A 45,000-mol-wt Protein from Unfertilized Sea Urchin Eggs Severs Actin Filaments in a Calcium-dependent Manner and Increases the Steady-state Concentration of Nonfilamentous Actin

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ABSTRACT A 45,000-mol-wt protein has been purified from unfertilized sea urchin (*Strongylocentrotus purpuratus*) eggs. The isolation scheme includes DEAE cellulose ion-exchange chromatography, gel filtration, and hydroxylapatite chromatography. The homogeneity of the isolated protein is >90% by SDS PAGE. The 45,000-mol-wt protein reduces the viscosity of actin filaments in a Ca^{2+}-dependent manner. The free calcium concentration required for the activity of this protein is in the micromolar range. Electron microscopic studies reveal that the formation of short filaments parallels the decrease in viscosity. Energy transfer and sedimentation experiments indicate a net disassembly of actin filaments and an increase in the steady-state nonfilamentous actin concentration in the presence of Ca^{2+} ions and the 45,000-mol-wt protein. The increase in the steady-state nonfilamentous actin concentration is proportional to the amount of 45,000-mol-wt protein added. The actin molecules disassembled by the addition of the 45,000-mol-wt protein are capable of polymerization.

The organization of the contractile proteins in nonmuscle cells is a subject of great interest in biological research. A number of proteins that interact with actin in several different modes and alter the assembly of purified actin have been isolated from a wide variety of cells (for reviews see references 9, 20, 32, 46). These proteins are believed to play a regulatory role in the cytoskeletal changes that occur during cell locomotion, secretion, cytoplasmic streaming, and cytokinesis.

Sea urchin eggs provide an excellent system to study the molecular mechanism of actin assembly because it is easy to obtain a large homogenous population undergoing a synchronized change in cytoskeleton arrangement upon fertilization (8, 10). In unfertilized sea urchin eggs of *S. purpuratus*, there is an actin concentration of ~3 mg/ml and ~40% of this actin appears to be monomeric (24). Otto et al. (26) reported that in *Tripneustes gratilla*, a Hawaiian sea urchin, ~90% of the actin is nonsedimentable at 100,000 *g* for 60 min in an isotonic extract of unfertilized eggs. An identical extract of fertilized *T. gratilla* eggs has 60–65% nonsedimentable actin.

The state of the actin in the cortex of sea urchin eggs changes dramatically upon fertilization. The numerous short microvilli that cover the surface of the unfertilized egg elongate greatly (33, 34), and each microvillus contains a bundle of actin filaments (6). Upon fertilization there is also a rapid Na^+ influx, an increase in intracellular free Ca^{2+} concentration, and a pH increase (for reviews, see references 11, 12, 41). The elongation and formation of actin filament bundles have been suggested to be Ca^{2+} and pH regulated (1). In that the in vitro steady-state, nonfilamentous concentration of purified sea urchin egg actin is 0.02–0.05 mg/ml (24), and the concentration of total actin in unfertilized eggs is much greater, the regulation of the state of actin assembly may involve accessory proteins.

Several actin-binding proteins have been isolated from eggs. Fascin, a 58,000-mol-wt protein, has been shown to cross-link actin filaments and form bundles (5). Immunofluorescent antibody staining has located fascin in the fertilized egg cortex (26) and in the pseudopods of sea urchin coelomocytes (25). Fascin cross-linked actin-filament bundles prepared in vitro have a transverse-stripe pattern of 11-nm axial periodicity, which is similar to that observed in sea urchin microvilli in situ (6, 37). These needle-shaped bundles form a three-dimensional gel when a 220,000-mol-wt egg protein is added (5). The isolations of fascin and the 220,000-mol-wt protein start

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from a temperature-induced actin gel from an egg extract (18, 19). The gelation of the crude extract is Ca^{2+} sensitive, but the formation of a reconstituted gel from purified actin, fascin, and the 220,000-mol-wt protein is not (5). Thus there must be yet another component that is involved in this Ca^{2+} dependence. A profilin-like protein and depactin have been isolated from echinoderm eggs (16, 22, 23), but there is no known pH or Ca^{2+} dependence on the action of these proteins.

An activity from unfertilized sea urchin eggs, which modulates actin assembly in a Ca^{2+}-dependent manner, was previously reported (35). Here we report the purification of this activity to electrophoretic homogeneity. The activity resides in a 45,000-mol-wt protein, which severs actin filaments in the presence of Ca^{2+} and increases the steady-state nonfilamentous actin concentration (43).

MATERIALS AND METHODS

Purification of the 45,000-mol-wt Protein: Sea urchin (*S. purpuratus*) eggs were shed into artificial seawater (Instant Ocean purchased from Aquarium Systems, Mentor, OH) by intracoelomic injection of 0.5 M KCl. The unfertilized eggs were washed three times in at least 10-fold vol of a pH 7.0 isotonic solution free of divalent cations; 19 parts of 0.53 M NaCl solution were mixed with one part of 0.53 M KCl, and neutralized EGTA was added to a final concentration of 0.2 mM (18). The washed unfertilized eggs were sedimented by hand centrifugation (packed volume of eggs was 8 ml) and homogenized using a type A pestle into 10-fold (vol/vol) ice-cold homogenization buffer (0.1 M *N*-morpholino-ethanesulfonic acid, 5 mM EGTA, 0.9 M glycerol, 0.5 mM ATP, 0.2 mM dithiothreitol (DTT),[1] 10 mM *p*-tosyl-L-arginine methyl ester, 1 mM phenylmethylsulfonyl fluoride, 0.05% NaN_3, pH 6.9 at 4°C) by 7–10 passes in a Dounce homogenizer on ice. Changing the buffer/egg volume ratio from 10 to 5 or even to 2 did not affect the specific activity in the extract. There was no noticeable proteolysis in the process of preparing the egg homogenate, as indicated by SDS PAGE of the extract compared with washed eggs dissolved quickly in boiling 2% SDS solution. All of the following procedures were carried out at a temperature between 0 and 4°C unless otherwise specified.

The egg homogenate was centrifuged for 60 min at 15,000 *g*. The low-speed supernatant (LSS) was centrifuged again at 150,000 *g* for 1 h to yield the first high-speed supernatant (HSS1). HSS1 was then dialyzed against 1 liter of low salt buffer containing 10 mM Tris, 0.1 mM EGTA, 0.5 mM ATP, 0.2 mM DTT, 0.05 mM $MgCl_2$, 1 mM *p*-tosyl-L-arginine methyl ester, 1 mM phenylmethylsulfonyl fluoride, 0.05% NaN_3, pH 8.0 at 4°C, with three changes of buffer at intervals of 6–8 h. The dialyzed HSS1 was clarified by centrifugation at 150,000 *g* for 1 h to yield the second high-speed supernatant (HSS2).

Pre-swollen DEAE cellulose (Whatman DE52, Whatman Chemical Separation Inc., Clifton, NJ) was packed into a column (2.6 × 15 cm) after washing in DEAE buffer containing 10 mM Tris, 0.1 mM EGTA, 0.5 mM ATP, 0.2 mM DTT, 0.05 mM $MgCl_2$, 0.05% NaN_3, pH 8.0 at 4°C. HSS2 (84 ml) at a protein concentration of 2 mg/ml was loaded onto the column. The column was washed with 200 ml DEAE buffer before eluting with a 520-ml NaCl gradient from 0 to 0.3 M in the same buffer. Fractions were assayed for inhibition of actin polymerization by rolling ball viscometry (see below). The activity peak was pooled (DEAE pool), dialyzed, and concentrated by vacuum dialysis against DEAE buffer using a 10,000-mol-wt cutoff collodion bag (Schleicher & Schuell, Inc., Keene, NH).

