A 45,000-mol-wt Protein–Actin Complex from Unfertilized Sea Urchin Egg Affects Assembly Properties of Actin

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ABSTRACT A one-to-one complex of a 45,000-mol-wt protein and actin was purified from unfertilized eggs of the sea urchin, Hemicentrotus pulcherrimus, by means of DNase I–Sepharose affinity and gel filtration column chromatographies. Effects of the complex on the polymerization of actin were studied by viscometry, spectrophotometry, and electron microscopy. The results are summarized as follows: (a) The initial rate of actin polymerization is inhibited at a very low molar ratio of the complex to actin. (b) Acceleration of the initial rate of polymerization occurs at a relatively high, but still substoichiometric, molar ratio of the complex to actin. (c) Annealing of F-actin fragments is inhibited by the complex. (d) The complex prevents actin filaments from depolymerizing. (e) Growth of the actin filament is inhibited at the barbed end. In all cases except b, a molar ratio of less than 1:100 of the 45,000-mol-wt protein–actin complex to actin is sufficient to produce these significant effects. These results indicate that the 45,000-mol-wt protein–actin complex from the sea urchin egg regulates the assembly of actin by binding to the barbed end (preferred end or rapidly growing end) of the actin filament. The 45,000-mol-wt protein–actin complex can thus be categorized as a capping protein.

Actin is one of the most abundant proteins in eucaryotic cells. It is an essential component of muscle and contractile structures and cytoskeletons of nonmuscle cells. Nonmuscle cell extracts prepared under physiological salt conditions contain actin in both monomeric and filamentous forms (1, 7, 10, 14, 31, 33, 43). It is generally agreed that the filamentous actin plays a major role in cell motility; it is also assumed that transformation from the monomeric to filamentous form takes place when cells form contractile and/or cytoskeletal structures.

In the unfertilized sea urchin egg, about half of actin is in the monomeric form (31). Fertilization induces polymerization of actin in the cortical layer to form microvillar actin bundles and the cortical actin meshwork (2, 9, 30, 40). Formation of the contractile ring filaments (37) also seems to be related to modulations in the state of actin filaments within the cell. To clarify the mechanism by which these polymerized actin filaments are formed in the cell, the systems that modulate actin assembly must be studied. Accordingly, we have found several G-actin-binding proteins in the unfertilized sea urchin egg (19). Two of these proteins which depolymerize actin filaments have been purified and characterized so far (19, 28).

In this paper, we report the isolation of a newly discovered 45,000-mol-wt protein–actin complex (45K-A). This complex influences the rates of actin polymerization and depolymerization, inhibits the interaction between actin filaments, and caps the barbed end of the filament, thereby arresting filament growth at this end. A preliminary account of this study has already been published (20, 29).

MATERIALS AND METHODS

**Buffer Solutions:** The buffer solutions used were composed as follows: F buffer-I—0.1 M KCl, 10 mM HEPES (pH 7.5), 2 mM MgCl₂, 0.2 mM ATP, 0.2 mM diithiothreitol (DTT), 10 mM p-tosyl-L-arginine methyl ester, 1 μg/ml leupeptin; F buffer-2—F buffer-I plus 1 mM EGTA; F-E buffer—F buffer-I plus 2 mM CaCl₂; F-Gu buffer—F buffer-I plus 3 M guanidine hydrochloride.

**Preparation of the Egg Extract:** Eggs of the sea urchin, Hemicen-
**Preparation of Actin:** Sea urchin egg actin was prepared as described by Mabuchi and Spudich (31). Rabbit skeletal muscle actin was prepared by the method of Spudich and Watt (41) with slight modifications (19) and further purified by gel filtration on a Sephadex G-100 column (Pharmacia Fine Chemicals, Uppsala, Sweden) preequilibrated with 2 mM HEPES (pH 7.8), 0.2 mM ATP, and 0.2 mM DTT. The purified G-actin was stored up to 2 wk at 0°C in the presence of 2 mM NaN3.

**Affinity Chromatography with a DNase I Column:** DNase I (Boehringer, grade II, Mannheim, Federal Republic of Germany) was coupled to Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA) according to the Bio-Rad instructions (4). After the coupling was completed, precautionary blocks of any remaining active esters were accomplished by adding 1 M ethanolamine-HCl (pH 8.0). The DNase I-coupled agarose gels were then transferred into a glass cylinder (1 × 5 cm), washed with F-Gu buffer and equilibrated with F-C buffer.

