Alpha-Actinin from Sea Urchin Eggs: Biochemical Properties, Interaction with Actin, and Distribution in the Cell during Fertilization and Cleavage

ISSEI MABUCHI, YUKIHISA HAMAGUCHI,* TAKAAKI KOBAYASHI,[‡]
HIROSHI HOSOYA[§], SACHIKO TSUKITA[‖], and SHOICHIRO TSUKITA[‖]
*Department of Biology, College of Arts and Sciences, University of Tokyo, Meguro-ku, Tokyo 153 and Department of Cell Biology, National Institute for Basic Biology, Okazaki, Aichi 444; *Department of Biology, Tokyo Institute of Technology, Meguro-ku, Tokyo 152; ‡Department of Biochemistry, Jikei University School of Medicine, Minato-ku, Tokyo 105; and §Department of Biochemistry and Biophysics, Faculty of Science, University of Tokyo and ‖Department of Anatomy, University of Tokyo School of Medicine, Bunkyo-ku, Tokyo 113, Japan*

ABSTRACT A protein similar to alpha-actinin has been isolated from unfertilized sea urchin eggs. This protein co-precipitated with actin from an egg extract as actin bundles. Its apparent molecular weight was estimated to be ~95,000 on an SDS gel: it co-migrated with skeletal-muscle alpha-actinin. This protein also co-eluted with skeletal muscle alpha-actinin from a gel filtration column giving a Stokes radius of 7.7 nm, and its amino acid composition was very similar to that of alpha-actinins. It reacted weakly but significantly with antibodies against chicken skeletal muscle alpha-actinin. We designated this protein as sea urchin egg alpha-actinin. The appearance of sea urchin egg alpha-actinin as revealed by electron microscopy using the low-angle rotary shadowing technique was also similar to that of skeletal muscle alpha-actinin. This protein was able to cross-link actin filaments side by side to form large bundles. The action of sea urchin egg alpha-actinin on the actin filaments was studied by viscometry at a low-shear rate. It gelled the F-actin solution at a molar ratio to actin of more than 1:20, at pH 6–7.5, and at Ca ion concentration <1 μM. The effect was abolished by the presence of tropomyosin. Distribution of this protein in the egg during fertilization and cleavage was investigated by means of microinjection of the rhodamine-labeled protein in the living eggs. This protein showed a uniform distribution in the cytoplasm in the unfertilized eggs. Upon fertilization, however, it was concentrated in the cell cortex, including the fertilization cone. At cleavage, it seemed to be concentrated in the cleavage furrow region.

Actin is a protein that forms filamentous polymers at physiological salt concentrations. In nonmuscle cells, motility and maintenance of cell shape are largely dependent on actin-based structures. These structures show a variety of morphologies: some are ordered arrays of actin filaments and others are random networks. These three-dimensional structures are considered to be constructed of actin plus actin cross-linking proteins since purified actin does not form such structures in vitro under physiological salt conditions. One of these proteins is alpha-actinin. This protein was discovered by Ebashi and colleagues (8, 32) in rabbit skeletal muscle and was localized to the Z disk of the sarcomere structure (33). In nonmuscle cells, its presence in the stress fibers of cultured mammalian cells was strongly suggested by an immunofluorescence study using antibodies against skeletal muscle alph-actinin (23). Since then, proteins that are similar to skeletal or smooth muscle alpha-actinin have been isolated from various types of mammalian tissues and cells: brain (42), Ehrlich tumor cells ("actinogelin," reference 35), HeLa cells (5), platelets (40), chromaffin granules (1), kidney (20), and Sarcoma 180 ascites (53). Such proteins have also been isolated from primitive organisms such as *Acanthamoeba* (38) and *Dictyostelium* (6, 10). These proteins may be important in the organization of actin filaments into contractile or cytoskeletal structures which are assembled and dissembled cyclically in the course of the cell cycle.

We have isolated from unfertilized sea urchin eggs a protein similar to alpha-actinin. Sea urchin eggs are known to form cytoskeletons upon fertilization (2, 4, 27, 44) and also to cyclically form contractile rings in the cleavage furrow (43). We localized this protein in the egg during fertilization and cleavage and observed its accumulation in the fertilization cone and cell cortex. The possible function of this protein in forming these actin-based structures in these cells is discussed.

MATERIALS AND METHODS

Purification of the 95,000-mol-wt Protein: Eggs of a sea urchin, *Hemicentrotus pulcherrimus*, were obtained by KCl-induced spawning and dejellied in acidified sea water. The temperature was kept at 0–4°C throughout the following procedure unless otherwise specified. The eggs were washed once with Ca-free artificial sea water and once with 0.5 M glycerol, 0.2 M NaCl, 10 mM $NaHCO_3$ and packed by centrifugation at 1,000 *g* for 8 min. An equal volume of 0.7 M glucose, 0.1 M KCl, 5 mM EGTA, 2 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol (DTT),[1] 10 μg/ml leupeptin, 10 mM 3-(*N*-morpholino)propane sulfonic acid (MOPS) buffer (pH 6.9) was added to the packed eggs, and the suspension was homogenized with a motor-driven Teflon-glass homogenizer for five strokes. The homogenate was centrifuged at 20,000 *g* for 10 min and then at 190,000 *g* for 1.5 h. The final supernatant was called extract and stored frozen at −80°C until use.