The concentrated activity peak in 4 ml at a protein concentration of 2 mg/ml was fractionated on a 1.5 × 76 cm Sephadex G-200 column (Pharmacia Fine Chemicals, Uppsala, Sweden). The activity, assayed by rolling ball viscometry, was pooled and dialyzed against hydroxylapatite (HAP) buffer (10 mM KH_2PO_4, 0.5 mM ATP, 0.2 mM DTT, 0.05% NaN_3, pH 7.0 at 4°C). The dialyzed G-200 pool, 1.5 ml at a protein concentration of 1 mg/ml, was loaded onto a 1 × 5-cm HAP (Bio-Gel HTP, Bio-Rad Laboratories, Richmond, CA) column packed and equilibrated with HAP buffer. This column was eluted with a phosphate gradient from 0.01 to 0.3 M in the same buffer.

[1] *Abbreviations used in this paper:* C_C, critical concentration; C_{HSS}, concentration of actin remaining the high-speed supernatant after centrifugation; DTT, dithiothreitol; FITC, fluorescein isothiocyanate; HAP, hydroxylapatite; HSS1, first high-speed supernatant; HSS2, second high-speed supernatant; IAENS, iodoacetamidoethyl aminonaphthalene sulfonic acid; LSS, low-speed supernatant.

The HAP fractions that contained highly purified 45,000-mol-wt protein were pooled, dialyzed against 10 mM Tris, 0.5 mM ATP, 0.2 mM DTT, and 0.05% NaN_3, pH 7.0 at 4°C, and stored on ice.

Rolling Ball Viscometry Assay: The viscosity was measured in a rolling ball apparatus similar to that described by Pollard (29) in the presence of 0.2 mM $CaCl_2$ or 0.2 mM EGTA with or without the 45,000-mol-wt protein. Rabbit skeletal muscle G-actin was prepared by the method of Spudich and Watt (38) and further purified by the recycling procedure of Pardee and Spudich (28). This actin (100 μl, 0.3 mg/ml) was incubated at 22°C for 10 min with 12.5 μl of egg extract in an assay buffer (10 mM Tris, 0.2 mM ATP, 0.2 mM DTT, 0.005% NaN_3, 0.05 mM $MgCl_2$, pH 7.8 at 25°C, containing either 0.2 mM $CaCl_2$ or 0.2 mM EGTA). Actin polymerization was induced by addition of 12.5 μl of a stock solution of 1 M KCl and 20 mM $MgCl_2$ to give final concentrations of 0.1 M KCl and 2 mM $MgCl_2$. An equal volume of buffer was added to the control samples. The samples were drawn into a 100-μl micropipette (Clay-Adams, Parsippany, NJ) after actin polymerization had reached steady state. A microball of diameter 0.64 mm, grade 10, 440C (Microball Co., Peterborough, NH) was inserted into the micropipette and passed back and forth several times before taking the first reading. The time for the ball to roll a distance of 6 cm at a 20° angle declination from horizontal at room temperature (22°C) was recorded, and the percentage of inhibition of actin polymerization was calculated as follows:

$$\% \text{ of inhibition} = 100 - \left[\frac{(\text{flow time of F-actin + sample}) - (\text{flow time of G-actin + sample})}{(\text{flow time of F-actin + buffer}) - (\text{flow time of G-actin + buffer})}\right] \times 100.$$

The flow time of G-actin plus samples was always essentially the same as the flow time of G-actin plus buffer. The presence of EGTA or Ca^{2+} did not significantly affect the flow time of F-actin alone. Material that gave 50% inhibition was designated arbitrarily to contain 1 U of activity.

Determination of the Required Concentration of Free Ca^{2+}: Actin was recycled into TEOLA-F-buffer (20 mM triethanolamine, 0.2 mM ATP, 0.005% NaN_3, 0.5 mM β-mercaptoethanol, 0.1 M KCl, 2 mM $MgCl_2$, pH 7.4 at 23°C) containing various Ca/EGTA ratios (EGTA was maintained at 0.5 mM). Calcium chloride (20 mM solution purchased from Dade Diagnostics, Inc., Miami, FL) was added to obtain the desired ratio of Ca^{2+} to EGTA. The 45,000-mol-wt protein was dialyzed into TEOLA-F-buffer without added $CaCl_2$ and EGTA. An appropriate concentration of the 45,000-mol-wt protein (that which gave ~60% of maximal inhibition) was determined by using the viscometry assay at 0.2 mM $CaCl_2$. That concentration was then added to F-actin in TEOLA-F-buffer containing various Ca/EGTA ratios. The free Ca^{2+} was calculated using the apparent affinity constant determined by Harafuji and Ogawa (14).

SDS PAGE: SDS-polyacrylamide slab gels were run using the methods of Laemmli (21).

Preparation of Fluorescein Isothiocyanate-labeled Actin and Iodoacetamidoethyl Aminonaphthalene Sulfonic Acid-labeled Actin: Muscle actin, freshly recycled as described by Pardee and Spudich (28), was labeled with fluorescein-5-isothiocyanate isomer 1 (FITC) and 5-(iodoacetamidoethyl) aminonaphthalene-1-sulfonic acid (IAENS) (Molecular Probes, Inc., Junction City, OR) according to the methods of Pardee et al. (27). The labeling stoichiometries were 0.8–1.1 for FITC-actin and 0.9–1.2 for IAENS-actin. The labeled protein was stored on ice in the dark in imidazole F-buffer (2 mM imidazole, 0.5 mM ATP, 0.5 mM β-mercaptoethanol, 0.005% NaN_3, 2 mM $MgCl_2$, 0.1 M KCl, pH 7.4 at 23°C). FITC-actin and IAENS-actin were recycled (27) immediately before the experiments. Briefly, IAENS-actin and FITC-actin were centrifuged in a Beckman airfuge (Beckman Instruments, Inc., Fullerton, CA) at 30 psi for 20 min. The pellets were resuspended into imidazole G-buffer (2 mM imidazole, 0.5 mM ATP, 0.5 mM β-mercaptoethanol, 0.005% NaN_3, 0.05 mM $MgCl_2$, pH 7.4 at 23°C) or TES G-buffer (2 mM TES, 0.05 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM β-mercaptoethanol, 0.005% NaN_3, pH 7.2 at 25°C). After 2 h of dialysis at a concentration of ~2 mg/ml in a 10,000-mol-wt cutoff collodion bag, the actin solutions were clarified by airfuge centrifugation (20 min at 30 psi). The extinction coefficients of fluorescent labels were determined in imidazole G-buffer. For FITC, $\epsilon^{M}_{490} = 6.6 \times 10^4\ cm^{-1}$, and for IAENS, $\epsilon^{M}_{335} = 5.2 \times 10^3\ cm^{-1}$, which were extremely close to the values determined in TES G-buffer (FITC, $\epsilon^{M}_{490} = 6.4 \times 10^4\ cm^{-1}$; IAENS, $\epsilon^{M}_{335} = 5.1 \times 10^3\ cm^{-1}$) by Pardee et al. (27). The presence of KCl and $MgCl_2$ up to 0.1 M and 2 mM did not affect the extinction coefficients.

