad Laboratories, Inc.). Minor contaminants that can be observed on overloaded gels of the final preparation do not comigrate with activity. The gel filtration chromatography (Fig. 4) demonstrates that in the absence of actin the 40,000-dalton protein migrates as a monomer under nondenaturing conditions.

The 40,000-dalton protein has dramatic effects on F-actin sedimentability (Fig. 5). Low concentrations of factor produced a limited reduction in sedimentability. At higher concentrations, there was a further gradual decrease in sedimentability, reaching completion at roughly a 1:10 ratio (see footnote 1) of factor to actin monomer. The curves obtained as a function of the concentration of 40,000-dalton protein were identical at 30 and 90 min of incubation before sedimentation, demonstrating that the extent of reduction in sedimentability had reached completion for each factor concentration. Thus, the factor acts stoichiometrically rather than catalytically. This factor-induced reduction in sedimentability showed an absolute dependence on Ca^{2+}.

The 40,000-dalton protein also inhibits the incorporation of radioactivity into F-actin (see *Assays* in Materials and Methods). This inhibition occurs at a low stoichiometry; one 40,000-dalton molecule per ~80 actin monomers was required to give maximal inhibition. This inhibition occurs only in the presence of Ca^{2+} (Fig. 5).

We also assayed the effect of the 40,000-dalton protein on the rate of actin assembly (Fig. 6). Under buffer conditions similar to those of Isenberg et al. (15) we found that the factor accelerated the rate of assembly. As with the other assays, this effect was Ca^{2+}-dependent.

We showed by SDS PAGE that the 40,000-dalton protein does not exert its effects by proteolyzing actin (Fig. 7). Furthermore, the 40,000-dalton protein is not a proteolytic fragment of actin. We digested parallel aliquots of this protein and

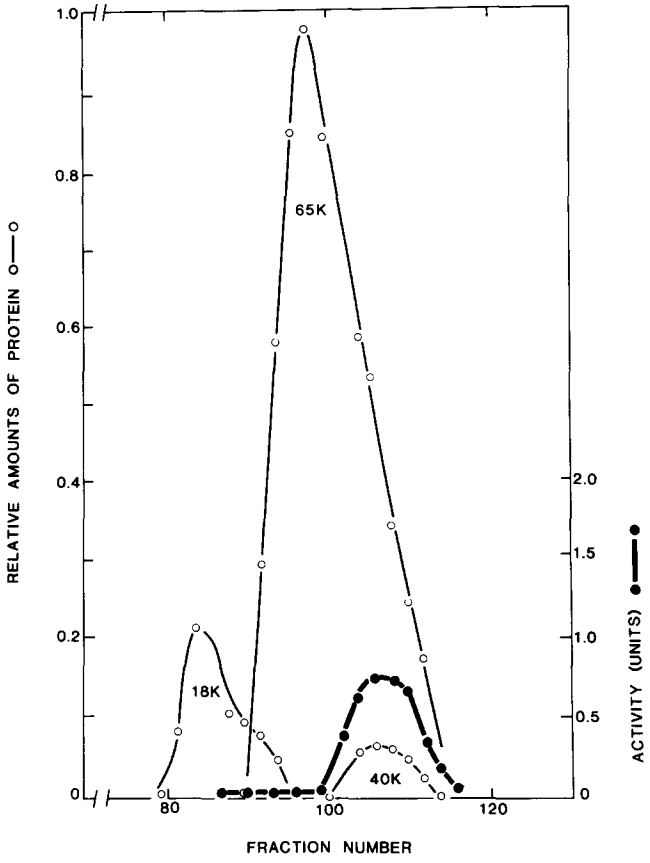

FIGURE 4 P-100 gel filtration chromatography. A 0.5-ml sample (obtained from a hydroxyapatite column run with a phosphate instead of a KCl gradient) was loaded onto a 50 × 1.5 cm P-100 column (100–200 mesh, Bio-Gel) in 25 mM triethanolamine, pH 7.5, 0.2 mM DTT, 50 mM KCl. The flow rate was 6 ml/h, and 0.3-ml fractions were collected and analyzed by SDS PAGE. Three bands (65,000, 40,000, and 18,000 daltons) were present in sufficient amounts to be quantitated by gel scanning. The relative amounts of these proteins were obtained by cutting out and weighing the peaks from the gel tracings. The column was calibrated using Blue Dextran-2000 (void volume), BSA (67,000 daltons), and ovalbumin (43,000 daltons). The 65,000- and 40,000-dalton components migrated as monomers, whereas the 18,000-dalton protein migrated as a higher molecular weight component. These fractions were assayed, and activity (see Materials and Methods) comigrated with the 40,000-dalton protein.