The soluble cytoplasmic protein fraction was dialyzed against F-buffer-1, and aggregates were removed by centrifugation at 10,000 g for 20 min. The supernatant was supplemented with CaCl2 to give a final concentration of 2 mM and applied to the DNase I-bound agarose column. After collection of the unadsorbed materials, the column was thoroughly washed with F-C buffer, and the proteins were eluted successively with F-E buffer, F-K buffer, and F-Gu buffer, as previously described (19).

**Assay of the Actin Polymerization:** Actin polymerization was assayed either by spectrophotometry (17) or by viscometry. A G-actin solution was supplemented with KCl and MgCl2 or with KCl alone at 20°C to initiate polymerization. When the effect of 45K-A was studied, 45K-A was mixed with G-actin and the salt was soon added. The changes in absorbance at 237 or 238 nm were monitored with a Gilford model 260 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH) equipped with an Endo Cal thermostabilizer (NESLAB Instruments, Inc., Portland, NH) regulated at 20°C and Shimadzu CS 910 dual wavelength chromatoscanner (Shimadzu Seisakusho Ltd., Tokyo). Viscosity was measured at 20°C in an Ostwald-type viscometer (Makuhari Rikagaku Kiki, Chiba, Japan) with an outflow time of 68 s for water.

**DNase I Inhibition Assay:** The DNase I inhibition assay for the measurement of actin content was performed as described by Mabuchi and Spudich (31).

**SDS PAGE:** Polyacrylamide slab gel electrophoresis in the presence of SDS was performed as described by Laemmli (24). The acrylamide concentration was 15% unless otherwise stated. Before electrophoresis, protein samples were boiled for 3-6 min in a solution of 1.6% SDS, 0.06 M Tris-HCl (pH 6.8), 5% (vol/vol) 2-mercaptoethanol, and 8% glycerol. The molecular weights of proteins were determined using the following marker proteins: rabbit muscle phosphorylase b (95,000), BSA (68,000), hen ovalbumin (45,000), rabbit skeletal muscle actin (42,000), bovine pancreas DNase I (31,000), soybean trypsin inhibitor (20,000), and horse heart cytochrome c (13,000). Gels were stained with 0.025% Coomassie Brilliant Blue R-250 dissolved in 25% (vol/vol) isopropanol and 10% (vol/vol) acetic acid, and destained in 7% acetic acid. Denaturation of stained gels was carried out using a Beckman model R-112 scanning spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). It was assumed that all electrophoresed proteins bound the dye equally.

**Protein Determination:** Protein was determined according to the method of Lowry et al. (25) using BSA as a standard.

**Electron Microscopy:** Sample solutions were mounted on a carbon-coated Formvar grid, stained negatively with 1% uranylacetate, and viewed with a JEOL JEM 100CX (JEOL Ltd., Tokyo) or Hitachi H-300 electron microscope (Hitachi Ltd. Tokyo).

**RESULTS**

**Purification of the 45K-A**

The soluble cytoplasmic protein fraction (1.555 mg of protein) of Hemicentrotus eggs was loaded on a DNase I affinity column in the presence of Ca2+. The column was washed first with the F-C buffer and then with the F-E buffer containing EGTA. Proteins still retained in the column were eluted with F-K buffer containing 1 M KCl; (lane 4) protein eluted with F-Gu buffer containing 3 M guanidine-HCl; and (lane 5) muscle actin. M represents molecular weight marker proteins (values × 103) (see Materials and Methods).

![FIGURE 1 SDS PAGE of the DNase I column eluates of the soluble cytoplasmic protein fraction. Proteins recovered from the DNase I column were precipitated with 6% trichloroacetic acid in the presence of 0.017% sodium deoxycholate (3). After adjusting pH to neutral with 1 M Tris (pH 9.3) solution, the proteins were further processed for electrophoresis as described in Materials and Methods. (lane 1) The soluble cytoplasmic protein fraction; (lane 2) proteins eluted with F-E buffer containing 5 mM EGTA; (lane 3) proteins eluted with F-K buffer containing 1 M KCl; (lane 4) protein eluted with F-Gu buffer containing 3 M guanidine-HCl; and (lane 5) muscle actin.](https://example.com/figure1.png)
far not possible to separate them by DEAE-cellulose, hydroxyapatite, or gel-filtration column chromatography. The F-K buffer eluate was further fractionated with a Sephadex G-200 column (1.2 × 101 cm) to purify the 45K-A (Fig. 3). The 45K-A was co-eluted from the column at a $K_v$ of 0.33. From this value and using marker proteins (Fig. 4), the Stokes radius of 45K-A was estimated to be 3.8 nm (38). Furthermore, the molar ratio of the 45K-A to actin in the purified fraction was 1:1 by estimation with densitometric scans. These results demonstrate that the two proteins form a one-to-one complex. 2.8 mg of the purified 45K-A was obtained.