Fluorescence Energy Transfer Experiments: The fluorescence energy transfer from IAENS-actin (fluorescent donor) to FITC-actin (fluorescent acceptor) was monitored with a Spex Fluorolog spectrofluorometer (Spex Industries, Inc., Metuchen, NJ) as described by Pardee et al. (27). The extent of energy transfer was determined from the decrease of IAENS-actin fluores-

cence at 470 nm with an excitation wavelength of 340 nm at room temperature. FITC-G-actin and IAENS-G-actin were mixed at a molar ratio of 10:1 in the presence of 0.1 mM $CaCl_2$ or 0.1 mM EGTA and co-polymerized into filaments by the addition of 50 mM KCl and 1 mM $MgCl_2$. Filament formation resulted in a 58% decrease of fluorescence. The presence of Ca^{2+} or EGTA did not affect the fluorescence of IAENS-G-actin or the fluorescence quench. The isolated 45,000-mol-wt protein added to the co-polymerized filamentous actin in the presence of Ca^{2+} at various molar ratios caused a recovery of fluorescence or relief of quench as a result of filament disassembly. The extent of filament disassembly was calculated from the relative percentage of quench relief. The disassembled actin was separated from the filaments by a 20-min 30-psi centrifugation in a Beckman airfuge. Protein concentrations in the supernatant were determined by the Bradford assay (3) using FITC-G-actin of known concentration as a standard.

Determination of Nonfilamentous Actin Concentration at Steady State: We refer to the nonfilamentous actin at steady state as HSS-actin (that is, that actin remaining in the high-speed supernatant after centrifugation; unless noted otherwise, the conditions were 45 min at 30 psi (~120,000 *g*) in a Beckman airfuge in an A-100/18 rotor at 22°C and the top 20% of the solution was removed to determine the actin concentration). We refer to the concentration of this HSS-actin as C_{HSS} to distinguish it from C_C (critical concentration) which has been used in reference to the threshold concentration of actin necessary for assembly to occur.

Reassembly of the Actin Disassembled by the 45,000-mol-wt Protein: Freshly recycled IAENS-G-actin was polymerized with 0.1 M KCl and 2 mM $MgCl_2$ in TES G-buffer. The 45,000-mol-wt protein was then added to generate a high concentration of HSS-actin. To this HSS-actin (300 μg/ml), unlabeled G-actin was added to 3.2 mg/ml. The final salt concentration was readjusted to 0.1 M KCl and 2 mM $MgCl_2$. The actin solution was incubated at 22°C for 40 min and centrifuged again. The protein concentration in the supernatant was measured using unlabeled G-actin as standard. The amount of IAENS-actin remaining in the supernatant was monitored by the fluorescence.

Protein Determination: During the process of the 45,000-mol-wt protein isolation, protein concentration was determined by the procedure of Hartree (15) using BSA as a standard. The crude homogenate was dissolved into hot 2% SDS solution before the determination. Fluorescently labeled and unlabeled muscle actin concentrations were monitored spectroscopically. For native actin, the extinction coefficient $\epsilon_{290}^{0.1\%} = 0.62\ cm^{-1}$ (17) was used. For FITC- and IAENS-actin, the absorbance at 290 nm was first corrected for the absorbance of the fluorophore alone. The Bradford assay (3) for protein concentration was applied in the C_{HSS} determinations using actin as a standard. Because the fluorescent tags on actin change the color development in the Bradford assay, FITC-actin and IAENS-actin were used as standards where appropriate.

Electron Microscopy: Samples were applied to carbon-coated nitrocellulose grids and negatively stained with 1% uranyl acetate aqueous solution. Stained samples were examined and photographed in a Philips 201 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ) at 80 kV.

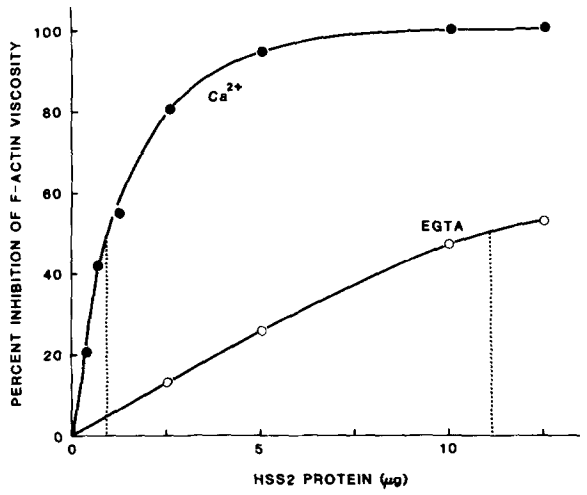

FIGURE 1 Rolling ball viscometry of F-actin in the presence of increasing concentrations of HSS2, which contains the 45,000-mol-wt protein, in the presence of 0.2 mM $CaCl_2$ (●) or 0.2 mM EGTA (○). G-actin (0.3 mg/ml, 100 μl) in the presence of Ca^{2+} or EGTA was incubated with or without HSS2 (12.5 μl), which had been diluted with assay buffer to different concentrations. Polymerization was subsequently initiated with 0.1 M KCl and 2 mM $MgCl_2$. The final viscosity, taken after minimum 40-min incubation, was lower for the F-actin samples with HSS2 and showed a decrease proportional to the amount of HSS2 added. The final viscosity was stable for many hours. Inhibitory activity was quantitated as described in Materials and Methods. In the presence of Ca^{2+}, ~0.7 μg HSS2 protein gave 50% inhibition. In the presence of EGTA, ~12 μg HSS2 protein was required to achieve the same amount of inhibition.

RESULTS

Purification of the 45,000-mol-wt Protein

Using viscometry as an assay, a Ca^{2+}-dependent inhibitory activity on actin assembly is present in crude egg extracts (Fig. 1). There is some detectable inhibitory activity in the presence of 0.2 mM EGTA. However, the amount of HSS2 needed for 50% inhibition is 10–20-fold higher in the presence of EGTA than with Ca^{2+}. We have purified a protein of 45,000 mol wt from unfertilized sea urchin eggs by following this activity (Table I).

The HSS2 had essentially the same specific activity as the

TABLE I

Purification of the 45,000-mol-wt Protein from Unfertilized Sea Urchin Eggs

	Volume	Protein concentration	Specific activity	Total activity
	ml	*mg/ml*	*U/mg × 10^{-3}*	*U × 10^{-3}*
Homogenate	88	8.5		
LSS	80	2.8	1.4	310
HSS1	78	2.1	1.5	250
HSS2	84	2.0	1.5	250
DEAE pool	24	0.4	6.2	60
G-200 pool	17	0.1	15	26
HAP pool	12	0.05	20	12

For this preparation 8 ml of packed unfertilized eggs were homogenized into 80 ml of buffer.
Details of the purification procedure are in Materials and Methods.