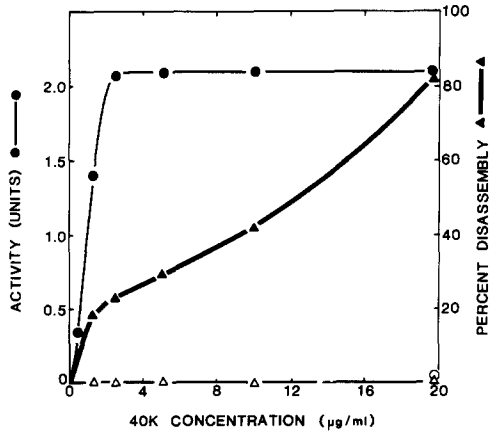

FIGURE 5 Reduction in sedimentability of F-actin by the 40,000-dalton protein. Sedimentability of actin (triangles) and the standard "activity" (see Materials and Methods) (circles) were measured under the following conditions: 200 μg/ml actin in 25 mM triethanolamine, pH 7.5, 0.2 mM DTT, 0.2 mM ATP, 50 mM KCl, 2 mM $MgCl_2$, 0.01% azide, and 0.1 mM $CaCl_2$ (closed symbols) or 0.5 mM EGTA (open symbols).

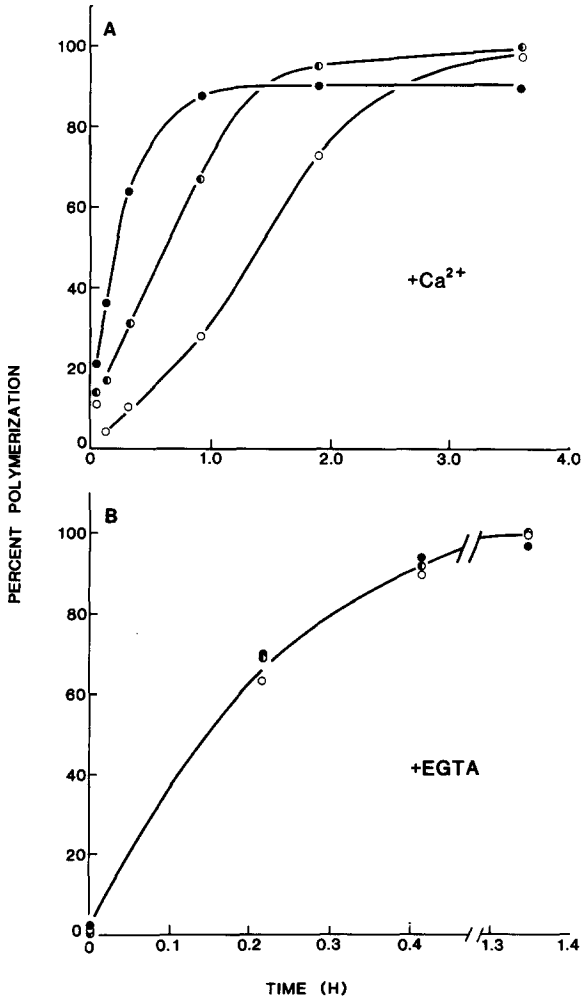

FIGURE 6 Acceleration of rate of actin assembly by the 40,000-dalton protein. 200 μg/ml actin was assembled in 25 mM triethanolamine, pH 7.5, 0.2 mM DTT, 0.2 mM ATP, 50 mM KCl, and 0.1 mM $CaCl_2$ (part *A*) or 0.5 mM EGTA (part *B*) in the presence of 0 μg/ml (open circles), 3 μg/ml (half-open circles), or 6 μg/ml (closed circles) 40,000-dalton protein. Polymerization was followed by sedimentation. "Percent polymerization" means percent of final control values of sedimented actin (where final control values are taken to be 100%). It does not mean that 100% of the actin is sedimentable under these conditions; in *A*, 37%, and in *B*, 13% of the actin remains in the supernate at 100% polymerization.