The extract (5.6 grams of protein) was dialyzed overnight against 10 vol of 0.12 M KCl, 0.1 mM EGTA, 1 mM $MgCl_2$, 0.1 mM ATP, 0.1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 mM MOPS buffer (pH 7.5), clarified by centrifugation at 20,000 *g* for 20 min, and applied to a DEAE-cellulose (DE-52, Whatman Ltd., Maidstone, Kent, U. K.) column (2.5 × 95 cm). Proteins were eluted by a linear KCl gradient from 0.12 to 0.4 M. Actin eluted as a broad peak at ~0.2 M KCl. Fractions that contained actin were pooled and mixed with solid ammonium sulfate to make 35% saturation with respect to ammonium sulfate. The solution was centrifuged at 10,000 *g* for 10 min and the pellets were suspended in and dialyzed overnight against 50 mM KCl, 1 mM EGTA, 2 mM $MgCl_2$, 0.1 mM ATP, 0.5 mM DTT, 5 μg/ml leupeptin, 10 mM MOPS buffer (pH 7.0). Precipitates were collected by centrifugation at 2,000 *g* for 10 min and dissolved by the addition of KCl to give a final concentration of 0.6 M. The solution was clarified by centrifugation and dialyzed overnight against the 50 mM KCl solution. Precipitates formed in this step aggregated into a cluster. They were shown by electrophoresis to contain exclusively actin, the 95,000-mol-wt protein, and a small amount of myosin as described in Results. They were collected by centrifugation at 2,000 *g* for 10 min and dissolved in and dialyzed against 1 mM MOPS buffer (pH 7.4), 0.05 mM $MgCl_2$, 0.1 mM EGTA, 0.2 mM ATP, 0.5 mM DTT, 5 μg/ml leupeptin.

Ammonium sulfate was then added to 50% saturation and the resultant precipitates were dissolved in 0.6 M KI, 0.2 mM $MgCl_2$, 1 mM EGTA, 0.2 mM ATP, 5 mM DTT, 5 μg/ml leupeptin, 10 mM MOPS buffer (pH 7.2) and loaded on a DNase I-Sepharose (Worthington Biochemical Corp., Freehold, NJ) column (7 ml). The flow-through fraction was collected and dialyzed overnight against a 50% saturated ammonium sulfate solution containing buffer A (0.2 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, 5 μg/ml leupeptin, 10 mM MOPS buffer, pH 7.2). Precipitates were dissolved in the 0.6 M KI solution and applied to a Sephacryl S-400 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (1.6 × 43 cm) preequilibrated with the same solution. The peak of the 95,000-mol-wt protein was collected and dialyzed overnight against buffer A and applied to a DEAE-cellulose column (2.5 ml). Proteins were eluted with a linear NaCl gradient of 0–0.3 M. The 95,000-mol-wt protein fraction was collected and immediately passed over the DNase I-Sepharose column (0.6 ml). The flow-through fraction was pooled and concentrated by dialysis overnight against 50%-saturated ammonium sulfate containing buffer A. The precipitates were finally dialyzed overnight against buffer A and clarified.

Preparation of Skeletal Muscle Actin, Alpha-Actinin, and Tropomyosin: Actin was prepared from rabbit skeletal muscle as described by Spudich and Watt (46) and purified by gel filtration using a Sephadex G-100 (Pharmacia Fine Chemicals) column. Alpha-actinin was prepared from chicken breast muscle by the method of Masaki and Takaiti (34) and purified by DEAE-cellulose column chromatography (39). Tropomyosin was prepared from rabbit skeletal muscle by the method of Ebashi et al. (9).

Viscometry: The viscosity of actin solutions was measured at a low-shear rate as described by MacLean-Fletcher and Pollard (30). G-actin (0.13 mg/ml) was mixed with the 95,000-mol-wt protein or skeletal muscle alpha-actinin, and 75 mM KCl, 1 mM $MgCl_2$, 2.6 mM Ca-EGTA buffer, 20 mM MOPS buffer, pH 7.0 (final concentrations) was added. The solution was immediately sucked into a 0.1-ml capillary pipette and then incubated from 2–3 h at 25°C. A stainless ball (0.6 mm diam) was dropped into the pipette inclined at an angle of 55° from the horizontal, and the time required for the ball to drop a certain distance was recorded. The apparent viscosity values were obtained after calibration of the viscometer with aqueous glycerol. The Ca ion concentration in the mixture was calculated using stability constants listed in reference 31 as described by Hamaguchi and Hiramoto (14). The viscosity of actin solutions at a high-shear rate was measured using an Ostwald-type viscometer of 0.28-ml capacity and water outflow time of 55 s at 25°C.

Electrophoresis: Electrophoresis was performed on a 10% polyacrylamide slab gel in the presence of SDS according to Laemmli (21). The gel was stained in 0.025% Coomassie Brilliant Blue, 25% isopropanol, 10% Acetic acid and destained in 10% acetic acid. Densitometry was carried out using a Shimadzu CS-910 dual wave length chromatoscanner (Shimadzu Corp., Kyoto, Japan).

Immunoblotting: Proteins on an SDS gel were transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH, 0.45-μm pore size) in 25 mM Tris, 130 mM glycine, 5% methanol at 100 mA for 2 h (19). The membrane was processed for immunoreaction with a diluted anti-chicken skeletal muscle alpha-actinin serum and for visualization using biotinyl Protein A-horseradish peroxidase-avidin D system (both from Vector Laboratories, Inc., Burlingame, CA) as described by Katayama et al. (19).