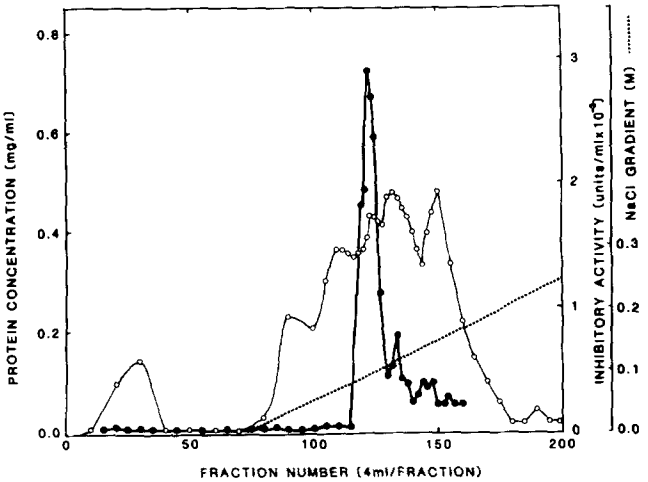

FIGURE 2 Protein (○) and Ca^{2+}-dependent inhibitory activity (●) profiles of the DE52 chromatograph of HSS2. HSS2 (84 ml, 2 mg/ml) in DEAE buffer was loaded onto a DE52 column (2.6 × 15 cm) equilibrated in the same buffer. The column with retained protein was then washed with 200 ml of DEAE buffer, and fractions were collected starting at the beginning of the wash. The column was then eluted with a linear NaCl gradient (0–0.3 M; 520 ml total) in DEAE buffer. Inhibitory activity was determined by rolling ball viscometry in the presence of either 0.2 mM $CaCl_2$ or 0.2 mM EGTA as described in Materials and Methods.

LSS and HSS1. We used Triton X-100 to solubilize the low-speed pellet; the activity in this Triton fraction was <10% of that in the HSS2. The HSS2 was chromatographed on a DEAE-cellulose column (Fig. 2). The Ca^{2+}-dependent inhibitory activity peaked at ~0.1 M NaCl whereas actin eluted at a higher salt concentration. In some preparations, the activity eluted as two peaks close together. The fractions containing peak activity were pooled and dialyzed against DEAE buffer without 0.1 mM EGTA. Before applying it to the gel filtration column, the DEAE pool was concentrated by vacuum dialysis. The recovery of activity from vacuum dialysis was >90%.

After filtration through a G-200 Sephadex column, the 45,000-mol-wt protein is the major component of the activity peak (Fig. 3). The K_{av} of the activity on a sizing column is ~0.6 in G-200 and ~0.25 in G-150 superfine Sephadex, which suggests that the 45,000-mol-wt protein is a monomer in its native state. The low-molecular-weight contaminants in the active fractions (Fig. 3) were largely removed by HAP column chromatography (Fig. 4). In some preparations one polypeptide of mol wt ~55,000 co-purified with the 45,000-mol-wt protein. In those cases, the HAP column gave some, but not complete, separation of these two proteins. The fractions containing only the 45,000-mol-wt protein were pooled for experiments. The rest of the fractions containing the 45,000-mol-wt protein were pooled and applied to a second DE52 column which was eluted with a 0–0.3 M NaCl gradient in 10 mM Tris, 0.5 mM ATP, 0.2 mM DTT, 0.05% NaN_3, pH 7.0 at 4°C. ~50% of the 45,000-mol-wt protein was separated

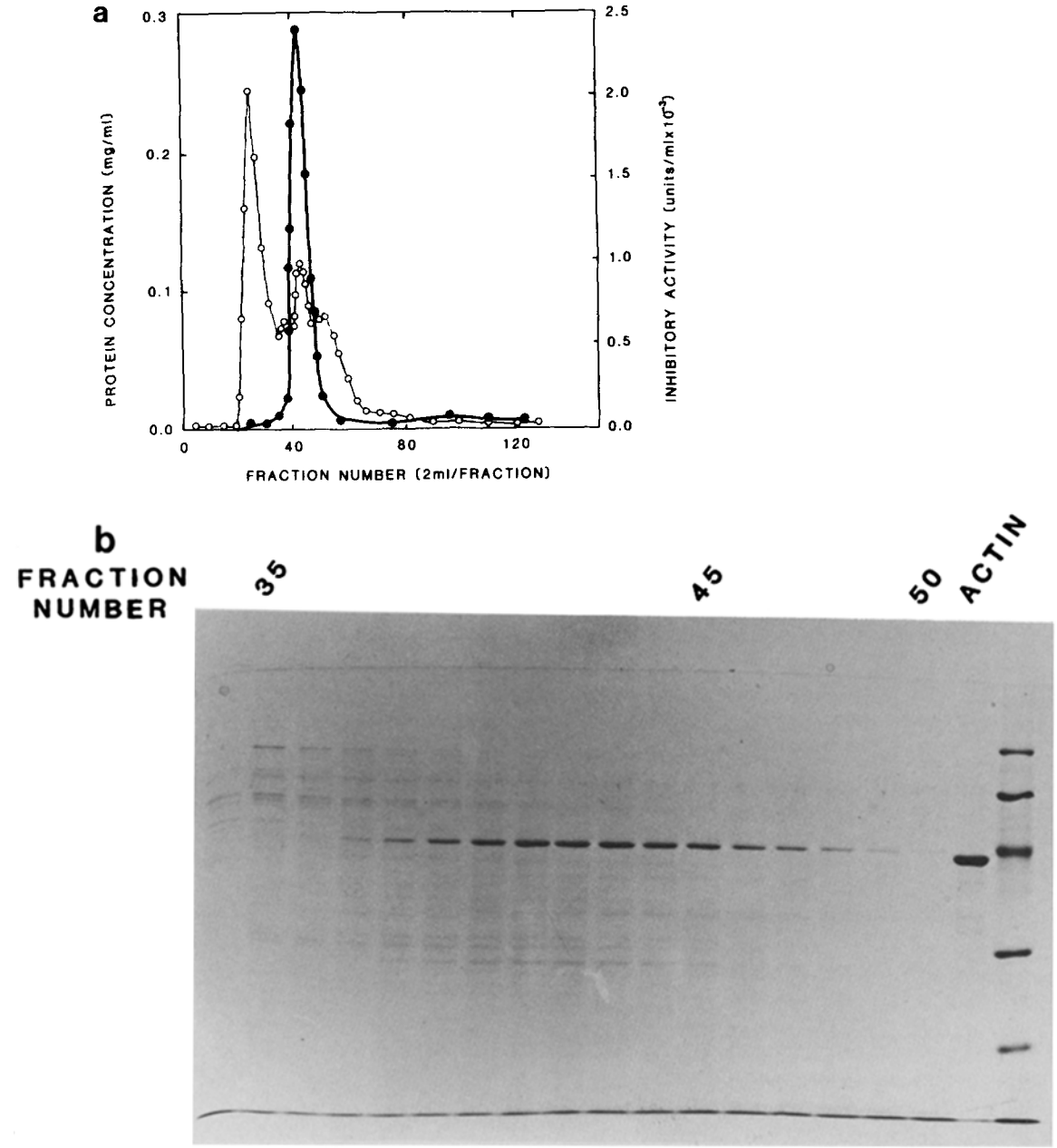

FIGURE 3 (*a*) Protein (○) and Ca^{2+}-dependent inhibitory activity (●) profiles of the G-200 Sephadex chromatograph. Concentrated DEAE pool (4 ml, 2 mg/ml) was applied to a 1.5 × 76 cm column. Protein was then eluted with DEAE buffer. (*b*) For the fractions containing the activity peak, protein composition was demonstrated by 10% SDS PAGE. The 45,000-mol-wt protein was shown to be the most predominant band by Coomassie Blue stain in the fractions. The gel is 10% acrylamide with a 5% stacking gel. Gel markers are of molecular weights (× 10^{-3}): 94, 67, 43, 30, and 20.1.