Effects of 45K-A on Actin Polymerization

Fig. 5 shows that 45K-A at very low concentrations affected both the initial rate of polymerization and the final viscosity level of the polymerized actin. The initial rate of viscosity increase was accelerated by the presence of 45K-A. This effect was observed in both the presence and absence of Ca$^{2+}$. (Data for the presence of 0.5 mM EGTA are not shown.) The viscosity increase was more clearly observed in the former case because the control polymerization was slower in the presence of Ca$^{2+}$ than in its absence. The final steady-state viscosity level was reduced by the presence of 45K-A. The effect was observed with a substoichiometric amount of 45K-A and was dependent on 45K-A concentration (Fig. 5). The final level was the same in both the presence and absence (not shown) of Ca$^{2+}$ provided that the 45K-A concentration was the same.

The monomeric actin concentration was measured by means of DNase I inhibition assay after the steady-state viscosity was attained. It was 18, 67, and 69 μg/ml (average of three determinations each) for samples a, b, and c in Fig. 5, respectively. It can be said that, first, addition of a substoichiometric amount of 45K-A resulted in the increase in the monomeric actin concentration to a large extent. Second, an increase in the 45K-A concentration from $\frac{1}{200}$ to $\frac{1}{50}$ of actin (in molar ratio) did not make any significant difference in the concentrations.
amount of the monomeric actin. The latter result, together with the viscosity data (Fig. 5), suggests that the low level of the final viscosity produced by the addition of 45K-A cannot be explained solely in terms of the depolymerization of actin.

A spectrophotometric study was also performed because of its efficacy in keeping actin solutions under no-shear stress conditions. By measuring the increase in absorption at 237 or 238 nm, which represents the increase in mass concentration of actin filament (17), the extent of actin polymerization could be determined. In the measurements shown in Fig. 6, 45K-A was mixed with G-actin (0.3 mg/ml) at varying molar ratios and the polymerization was initiated by adding salts. The presence of 45K-A showed a concentration-dependent biphasic effect on the rate of actin polymerization. At very low concentrations, 45K-A reduced the initial rate of polymerization, in both the absence of Ca\(^{2+}\) (45K-A/actin \(\leq 1:500\)) and its presence (45K-A/actin = 1:2000) (Fig. 6). At higher concentrations (45K-A/actin \(\geq 1:200\)), however, 45K-A accelerated actin polymerization, in both the presence and absence of Ca\(^{2+}\) (Fig. 6).

The final steady-state level was examined on samples in which the absorbance, \(A_{237}\) nm, reached a plateau at 60 min after addition of salts, that is, curves a, d, and e in Fig. 6. Samples containing two different amounts of 45K-A (45K-A/actin = 1:50 and 1:200) converged on the same level, which was a little lower than the control level. This difference in the steady-state absorbance from the control corresponded to actin concentration of 34 \(\mu\)g/ml when we take the critical actin concentration for polymerization as 30 \(\mu\)g/ml under present conditions (see below).

Electron microscopic observation was performed on the same samples as used in the spectrophotometry. It revealed that many short filaments were produced in samples containing 45K-A both in the absence (Fig. 7) and presence of Ca\(^{2+}\) (not shown), which was consistent with the low viscosity (Fig. 5).

Next, the effect of 45K-A on the elongation step of actin polymerization was studied. The addition of F-actin nuclei, which had been sonicated in the presence of 45K-A, did not promote polymerization of actin (Fig. 8). When F-actin solution was sonicated without 45K-A, its addition to monomeric actin resulted in rapid polymerization of actin. This result can be explained by assuming that the end of the
filament capped with 45K-A loses the ability to initiate filament growth.

**Effects of the 45K-A on Actin Filaments**

Addition of substoichiometric amounts of 45K-A to F-actin solution (0.22 mg/ml) caused a gradual reduction in its viscosity. The extent of the reduction was the same even when the amount of the 45K-A was changed (Fig. 9). This result suggests that 45K-A does not fragment actin filaments.

In the next experiment, actin filaments at steady state were sonicated in the presence or absence of 45K-A, and then the recovery of viscosity was monitored (Fig. 10). In the absence of 45K-A, the viscosity returned to the original level within 15 min, indicating that annealing of the sonicated F-actin fragments took place. In contrast, in the presence of 45K-A, the recovery in viscosity did not take place (Fig. 9).