Electron Microscopy: Samples were mounted on carbon-coated Formvar grids and stained negatively with 1% uranyl acetate. The specimen was viewed with a JEOL JEM-100CX electron microscope (JEOL Ltd., Tokyo, Japan) at 100 kV.

Low-angle rotary shadowing specimens were prepared according to Tyler and Branton (52). They were viewed with a Hitachi 11-DS electron microscope (Hitachi Ltd., Tokyo, Japan) at 75 kV.

Light Scattering: Light scattering from actin solutions was measured with Shimadzu R-540 spectrofluorometer at 450 nm. KCl and $MgCl_2$ were added to a G-actin solution (0.2 mg/ml) to give final concentrations of 75 and 2 mM, respectively, and the change in the light scattering intensity was recorded.

Amino Acid Analysis: Proteins were hydrolyzed in 6 N HCl that contained 0.1% phenol at 110°C for 24 h. Amino acids were analyzed with a Hitachi 835 amino acid analyzer.

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

Fluorescence Labeling of Protein and Microinjection: Sea urchin egg 95,000-mol-wt protein, skeletal muscle alpha-actinin, or BSA was labeled with rhodamine as described by Feramisco (11). The labeled protein (1 mg/ml) was dialyzed against 0.1 M KCl, 1 mM $MgCl_2$, 0.5 mM EGTA, 2 mM MOPS buffer (pH 7.0) and microinjected in the *H. pulcherrimus* eggs as described by Hiramoto (16) under a Nikon fluorescence microscope (Nippon Kogaku, K. K., Tokyo, Japan). The temperature was controlled at 20 ± 1°C during microinjection and subsequent observations.

RESULTS

Purification of the 95,000-mol-wt Protein

Upon DEAE-cellulose column chromatography of the egg extracts, actin eluted at ~0.2 M KCl as a broad peak. When the 35% ammonium sulfate fraction of the actin peak was dialyzed against a 0.05 M KCl solution, a portion (~30 mg starting from 5.6 grams of extract protein) of the protein precipitated. These precipitates solubilized well in 0.6 M KCl, and precipitates (~10 mg of protein) formed again when the KCl concentration was again lowered to 0.05 M. We found that the precipitates were composed of only two major proteins: actin and a higher molecular weight protein that comigrated with chicken skeletal muscle alpha-actinin (Fig. 1 *a*). Since the apparent molecular weight of skeletal muscle alpha-actinin on an SDS gel has been determined to be 96,000 (48), we tentatively call this protein the 95,000-mol-wt protein. A small amount of a 200,000-mol-wt protein, which we tentatively identified to be myosin heavy chains, was also detected

[1] *Abbreviations used in this paper:* DTT, dithiothreitol; MOPS, 3-(*N*-morpholino)propane-sulfonic acid.

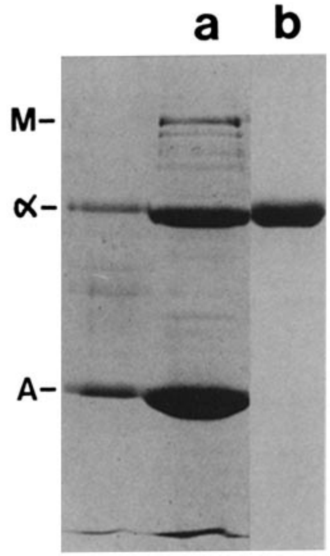

FIGURE 1 SDS PAGE of the 95,000-mol-wt protein. (*a*) 0.05 M KCl-precipitates from the 35% ammonium sulfate fraction. (*b*) Purified 95,000-mol-wt protein. The left lane represents marker proteins, chicken breast muscle alpha-actinin (α), and rabbit skeletal muscle actin (*A*). *M* indicates the position of myosin heavy chains.

(Fig. 1*a*). The molar ratio of the 95,000-mol-wt protein and actin in the second precipitates was fairly constant. Supposing that the molecular weight of the native 95,000-mol-wt protein particle is 200,000 (see below), the ratio was one 95,000-mol-wt protein molecule to 9.6 actin molecules (average of three determinations).

We examined these precipitates by electron microscopy using the negative staining technique. These precipitates appeared to be aggregates of dense actin bundles. Each of these bundles was several micrometers long (Fig. 2). However, the actin filaments within the bundles were short, ranging from 0.1 to 0.5 μm in length. Some filaments that seemed to have dropped out from the bundle were scattered around the bundle (Fig. 2). There were also some bipolar filaments 0.3–0.4 μm long which showed a characteristic feature of egg myosin filaments (26). These filaments scattered on the grid independent of the actin bundles. The one seen in Fig. 2 seemed to have attached to the bundle by chance.

The 95,000-mol-wt protein was purified from these precipitates as described in Materials and Methods. The procedure was devised mainly to remove actin by the use of a gel filtration column and a DNase I affinity column in the presence of 0.6 M KI. A trace amount of actin still remained even after these steps and was completely removed by DEAE-cellulose column chromatography and subsequent DNase I-affinity column chromatography in the absence of KI. The final 95,000-mol-wt protein fraction showed a purity of >95% as judged by densitometry of an SDS gel (Fig. 1*b*). The yield was 0.3 mg from 100 ml of packed eggs or 3.7 grams of extract protein.

Physicochemical and Immunological Properties of the 95,000-mol-wt Protein

When the purified 95,000-mol-wt protein was passed through a Sephacryl S-400 column, it eluted at a position of K_{av} = 0.42. Chicken skeletal muscle alpha-actinin eluted at the same position. This K_{av} gave a Stokes radius of 7.7 nm (22) which is the same value previously reported for rabbit skeletal muscle alpha-actinin (48). Since skeletal muscle alpha-actinin has been shown to be composed of two subunits of ~100,000 mol wt (48), it was considered that the 95,000-mol-wt protein also has a similar subunit configuration. This was supported by the electron microscopic observations, as shown below.