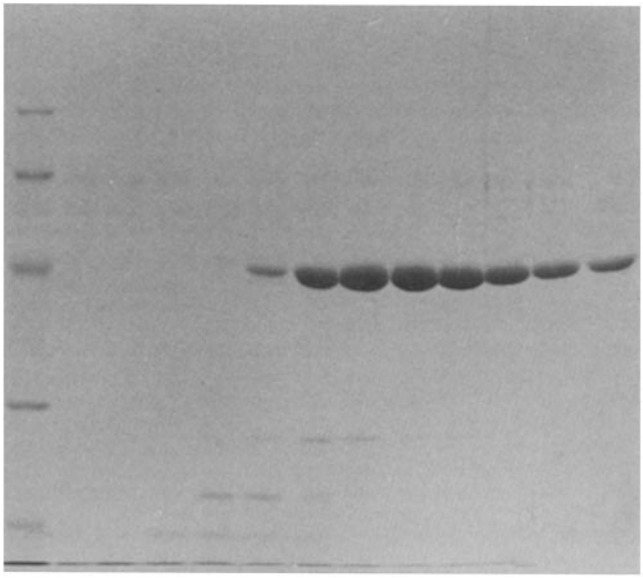

FIGURE 4 SDS PAGE of HAP-column fractions, which contain the Ca^{2+}-dependent inhibitory activity. The concentrated G-200 pool (1.5 ml, 1 mg/ml) in HAP-buffer was applied to a 1 × 5-cm HAP column. The column was washed with the same buffer before eluting with a linear potassium phosphate gradient (0.01–0.3 M). Most of the other proteins were present in the wash. The 45,000-mol-wt protein was eluted when the phosphate gradient was >0.05 M. The gel is 10% acrylamide with a 5% stacking gel. Molecular weights (× 10^{-3}) of the gel markers are 94, 67, 43, 30, and 20.1.

from the 55,000-mol-wt protein by this procedure. Filtration in higher ionic strength buffer did not seem to favor the separation; the 45,000-mol-wt protein eluted as a very broad peak in 1 M NaCl, which suggests some salt-dependent aggregation. DNase I affinity chromatography, which has been applied in the isolation of several actin binding proteins (16, 42), did not facilitate the 45,000-mol-wt protein purification under our conditions. The protein compositions at each step of the purification are displayed on SDS-polyacrylamide gels in Fig. 5.

Ca^{2+}-dependent Interaction of the 45,000-mol-wt Protein with Actin

The 45,000-mol-wt protein reduces the steady-state viscosity of F-actin in a Ca^{2+}-dependent, stoichiometric manner. The activity of the 45,000-mol-wt protein requires Ca^{2+} in the micromolar range (Fig. 6).

This lower viscosity could be due to a decrease either in the total number of filaments or in the length of individual filaments or both. We used electron microscopy to study the effect of the 45,000-mol-wt protein on the assembly state of actin in the presence and absence of Ca^{2+}. Actin alone in the presence of 0.2 mM $CaCl_2$ or 0.2 mM EGTA polymerized into long filaments (Fig. 7, *b* and *d*). In the presence of 0.2 mM EGTA, the addition of 45,000-mol-wt protein did not affect the assembly of long filaments (Fig. 7*c*). In contrast, only short filaments were observed when actin was polymerized in the presence of both 0.2 mM $CaCl_2$ and the 45,000-mol-wt protein (Fig. 7*a*). The length of the filaments decreased with an increasing ratio of the 45,000-mol-wt protein to actin (data not shown). Addition of 45,000-mol-wt protein to a pre-polymerized F-actin sample in the presence of Ca^{2+} caused an immediate decrease in viscosity and short filaments

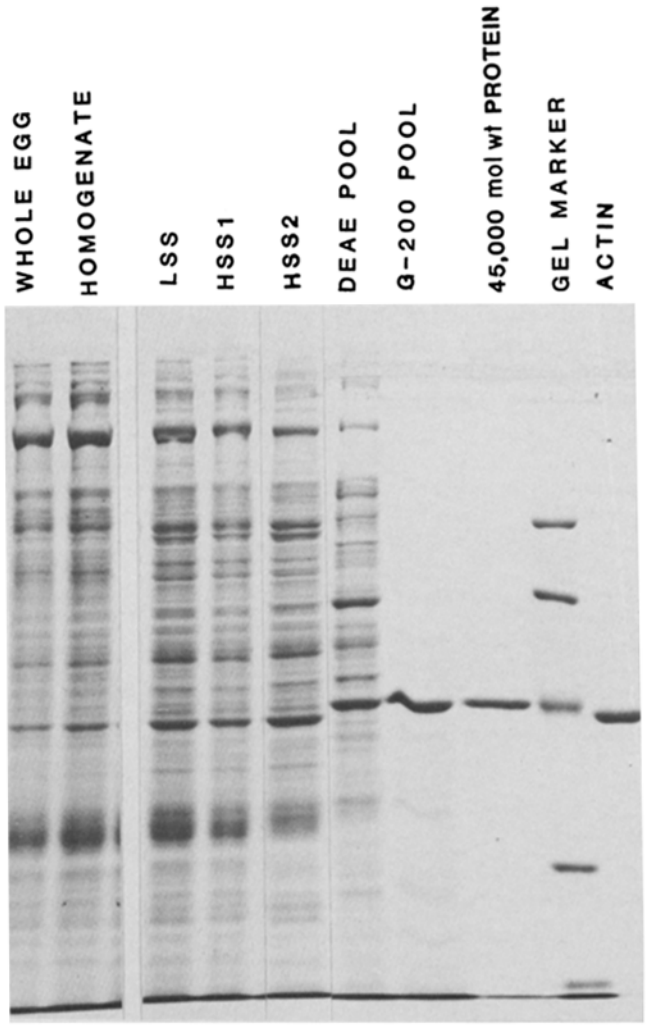

FIGURE 5 SDS PAGE illustrating protein composition of fractions during purification of the 45,000-mol-wt protein. ~20 μg of whole egg, homogenate, LSS, HSS1, HSS2; 10 μg of DEAE pool; 5 μg of G-200 pool; and 3 μg of purified 45,000-mol-wt protein were loaded. The gel is 10% acrylamide with a 5% stacking gel. Gel markers are of molecular weights (× 10^{-3}): 94, 67, 43, 30, and 20.1.

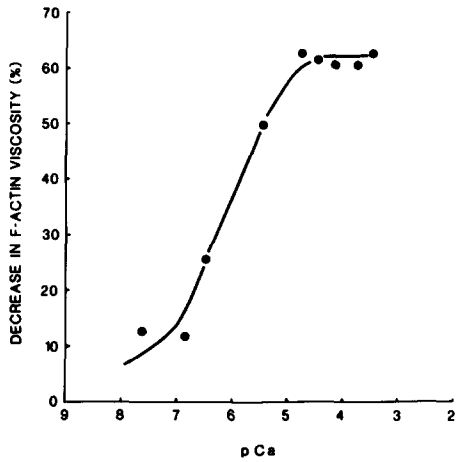

FIGURE 6 The 45,000-mol-wt protein (25 μl, 3.6 μg/ml) was added to F-actin (100 μl, 0.38 mg/ml) for each viscosity measurement. The molar ratio of the 45,000-mol-wt protein to F-actin was 1:565. The final EGTA concentration was 0.4 mM in all samples. The Ca/EGTA ratios were 0.4, 0.8, 0.9, 1.0, 1.05, 1.1, 1.2, 1.5, and 2.0. pCa was calculated using the apparent K_d of EGTA at pH 7.4 for Ca^{2+} of 3.981 × 10^{-8} (14).