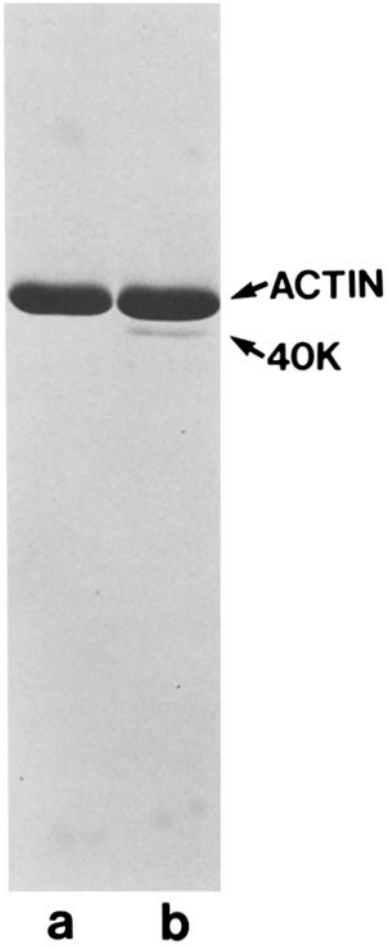

FIGURE 7 The 40,000-dalton protein does not affect the migration of actin on SDS gels. 200 μg/ml F-actin in 25 mM triethanolamine, 0.2 mM DTT, 0.2 mM ATP, 2 mM $MgCl_2$, 50 mM KCl, 0.2% sodium azide and 0.1 mM $CaCl_2$ was incubated for 0.5 h at room temperature in (*a*) the absence or (*b*) the presence of 25 μg/ml 40,000-dalton protein.

of actin with *S. aureus* V-8 protease and displayed the fragments on 15% SDS gels (5). The pattern of peptides was quite different, proving that the two proteins are different (Fig. 8). The protein doublet (see arrows, Fig. 8) derives from the V-8 protease preparation.

DISCUSSION

We have isolated a protein from *D. discoideum* which affects the assembly properties of actin in a Ca^{2+}-dependent manner. The protein reduces the sedimentability of F-actin, accelerates the assembly of G-actin under some conditions, and appears to inhibit exchange between G- and F-actin. Current evidence (Yamamoto and Spudich, manuscript in preparation) indicates that the most likely mechanism of this apparent inhibition of exchange is an increase in critical concentration of actin in the presence of the 40,000-dalton protein. This increase in critical concentration would lower the specific radioactivity of the G-actin pool and therefore could decrease the extent of incorpo-