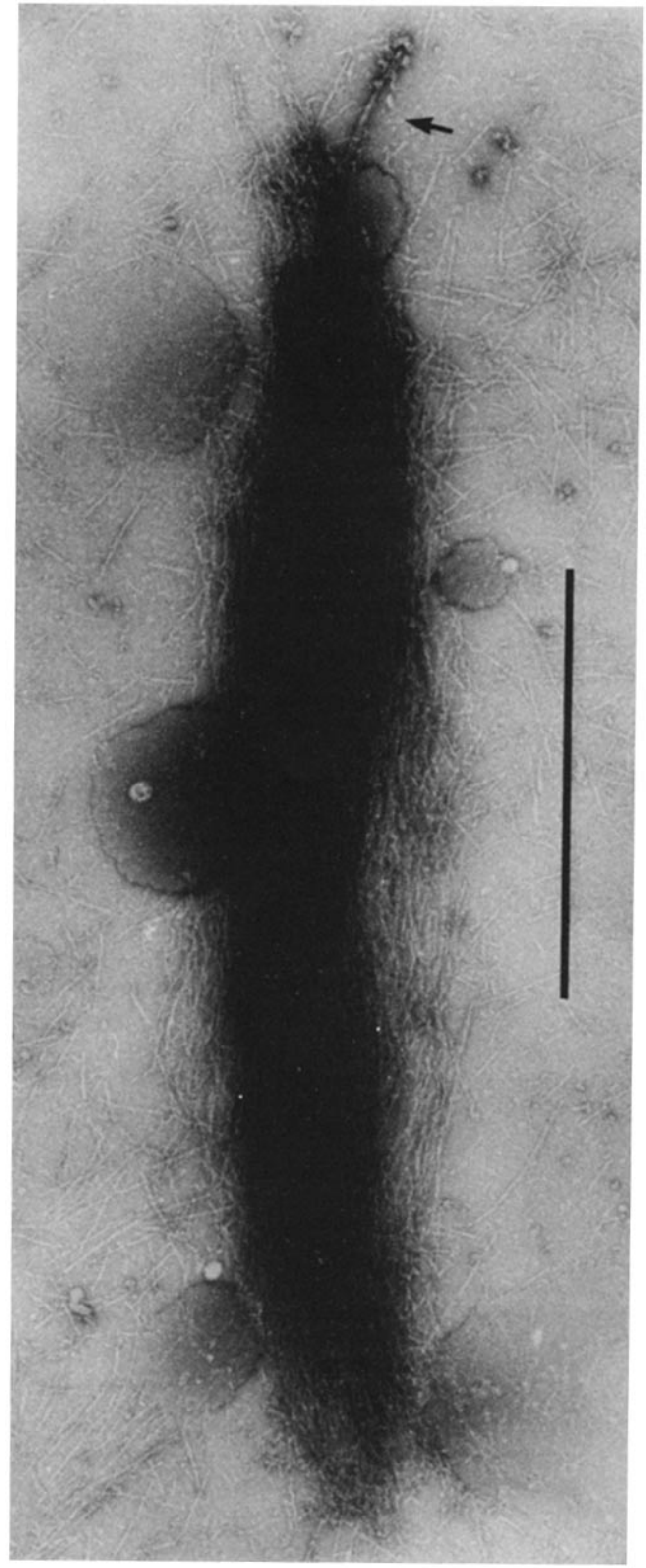

FIGURE 2 A small actin bundle in the 0.05 M KCl precipitates from the 35% ammonium sulfate fraction. The arrow points to a myosin filament. Bar, 1 μm. × 57,000.

By the use of the low-angle rotary shadowing technique, we found that the 95,000-mol-wt protein is a dumbbell-shaped molecule of ~50 nm in length (Fig. 3*a*). Both the length and

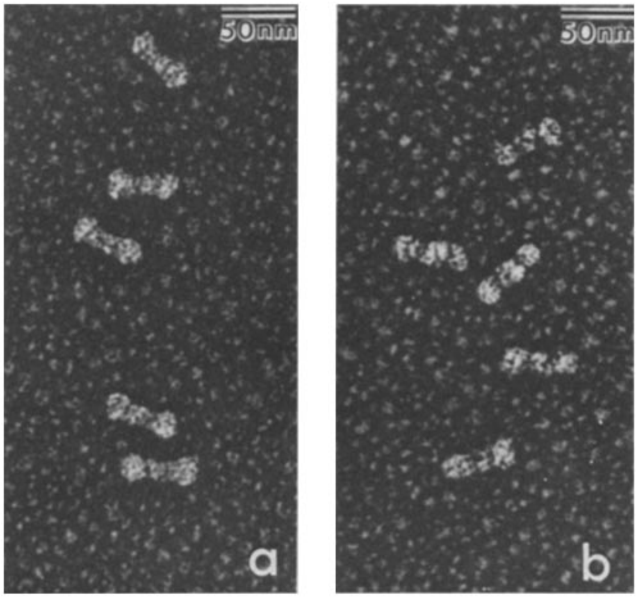

FIGURE 3 Rotary-shadowed specimen of the 95,000-mol-wt protein and alpha-actinin. (*a*) Sea urchin egg 95,000-mol-wt protein. (*b*) Chicken breast muscle alpha-actinin. × 200,000.