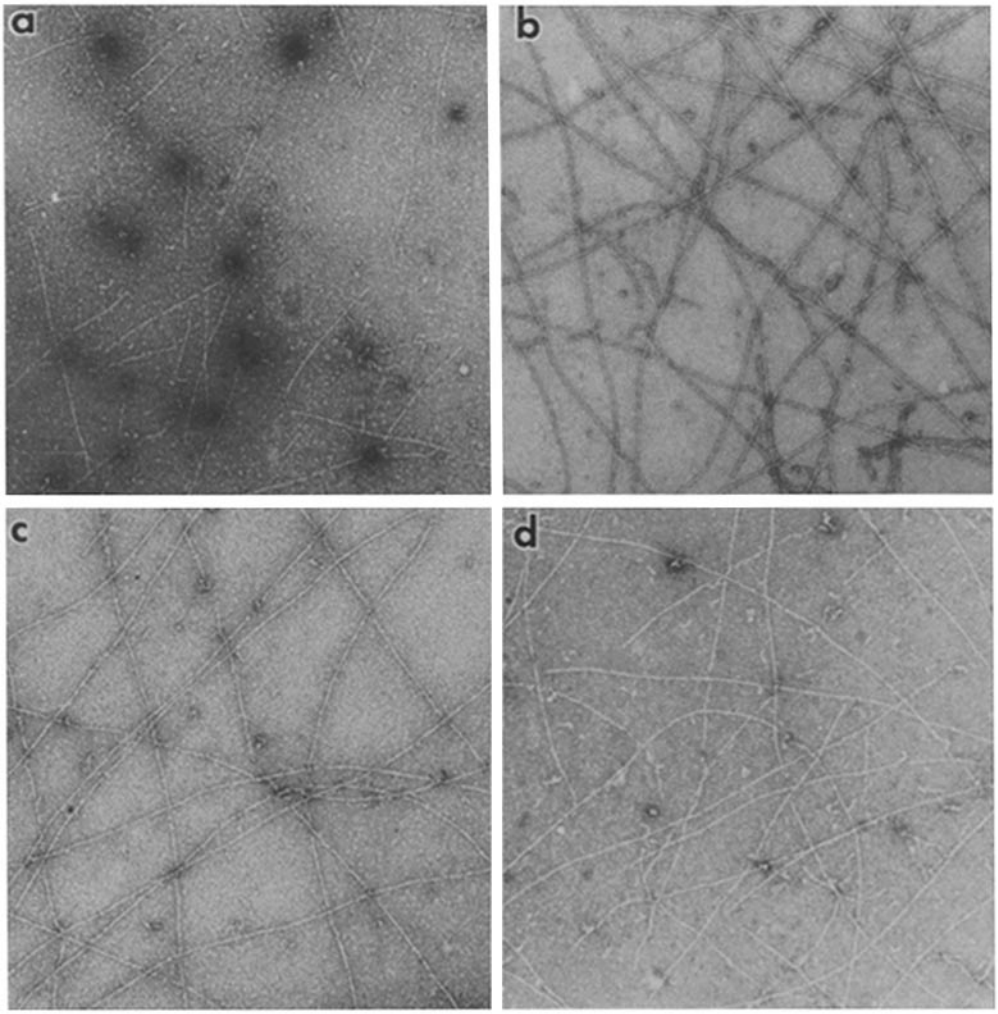

FIGURE 7 Electron microscopy of actin filaments under various conditions. G-actin at 0.3 mg/ml was polymerized by addition of 0.1 M KCl and 2 mM $MgCl_2$. (*a*) Actin polymerized in the presence of 45,000-mol-wt protein (~9 μg/ml) and 0.2 mM $CaCl_2$. (*b*) Actin polymerized in 0.2 mM $CaCl_2$. (*c*) Actin polymerized in the presence of 0.2 mM EGTA and 45,000-mol-wt protein. (*d*) Actin polymerized in 0.2 mM EGTA. × 50,000.

were seen in electron micrographs of samples. The addition of 2 mM EGTA to this sample did not restore either the viscosity or long filaments in 2 h.

The 45,000-mol-wt Protein Increases the Steady-state Concentration of HSS-actin

We have used high-speed centrifugation to show that the 45,000-mol-wt protein increases the steady-state level of actin in the HSS. Using 0.5 mg/ml actin, the increase in C_{HSS} (in this experiment we used 30 psi for 20 min in a Beckman airfuge) was approximately linear with respect to the ratio of 45,000-mol-wt protein to actin up to 1:100, where ~50% of the actin became nonsedimentable. We have also used fluorescence energy-transfer techniques (39), which were previously used to study actin assembly and exchange of actin monomers into the filament by Taylor et al. (40), Wang and Taylor (45), Pardee et al. (27), Yamamoto et al. (48), and Wang et al. (44). Co-polymerization of actin labeled with IAENS (donor) and actin labeled with FITC (acceptor) can be followed kinetically by monitoring quench of donor–actin fluorescence. Complete co-assembly of donor and acceptor actin equals 58% quench of donor fluorescence (Fig. 8). Addition of the 45,000-mol-wt protein to a steady-state population of co-polymerized IAENS- and FITC-labeled actin resulted in a Ca^{2+}-dependent decrease in fluorescence quench, qualitatively consistent with the increase in nonsedimentable actin measured by centrifugation. This effect is most likely due to a net disassembly of actin filaments to monomeric actin (see Discussion).

In the presence of Ca^{2+}, addition of 45,000-mol-wt protein to the F-actin at a molar ratio of 1:100 caused 18% decrease in the fluorescence quench, which corresponds to 31% depolymerization. This value is somewhat lower than that observed by the above 20-min sedimentation experiment, presumably because the sedimentation conditions did not eliminate all small oligomers from the solution. The fluorescence level reached the new steady state rapidly with a $t_{1/2}$ of ~3 min (Fig. 8). Higher ratios of 45,000-mol-wt protein to actin resulted in a larger percentage relief of quench (Fig. 8). Addition of 45,000-mol-wt protein to actin at molar ratios between 1:400 and 1:50 resulted in a proportional increase in the percentage of quench relief (Fig. 9). The corresponding percentage of actin disassembly was calculated assuming that the maximum quench represents 100% assembly.

The Actin Depolymerized by the Action of the 45,000-mol-wt Protein is Assembly Competent

An important question is whether the actin that is disassembled by the 45,000-mol-wt protein has altered assembly properties. When freshly recycled IAENS–G-actin in TES G-buffer was polymerized at 1 mg/ml with addition of 0.1 M KCl and 2 mM $MgCl_2$, the C_{HSS} was very low (32 μg/ml). In the presence of the 45,000-mol-wt protein, the C_{HSS} was raised to ~300 μg/ml as determined by both fluorescence and the