Depolymerization of actin in either the absence or presence of 45K-A was studied by measuring A 238 nm of F-actin solution after dilution with water. As shown in Fig. 11, the rate of reduction in A 238 nm was suppressed by 45K-A, which suggested that 45K-A inhibited the rate of depolymerization of actin.

**45K-A Blocks Filament Growth at the Barbed End**

Considering the effects of 45K-A on F-actin as described above, we conclude that the complex binds to the end of actin filament as do so-called end-blocking or capping proteins such as β-actinin (32), acumentin (39), Acanthamoeba capping protein (21), and fragmin (15, 18).

In order to determine the filament end to which the com-

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**Figure 7** Electron micrographs of negatively stained actin filaments. G-actin (0.15 mg/ml) was polymerized in the absence (A) or presence (B) of 45K-A (45K-A/actin, 1:50) by addition of 0.1 M KCl and 2 mM MgCl₂. The composition of the buffer solution is the same as described in the legend to Fig. 6. Bar, 1.0 μm. × 35,000.

**Figure 8** Effects of the 45K-A on nucleated actin filament growth. Steady-state samples of F-actin solution (0.3 mg/ml) were sonicated with a Branson sonicator (Branson Sonic Power Co., Danbury, CT) at the setting position of 5 in the absence or presence of 45K-A (3 μg/ml). 0.1 vol of the sonicated F-actin was then added to gel-filtered G-actin (0.3 mg/ml). Polymerization was induced by immediate addition of KCl up to 30 mM. (a) Control (G-actin plus KCl); (b) control plus 45K-A; (c) control plus sonicated F-actin; (d) control plus F-actin sonicated in the presence of 45K-A.
FIGURE 9 Effects of the 45K-A on the viscosity of F-actin solution. At the time indicated by the arrow, 45K-A (O, 1.6 μg/ml or ●, 3.6 μg/ml) was added to F-actin solution (0.22 mg/ml) in 10 mM HEPES (pH 7.5), 0.1 M KCl, 0.15 mM EDTA, 0.5 mM CaCl₂, 0.15 mM ATP, and 0.15 mM DTT. The changes in viscosity were monitored.

FIGURE 10 Effects of the 45K-A on the annealing of the F-actin fragments. At 0 min, F-actin solution (0.15 mg/ml) in F-buffer plus 0.5 mM EGTA was sonicated for 10 s in the absence (O) or presence (●) of 45K-A (6 μg/ml) with a Branson sonicator at the setting position of 4 at 0°C. At the time indicated by the arrow, the sonicated sample was injected into an Ostwald-type viscometer, and the changes in viscosity were monitored at 20°C. The broken line indicates the viscosity level before sonication.

complex binds, we employed the false discharge isolated from horseshoe crab sperm (5, 6, 27). G-actin freed from exogenous ATP by gel filtration using a Sephadex G-25 column was incubated with false discharge pieces in the absence or presence of 45K-A. Then myosin S-1 was added to determine the directionality of the actin filaments grown from the ends of the false discharge pieces (27). The sample was examined by electron microscopy. Fig. 12a shows that actin filaments normally grew bidirectionally with a strong bias for the barbed end in the absence of 45K-A. In the presence of 45K-A, however, little growth was observed at the barbed end (Fig. 12, b and c), whereas normal growth took place at the pointed end. Therefore, we concluded that 45K-A caps the barbed end of the actin filament.

DISCUSSION

We have suggested that there are several proteins that possess G-actin binding activity in the sea urchin egg (19). In the present study a complex of a 45,000-mol-wt protein and G-actin exhibited potent effects on actin filament assembly. The 45K-A showed a mode of action different from those of two actin-depolymerizing proteins, depactin (19, 26, 29) and profilin (28), which have already been purified and characterized from starfish or sea urchin eggs. More specifically, the complex acts on actin in a manner similar to that of the so-called end-blocking or capping proteins.

Electron microscopy revealed that the 45K-A blocked actin monomer addition to the barbed end of the actin filament in the acrosomal process of the horseshoe crab sperm. It was further shown by viscometry that the 45K-A blocked the annealing of actin filaments and lowered the rate of actin polymerization seeded by actin filament nuclei. Moreover, the 45K-A reduced the rate of the depolymerization of actin. These results are consistent with the conclusion that the 45K-A binds to and caps one end (the barbed end) of the actin filament.