TABLE I
Amino Acid Composition of the 95,000-mol-wt Protein

Amino acids	Sea urchin egg 95K* protein	Porcine red muscle alpha-actinin‡
	mol%	
Asp	12.1	11.9
Thr	4.6	4.8
Ser	6.0	5.1
Glu	14.0	17.1
Pro	4.2	3.7
Gly	5.7	5.7
Ala	8.9	9.4
Cys	0.57	1.3
Val	4.4	3.6
Met	3.5	2.8
Ile	5.2	4.4
Leu	10.6	9.7
Tyr	2.6	2.5
Phe	3.9	3.1
Lys	6.5	5.8
His	1.6	2.4
Arg	5.7	6.7

* *95K*, 95,000-mol-wt (protein).
‡ Calculated from Suzuki et al. (49).

the shape were very similar to the appearance of skeletal muscle alpha-actinin (Fig. 3*b*). The difference might exist in the middle part of the dumbbell: the shape of the 95,000-mol-wt protein seemed to be slightly more slender than that of alpha-actinin. However, molecules may appear larger than they are when viewed by this technique (52); the length of skeletal muscle alpha-actinin molecule estimated by negative staining technique is reported to be ~40 nm (49).

The amino acid composition of the 95,000-mol-wt protein is shown in Table I. It was very similar to that of skeletal muscle alpha-actinins (39, 49).

The immunological relationship between the 95,000-mol-wt protein and skeletal muscle alpha-actinin was investigated using antibodies against the latter protein by means of immunoblotting technique. A weak but significant reaction occurred between the antibodies and the 95,000-mol-wt protein (data not shown).

Interaction of the 95,000-mol-wt Protein with Actin

Interaction of the 95,000-mol-wt protein with actin was first studied by viscometry at a low-shear rate. Rabbit skeletal muscle G-actin was supplemented with 75 mM KCl and 2 mM $MgCl_2$ in the presence or absence of the 95,000-mol-wt protein or skeletal muscle alpha-actinin. The solution was sucked up into a capillary pipette, and the falling ball assay was performed after 3 h. First, we studied the effect of the 95,000-mol-wt protein concentration on the viscosity of filamentous actin. The viscosity of actin increased gradually with increasing concentration of the 95,000-mol-wt protein, but it then increased abruptly at a point where the ratio of the 95,000-mol-wt protein to actin was 1:20 (Fig. 4*a*). The "critical gelling ratio" for skeletal muscle alpha-actinin was 1:10 (Fig. 4*a*).

We studied the effects of pH and Ca ion concentration on the activity of the 95,000-mol-wt protein to induce actin gel to elucidate the significance of this phenomenon in the eggs. At pH values between 6.0 and 7.5, the 95,000-mol-wt protein induced actin to form gel (Fig. 4*b*). However, no obvious gel was formed above pH 8.0. This was not due to the use of Tris buffer as gelation was unaffected by Tris at pH 7.5. The Ca ion concentration showed a marked effect on the actin viscosity at a low-shear rate (Fig. 4*c*). Below 0.24 μM Ca^{2+}, the 95,000-mol-wt protein induced actin gel. Above 25 μM Ca^{2+}, however, it did not induce gelation. The transition point seemed to be ~1 μM.

Since the action of skeletal muscle alpha-actinin on actin is abolished by tropomyosin (13), we examined the action of the 95,000-mol-wt protein on actin in the presence of this protein (Fig. 4*d*). Its effect appeared at a tropomyosin/actin ratio of as low as 1:84 (2.5 μg/ml tropomyosin in Fig. 4*d*), and it completely abolished the effect of the 95,000-mol-wt protein at 1:10 (not shown).

Whether the 95,000-mol-wt protein can sever actin filaments in the presence of Ca ions, as has been reported for villin (3) or gelsolin (54) which have similar subunit molecular weights, was investigated by high-shear viscometry. Addition of the 95,000-mol-wt protein to an F-actin solution in the presence of 0.2 mM $CaCl_2$ resulted in a slight increase in viscosity (not shown). This result led us to conclude that the 95,000-mol-wt protein does not sever actin filaments. A further addition of 3 mM EGTA caused a rapid (within 2 min) increase of the high-shear viscosity (not shown), which confirms the results of the falling ball viscometry.

Next, we investigated the interaction of the 95,000-mol-wt protein with skeletal muscle actin by electron microscopy. Actin filaments were examined by the negative staining technique after the addition of the 95,000-mol-wt protein. Long loose bundles of actin filaments were observed (Fig. 5*a*). These unit filaments were long although we could not follow their entire length; they seemed to have a normal length of the F-actin in contrast to the filaments in the crude precipitates (Fig. 2).