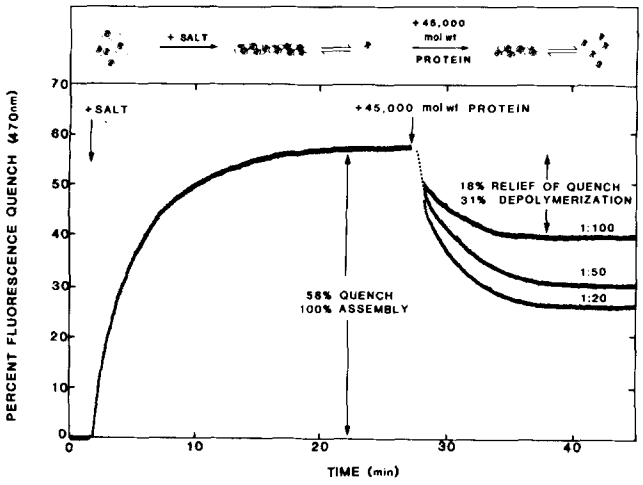

FIGURE 8 Depolymerization of F-actin caused by the addition of 45,000-mol-wt protein in the presence of 0.2 mM Ca^{2+}. A 10:1 ratio of rabbit skeletal muscle FITC-G-actin to IAENS-G-actin in imidazole G-buffer was co-assembled by the addition of 50 mM KCl and 1 mM $MgCl_2$. Total actin concentration was 0.5 mg/ml. The IAENS-actin fluorescence was measured at 340-nm excitation and 470-nm emission. Complete polymerization of actin results in 58% quench of IAENS-G-actin fluorescence. There is a quick and significant decrease in fluorescence quench after the addition of 45,000-mol-wt protein. For example, at a 1:100 molar ratio of 45,000-mol-wt protein to actin, there is an 18% relief of quench; this quench is Ca^{2+} dependent. Adding the 45,000-mol-wt protein to G-actin has no effect on fluorescence (not shown).

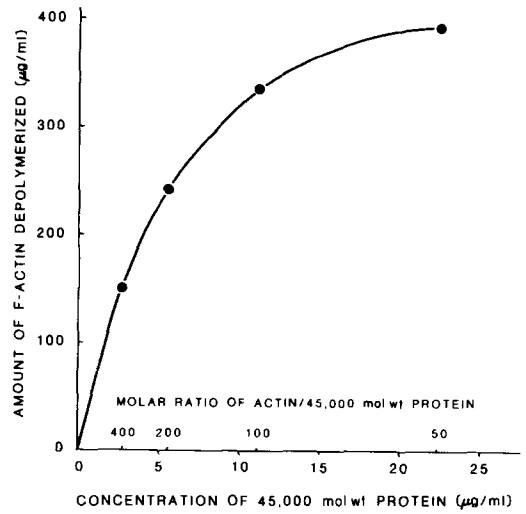

FIGURE 9 Quantitation of depolymerization of F-actin by 45,000-mol-wt protein in the presence of 0.2 mM Ca^{2+}. F-actin, at a final concentration of 1 mg/ml, was polymerized with 50 mM KCl and 1 mM $MgCl_2$. The molar ratio of FITC-G-actin to IAENS-G-actin was 10:1. The 45,000-mol-wt protein was added to F-actin at a molar ratio of 1:400, 1:200, 1:100, and 1:50. Relief of quench was monitored as in Fig. 7. The amount of depolymerization was calculated as described in Materials and Methods.

Bradford assay (5) (Table II). The assembly competency of this large amount of HSS-actin was tested as follows. Unlabeled G-actin was combined with the HSS-actin to a final actin concentration of 3.5 mg/ml and the salt concentration was restored to 0.1 M KCl and 2 mM $MgCl_2$. After 40 min of incubation at 22°C and centrifugation in an airfuge, a new C_{HSS} was determined. >90% of the IAENS-actin that had been solubilized by the action of the 45,000-mol-wt protein was now in sedimentable form.

TABLE II

Effect of the 45,000-mol-wt protein on C_{HSS}

Components	Actin concentration before centrifugation	C_{HSS}	Nonsedimentable* IAENS-actin
	mg/ml	*μg/ml*	*μg/ml*
IAENS-F-actin	1.0	32	35
Unlabeled Actin	3.0	43	—
IAENS-F-actin + 45,000-mol-wt protein‡	1.0	300	320
Disassembled actin induced by 45,000-mol-wt protein + excess unlabeled actin	0.3 3.2	92	16

* Values calculated from fluorescence measurements at 470 nm, exciting at 340 nm.

‡ The 45,000-mol-wt protein was added to a final concentration of 36 μg/ml. For experimental details, see Materials and Methods.

DISCUSSION

The isolated 45,000-mol-wt protein from unfertilized sea urchin eggs interacts with actin in a Ca^{2+}-dependent manner. It resembles severin, a Ca^{2+}-mediated actin-binding protein from *Dictyostelium discoideum* (4, 47) in many ways. Both of these proteins decrease steady-state actin viscosity, sever actin filaments, and interestingly, increase C_{HSS} greatly. Binding to G-actin and sequestering it in a nonpolymerizable complex in the way that profilin does (7, 31) cannot explain the large increase in C_{HSS} by the 45,000-mol-wt protein. For example, as shown in Fig. 9, when the 45,000-mol-wt protein is added to an F-actin solution (1 mg/ml) to a final concentration of 5.6 μg/ml (0.12 μM) the C_{HSS} increases by 240 μg/ml (5.8 μM) which is more than 40 times the 45,000-mol-wt protein concentration. The quench relief caused by the 45,000-mol-wt protein is most likely due to a net disassembly of actin filaments to monomeric actin since the ratio of FITC-actin to IAENS-actin is 10:1 in these experiments. Thus, even small oligomers should retain neighboring acceptor-donor pairs for efficient energy transfer.

There is the conventional argument (20) that the increase in G-actin concentration in the presence of a barbed end blocking agent such as cytochalasin or various proteins (e.g., villin, fragmin, and gelsolin) is due to a difference of "critical concentration" between the pointed and barbed ends of F-actin. However, the largest expected difference of critical concentration between the ends, derived from the measurements of Pollard and Mooseker (30), Wegner (47), and Bonder et al. (2) does not appear to be nearly large enough to account for the large increase in C_{HSS} caused by the 45,000-mol-wt protein.

Results analogous to those described here have been obtained with severin, a protein from *Dictyostelium discoideum* that has a very high affinity ($K_a \geq 10^{10}$) for actin and severs actin filaments (13, 48). An increasing concentration of severin in the presence of Ca^{2+} causes a concomitant and large increase in C_{HSS}. Understanding the mechanism of this C_{HSS} increase will be important to elucidate the mechanism of action of the 45,000-mol-wt egg protein and of severin.

In hypothesizing the biological function of the 45,000-mol-

wt protein in eggs, one possibility is that the transient Ca^{2+} increase upon fertilization activates this protein, which results in the release of actin from a storage form (36). This released actin would then be available for subsequent polymerization of actin filaments and the formation of long microvilli. It will be of interest to see what, if any, changes occur in the 45,000-mol-wt protein when eggs are fertilized. Some modification might be expected if this protein is involved in the regulatory changes that occur in the cytoskeleton upon fertilization. We have observed an inhibitory activity on F-actin viscosity in extracts of fertilized sea urchin eggs, and we have begun to isolate this activity for comparison with the 45,000-mol-wt protein from unfertilized eggs.