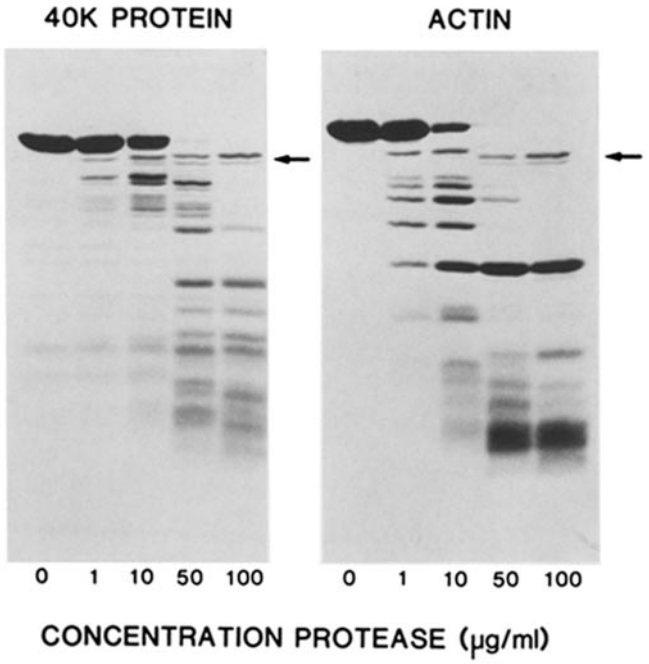

FIGURE 8 Peptide mapping of the 40,000-dalton protein vs. actin. 600 μg/ml of *Dictyostelium* actin or 40,000-dalton protein in 125 mM Tris Cl, pH 6.8, 0.5% SDS, 10% glycerol was heated to 100°C for 2 min, then incubated at 37°C with *S. aureus* V-8 protease (Miles Laboratories Inc.) for 1 h at a final concentration of 0, 1, 10, 50, and, 100 μg/ml. The samples were brought to 2% SDS and 10% 2-mercaptoethanol, incubated for 2 min at 100°C and run on a 15% gel with a 5% stacking gel. Note especially the prominent 26,000-dalton band in the actin digest which is not seen in the 40,000-dalton protein digest. The doublet near the top of the gel (see arrows) derives from the V-8 protease preparation.

ration of radioactivity into the F-actin without decreasing the amount of G-actin incorporated.

A comparison of this 40,000-dalton protein with previously described factors from different cell types shows both similarities and differences. The properties of this *Dictyostelium* protein most closely resemble those of the 45,000-dalton protein called fragmin from *Physarum* (11, 13, 14). Both proteins disassemble (see footnote 2) actin filaments in a Ca^{2+}-dependent manner. Furthermore, under certain conditions, both proteins can enhance the initial rate of assembly of actin, as if they have the ability to generate nuclei for assembly. *Physarum* fragmin differs from this *Dictyostelium* 40,000-dalton protein in that it comigrates with actin on an SDS-gel (11). Moreover, fragmin is devoid of cysteine residues (11) whereas the 40,000-dalton protein has five (Yamamoto and Spudich, manuscript in preparation). Isenberg et al. (15) have described a "capping protein" from *Acanthamoeba*. They demonstrated by electron microscopy that the capping protein blocks assembly at the "barbed" end of actin filaments. Capping protein can enhance the initial assembly rate of actin under certain conditions. Unlike the *Acanthamoeba* capping protein, however, the *Dictyostelium* 40,000-dalton protein is Ca^{2+}-dependent, does not bind to DEAE, and behaves as a 40,000-dalton monomer rather than an oligomer with 28,000- and 31,000-dalton subunits. An activity has been reported in platelets which competes with cytochalasin binding to filaments (10). Because cytochalasin appears to bind at the "barbed" end of the filament, this activity is likely to be due to a protein that, like capping protein, binds at the barbed end. Villin (95,000 daltons; reference 3) and gelsolin (91,000 daltons; reference 22 and 23) apparently act by reducing actin filament length. Like fragmin and the protein described in this report, these two proteins are Ca^{2+}-dependent. Villin has also been reported to accelerate actin assembly and to cap the barbed filament end (7).

The extent to which the *Dictyostelium* 40,000-dalton protein is analogous to these other proteins requires further characterization of all the factors that have been identified. We believe that it is premature to try to come to any conclusions about whether any of the above proteins serve the same function in vivo. In fact, it is not even clear what the in vivo function of most of these proteins is. We predict that there will be a large number of components in a single cell type which affect assembly and disassembly of actin filaments. There are likely to be factors that enhance and others that inhibit nearly every step of assembly-disassembly processes occurring in the contractile apparatus. An understanding of the physiological role of our 40,000-dalton protein may have to await a complete characterization of all the actin regulatory proteins in *Dictyostelium*. So far, only gelation factors (which are also Ca^{2+}-regulated) had been purified from this organism (6, 12). We have shown in collaboration with D. L. Taylor (Harvard University, Cambridge, MA) that our 40,000-dalton protein has no gelation activity. We are currently purifying other activities that affect actin assembly and which separated away from the 40,000-dalton protein during its purification.

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