The interaction was further investigated using the low-angle rotary shadowing technique. Filaments ~0.3 μm in length, shown in Fig. 5, *b* and *c*, were identified as actin filaments in that those of the same appearance were observed in a pure F-

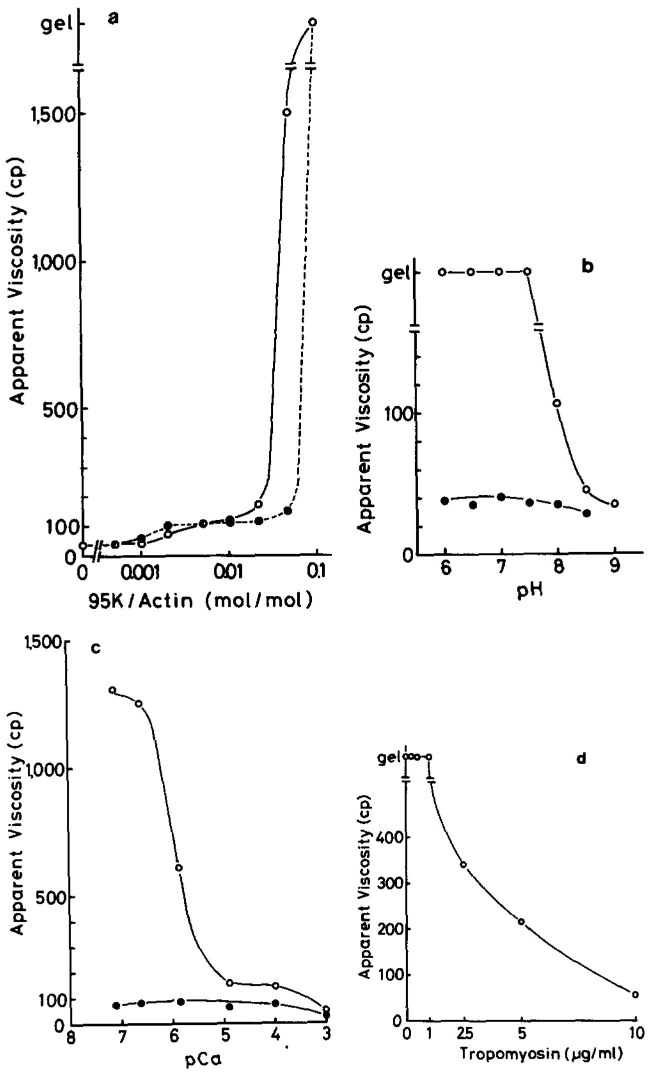

FIGURE 4 Interaction of the 95,000-mol-wt protein (*95K*) with actin filaments as studied by low-shear viscometry. The 95,000-mol-wt protein or skeletal muscle alpha-actinin was mixed with G-actin followed by the addition of salts to induce polymerization of actin. The viscosity was measured as described in Materials and Methods. The basic mixture consisted of 0.1 M KCl, 1 mM $MgCl_2$, 20 mM MOPS buffer (pH 7.0), 2.6 mM Ca-EGTA buffer (free Ca ion concentration, 0.078 μM), 0.13 mg/ml actin, and 62 μg/ ml 95,000-mol-wt protein. (*a*) Effects of the various concentrations of the 95,000-mol-wt protein or skeletal muscle alpha-actinin on the viscosity of F-actin. The molar ratios were calculated assuming the molecular weight of the 95,000-mol-wt protein to be 200,000. O, 95,000-mol-wt protein; ●, chicken skeletal muscle alpha-actinin. (*b*) Effects of pH. The following buffer (33 mM) were used instead of 20 mM MOPS. pH 6 and 6.5, 2-(*N*-morpholino)ethane sulfonic acid; pH 7 and 7.5, MOPS; pH 8–9, Tris. O, Actin plus 95,000-mol-wt protein; ●, actin only. (*c*) Effects of Ca ions. O, Actin plus 95,000-mol-wt protein (26 μg/ml); ●, actin only. (*d*) Effects of tropomyosin.

actin preparation and that they were very similar to the actin filaments reported by Tyler and Branton (52) using the same technique. Rod-shaped particles similar to the 95,000-mol-wt protein molecules (Fig. 3) attached to the actin filaments in the mixture of F-actin and the 95,000-mol-wt protein (Fig. 5). Two types of attachment were observed. In one case, particles attached to the side of the actin filament (Fig. 5*b*), and in the other case, the 95,000-mol-wt protein-like particle cross-linked two actin filaments (Fig. 5*c*). The cross-link corresponded to the length of the particle. The former image might be produced from the latter case as a result of detachment of an actin filament from one end of the 95,000-mol-wt protein by a shearing force when the solution was sprayed onto mica (52).

Because the actin filaments in the crude precipitates were short, we considered the possibility that the 95,000-mol-wt protein possesses an ability to bind to the ends of the actin filaments. If so, the 95,000-mol-wt protein would affect the assembly process of actin. We checked this by testing, by light scattering measurements (Fig. 6), whether the purified 95,000-mol-wt protein has any effect on the time course of nucleated polymerization of actin. Polymerization of rabbit skeletal muscle G-actin was induced by the addition of sonicated F-actin with or without the 95,000-mol-wt protein protein or skeletal muscle alpha-actinin (molar ratio to F-actin, 1/14). Rapid polymerization took place after addition of sonicated F-actin (Fig. 6). Neither the 95,000-mol-wt protein nor skeletal muscle alpha-actinin had a significant effect on the nucleated polymerization of actin. In addition, actin polymerization in the absence of added nuclei was also unaffected by these proteins at the concentration used (not shown). Electron microscopy also showed that F-actin polymerized from G-actin in the presence of the 95,000-mol-wt protein were essentially as long as control filaments (not shown).