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REFERENCES

1. Begg, D. A., L. I. Rebhun, and H. Hyatt. 1982. Structural organization of actin in the sea urchin egg cortex: microvillar elongation in the absence of actin filament bundle formation. *J. Cell Biol.* 93:24–32.
2. Bonder, E. M., D. J. Fishkind, and M. S. Mooseker. 1983. Direct measurement of critical concentrations and assembly rate constants at the two ends of an actin filament. *Cell.* 34:491–501.
3. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
4. Brown, S. S., K. Yamamoto, and J. A. Spudich. 1982. A 40,000-dalton protein from *Dictyostelium discoideum* affects assembly properties of actin in a Ca^{2+}-dependent manner. *J. Cell Biol.* 93:205–210.
5. Bryan, J., and R. E. Kane. 1978. Separation and interaction of the major components of sea urchin actin gel. *J. Mol. Biol.* 125:207–244.
6. Burgess, D. R., and T. E. Schroeder. 1977. Polarized bundles of actin filaments within microvilli of fertilized sea urchin eggs. *J. Cell Biol.* 74:1032–1037.
7. Carlsson, L., L.-E. Nyström, I. Sundkvist, F. Markey, and U. Lindberg. 1977. Actin polymerizability is influenced by profilin, a low molecular weight protein in non-muscle cells. *J. Mol. Biol.* 115:465–483.
8. Chandler, D. E., and J. Heuser. 1981. Postfertilization growth of microvilli in the sea urchin egg: new views from eggs that have been quick-frozen, freeze fractured, and deeply etched. *Dev. Biol.* 82:393–400.
9. Craig, S. W., and T. D. Pollard. 1982. Actin-binding proteins. *Trends Biochem. Sci.* 7:88–92.
10. Eddy, E. M., and B. M. Shapiro. 1976. Changes in the topography of the sea urchin egg after fertilization. *J. Cell Biol.* 71:35–48.
11. Epel, D. 1975. The program of and mechanisms of fertilization in the echinoderm egg. *Am. Zool.* 15:507–522.
12. Epel, D. 1978. Mechanism of activation of sperm and egg during fertilization of sea urchin gametes. *Curr. Top. Dev. Biol.* 12:185–246.
13. Giffard, R. G., A. G. Weeds, and J. A. Spudich. 1984. Ca^{2+}-dependent binding of severin to actin: a one-to-one complex is formed. *J. Cell Biol.* 98:1796–1803.
14. Harafuji, H., and Y. Ogawa. 1980. Re-examination of the apparent binding constant of ethylene glycol *bis*(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid with calcium around neutral pH. *J. Biochem. (Tokyo).* 87:1305–1312.
15. Hartree, E. F. 1972. Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Anal. Biochem.* 48:422–427.
16. Hosoya, H., I. Mabuchi, and H. Sakai. 1982. Actin modulating proteins in the sea urchin egg. I. Analysis of G-actin-binding proteins by DNase I—affinity chromatography and purification of a 17,000 molecular weight component. *J. Biochem. (Tokyo)* 92:1853–1862.
17. Houk, T. W., Jr., and K. Ue. 1974. The measurement of actin concentration in solution: a comparison of methods. *Anal. Biochem.* 62:66–74.
18. Kane, R. E. 1975. Preparation and purification of polymerized actin from sea urchin egg extracts. *J. Cell Biol.* 66:305–315.
19. Kane, R. E. 1976. Actin polymerization and interaction with other proteins in temperature-induced gelation of sea urchin egg extracts. *J. Cell Biol.* 71:704–714.
20. Korn, E. D. 1982. Actin polymerization and its regulation by proteins from nonmuscle cells. *Physiol. Rev.* 62:672–737.
21. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
22. Mabuchi, I. 1983. An actin-depolymerizing protein (Depactin) from starfish oocytes: properties and interaction with actin. *J. Cell Biol.* 97:1612–1621.
23. Mabuchi, I., and H. Hosoya. 1982. Actin-modulating proteins in the sea urchin egg. II. Sea urchin egg profilin. *Biomed. Res.* 3:465–476.
24. Mabuchi, I., and J. A. Spudich. 1980. Purification and properties of soluble actin from sea urchin eggs. *J. Biochem.* 87:785–802.
25. Otto, J. J., R. E. Kane, and J. Bryan. 1979. Formation of filopodia in coelomocytes: localization of fascin, a 58,000 dalton actin cross-linking protein. *Cell.* 17:285–293.
26. Otto, J. J., R. E. Kane, and J. Bryan. 1980. Redistribution of actin and fascin in sea urchin eggs after fertilization. *Cell Motil.* 1:31–40.
27. Pardee, J. D., P. A. Simpson, L. Stryer, and J. A. Spudich. 1982. Actin filaments undergo limited subunit exchange in physiological salt conditions. *J. Cell Biol.* 94:316–324.
28. Pardee, J. D., and J. A. Spudich. 1982. Purification of muscle actin. *Methods Enzymol.* 85:165–181.
29. Pollard, T. D. 1982. A falling ball apparatus to measure filament cross-linking. *Methods Cell Biol.* 24:301–311.
30. Pollard, T. D., and M. S. Mooseker. 1981. Direct measurement of actin polymerization rate constants by electron microscopy of actin filaments nucleated by isolated microvillus cores. *J. Cell Biol.* 88:654–659.
31. Reichstein, E., and E. D. Korn. 1979. Acanthamoeba profilin. A protein of low molecular weight from Acanthamoeba castellanii that inhibits actin nucleation. *J. Biol. Chem.* 254:6174–6179.
32. Schliwa, M. 1981. Proteins associated with cytoplasmic actin. *Cell.* 25:587–590.
33. Schroeder, T. E. 1978. Microvilli on sea urchin eggs: a second burst of elongation. *Dev. Biol.* 64:342–346.
34. Schroeder, T. E. 1979. Surface area change at fertilization: resorption of the mosaic membrane. *Dev. Biol.* 70:306–326.
35. Spudich, A., R. G. Giffard, and J. A. Spudich. 1982. Molecular aspects of cortical actin filament formation upon fertilization. *Cell Differ.* 11:281–284.
36. Spudich, A., and J. A. Spudich. 1979. Actin in Triton-treated cortical preparations of unfertilized and fertilized sea urchin eggs. *J. Cell Biol.* 82:212–226.
37. Spudich, J. A., and L. A. Amos. 1979. Structure of actin filament bundles from microvilli of sea urchin eggs. *J. Mol. Biol.* 129:319–331.
38. Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866–4871.
39. Stryer, L. 1978. Fluorescence energy transfer as a spectroscopic ruler. *Annu. Rev. Biochem.* 47:819–846.
40. Taylor, D. L., J. Reidler, J. A. Spudich, and L. Stryer. 1981. Detection of actin assembly by fluorescence energy transfer. *J. Cell Biol.* 89:362–367.
41. Vacquier, V. D. 1981. Dynamic changes of the Egg Cortex. *Dev. Biol.* 84:1–26.
42. Wang, L. L., and J. Bryan. 1981. Isolation of calcium-dependent platelet proteins that interact with actin. *Cell.* 25:637–649.
43. Wang, L. L., and J. A. Spudich. 1982. Isolation of a calcium-dependent actin-binding protein from sea urchin eggs. *J. Cell Biol.* 95:286a.
44. Wang, Y.-L., E. M. Bonder, M. S. Mooseker, and D. L. Taylor. 1983. Effects of villin on the polymerization and subunit exchange of actin. *Cell Motil.* 3:151–165.
45. Wang, Y.-L., and D. L. Taylor. 1981. Probing the dynamic equilibrium of actin polymerization by fluorescence energy transfer. *Cell.* 27:429–436.
46. Weeds, A. 1982. Actin-binding proteins—regulators of cell architecture and motility. *Nature (Lond.).* 296:811–816.
47. Wegner, A. 1982. Treadmilling of actin at physiological salt concentrations. An analysis of the critical concentrations of actin filaments. *J. Mol. Biol.* 161:607–615.
48. Yamamoto, K., J. D. Pardee, J. Reidler, L. Stryer, and J. A. Spudich. 1982. Mechanism of interaction of *Dictyostelium* severin with actin filaments. *J. Cell Biol.* 95:711–719.