When actin was polymerized to a steady state in the presence of substoichiometric amounts of 45K-A, the monomeric actin concentration was a little higher than that of the control. The A 237 nm in the presence of 45K-A was a little lower than that of the control, which corresponded to a small increment in the monomeric actin concentration. When 45K-A was added to F-actin solution, the viscosity decreased gradually. The extent of the viscosity decrease did not seem to be affected by the change in the concentration of the 45K-A added. All these changes in the state of actin can be explained by the barbed end-capping by 45K-A. It has been proposed that the critical concentration of actin is different at the two ends of the actin filament, high at the pointed end and low at the barbed end (47). Recently, this has been confirmed experimentally (5, 36). Therefore, the capping of the barbed end of the actin filament will result in an increase in the monomeric actin concentration up to the critical concentration at the pointed end.

The 45K-A accelerated the polymerization of actin under both high-shear and no-shear stress at relatively high concentrations. Considering that 45K-A was able to inhibit the elongation step of actin polymerization, 45K-A may have increased the number of nuclei. This explanation is supported by the fact that the actin filaments polymerized in the presence of 45K-A were short compared with the control.

FIGURE 11 Effects of the 45K-A on the depolymerization of F-actin. At 0 min, F-actin solution (2.0 mg/ml) in 20 mM KCl, 5 mM HEPES (pH 7.2), 0.2 mM ATP, and 0.2 mM DTT was diluted four times with water in the presence (a) or absence (b) of the 45K-A (10 μg/ml). The depolymerization was monitored by measuring absorption at 238 nm.
Two models have been hypothesized that explain the increase of the number of nuclei by 45K-A. One is the stabilization of the nuclei-actin oligomers of two to four actin molecules (12, 22, 34, 35, 44, 48). The other is the enhancement of the increase in the number of nuclei by the production of many filament ends by the cut filaments formed at an early stage of polymerization. We consider the former to be more likely because the complex did not seem to possess any significant actin filament-severing activity as judged by viscometry.

The spectrophotometric study revealed that the 45K-A suppressed the rate of actin polymerization at relatively low molar ratios. At high molar ratios, the rate was increased. Therefore, the effect of the 45K-A on the rate of actin polymerization was biphasic depending on its concentration. We must consider two modes of effects of the 45K-A on actin polymerization to explain these results. One is the increase in the number of the nuclei caused by stabilization as discussed above, which should result in the enhancement of the overall rate of polymerization. The other is the blockade of the barbed end of the actin filament leaving the pointed end free, lowering the elongation rate. When the amount of 45K-A is large, the former is applied; when small, the latter is emphasized.

Proteins that bind to one end of the actin filaments and thereby regulate the assembly of actin in vitro have already been demonstrated in skeletal muscle (β-actinin [32]), intestinal brush border (villin [8, 13]), brain (23), platelet (46), Acanthamoeba (21), Physarum (fragmin [15, 18]), and macrophage (gelsolin [49] and acumenin [39]). The present sea urchin egg 45K-A does not resemble gelsolin, villin, or fragmin, which sever actin filaments in the presence of Ca\(^{2+}\), but is similar to β-actinin from skeletal muscle and to brain and Acanthamoeba capping proteins inasmuch as they are heterodimers consisting of relatively small molecular-weight polypeptides and are not significantly Ca\(^{2+}\) sensitive. The protein most closely related to the present complexes, however, might
be a complex of fragmin and actin originally called *Physarum* actin (16). Both complexes are one-to-one complexes of G-actin and a protein having a molecular weight close to that of actin; they also do not seem to sever actin filaments (18). However, separation of the 45,000-mol-wt component from actin and its characterization remains to be studied for further identification of this protein and its mode of action on actin.

We do not have evidence at present that the 45,000-mol-wt protein and actin really form a complex in the egg cytoplasm. These proteins may have become associated during the extraction of proteins from eggs. However, it is reasonable to speculate that the complex was present in the cytoplasm inasmuch as the amount of the 45,000-mol-wt protein in the egg was considerably high and the association of these proteins was extremely tight.

The sea urchin egg undergoes morphological changes after fertilization, especially in the cell cortex, which could be based on changes in the supramolecular assembly of actin (reviewed by Vacquier [45]). A large amount of monomeric actin pooled in the unfertilized eggs (31), probably as complexed forms with depactin (19) and/or profilin (28), is considered a precursor of actin-based structures in the fertilized eggs. The 45K-A can act as a nucleus for the polymerization of actin from this pool. More importantly, this complex may determine the site of assembly of actin filaments and regulate their rate of assembly.

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