Distribution of the 95,000-mol-wt Protein in the Egg

When the 95,000-mol-wt protein and skeletal muscle alpha-actinin were labeled with rhodamine (11), their ability to cross-link actin filaments in the falling-ball assay was unaffected. Rhodamine-labeled 95,000-mol-wt protein, skeletal muscle alpha-actinin, or BSA was microinjected into the unfertilized *H. pulcherrimus* eggs. It took ~10 min for the rhodamine-95,000-mol-wt protein or -muscle alpha-actinin to diffuse into the entire cytoplasm, whereas it took only 3–4 min for rhodamine-BSA to diffuse. No particular localization of the injected buffer was observed (Fig. 7*a*). The fluorescence was weaker at the periphery of the egg than deep inside it because of the spherical shape of the egg. When the egg previously microinjected with the rhodamine-95,000-mol-wt protein was fertilized, we observed a marked concentration of the fluorescence in the cortical layer including the fertilization cone (Fig. 7*b*). This was especially clear when this egg was compared with a control specimen into which rhodamine-labeled BSA had been injected (Fig. 7*c*). The fluorescence due to labeled 95,000-mol-wt protein began to localize in the cortical layer ~1 min after fertilization. The cortical fluorescence seemed to reach a maximum 3–4 min after fertilization and weakened gradually 30 min after fertilization. However, when rhodamine-95,000-mol-wt protein was reinjected after this weakening of the signal, strong fluorescence reappeared in the cortical layer. The formation of the fertilization cone took place ~80 s after fertilization. This fluorescence persisted as long as the cone existed (for ~3 min).

Localization of this protein during the first cleavage was investigated on eggs injected with the labeled protein after fertilization. The fluorescence was concentrated throughout the cortical layer (Fig. 8). The fluorescent layer at the cleavage furrow region seemed slightly thicker than that in the polar region (Fig. 8). Similar results were obtained with rhodamine-

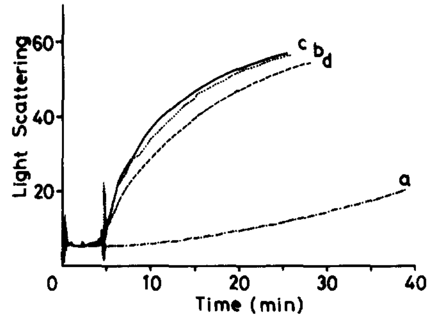

FIGURE 6 Nucleated polymerization of actin in the presence of the 95,000-mol-wt protein. Rabbit skeletal muscle G-actin (0.2 mg/ml) in 1 mM MOPS buffer, 0.5 mM EGTA, 0.2 mM DTT, 0.4 mM ATP, and 50 μM $MgCl_2$ (pH 7.4) was supplemented with 40 mM KCl at 0 min and the changes in the light scattering intensity were recorded. After ~5 min, sonicated F-actin (Ohtake sonicator, 20 W for 10 s) was added at a final concentration of 6 μg/ml with or without the 95,000-mol-wt protein or skeletal muscle alpha-actinin (final 2.0 μg/ml each, 1/14 in molar ratio to the sonicated F-actin). (a) Actin only; (*b*) actin plus nuclei; (c) actin plus nuclei plus 95,000-mol-wt protein (*d*) actin plus nuclei plus skeletal muscle alpha-actinin.

labeled skeletal muscle alpha-actinin, although the localization of the fluorescence was less distinct compared with the 95,000-mol-wt protein protein.

DISCUSSION

About 0.3 mg of the 95,000-mol-wt protein was obtained from 100 ml of the *Hemicentrotus* eggs by the present purification procedure. We could not estimate the amount of this protein in the egg because there were many protein bands on an SDS gel around the 95,000-mol-wt region when crude protein fractions were analyzed. A rough calculation, however, leads us to speculate that it comprises 2–4% of the total actin in the egg, taking the intracellular actin concentration of 3 mg/ml (29) and the interaction molar ratio of one 95,000-mol-wt protein to 10 (from the ratio in the 0.05 M KCl precipitates) to 20 (from the experiments shown in Fig. 4*a*) actins and supposing that the yield was 10%. It should be noted that not all the protein showing 95,000-mol-wt on an SDS gel precipitated with actin from the 35% saturated ammonium sulfate fraction; we might have purified only a part of the 95,000-mol-wt protein. On the other hand, this purification procedure is advantageous for this protein because the co-precipitation of this protein with actin as actin bundles seemed to be highly specific.

The sea urchin egg 95,000-mol-wt protein resembles alpha-actinin from skeletal or smooth muscle in the following ways.

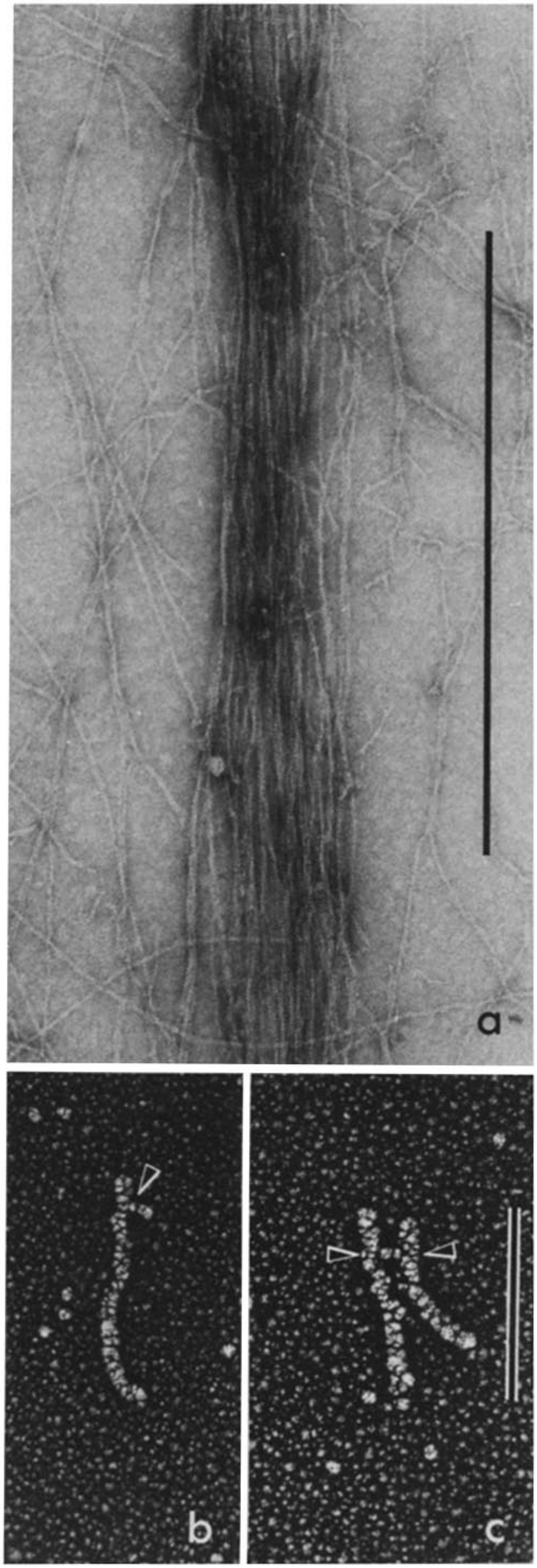

FIGURE 5 Electron micrographs of actin filaments cross-linked by the 95,000-mol-wt protein. (a) Rabbit skeletal muscle F-actin bundled in the presence of the 95,000-mol-wt protein. 0.2 mg/ml actin and 50 μg/ml 95,000-mol-wt protein in 0.1 M KCl, 1 mM $MgCl_2$, 0.5 mM EGTA, ;10 mM MOPS buffer, pH 7.0. Bar, 1 μm. × 98,600. (*b*) A rotary-shadowed image of a skeletal muscle actin filament to which a 95,000-mol-wt protein molecule (arrowhead) attached. (c) A rotary-shadowed image of two skeletal muscle actin filaments cross-linked (arrowheads) by a 95,000-mol-wt protein molecule. (*b* and c) 0.4 mg/ml actin and 0.1 mg/ml 95,000-mol-wt protein in 50% glycerol, 0.05 M KCl, 0.5 mM $MgCl_2$, 0.25 mM EGTA, 5 mM MOPS buffer, pH 7.0. Bar, 0.3 μm. × 100,000.

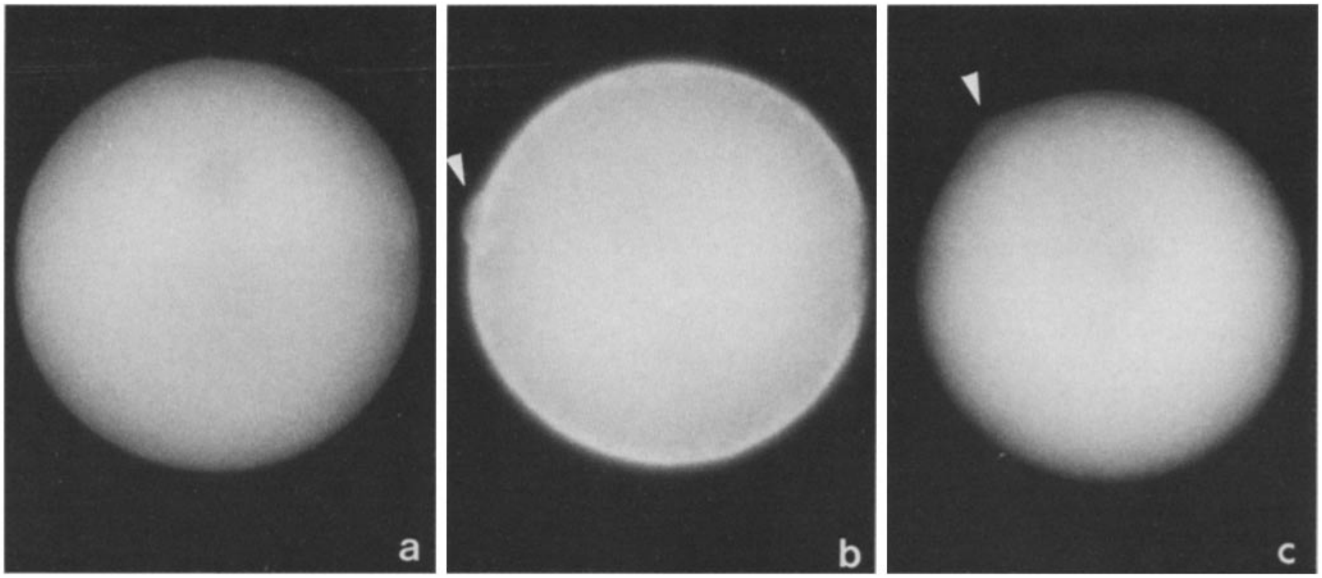

FIGURE 7 Distribution of the 95,000-mol-wt protein in the live egg before and after fertilization. (*a*) Rhodamine-labeled 95,000-mol-wt protein (1 mg/ml) was injected into an unfertilized *Hemicentrotus* egg; taken 13 min after microinjection. (*b*) The same egg as in a was then inseminated; taken 80 s after insemination. (*c*) An egg injected with rhodamine-labeled BSA 10 min before insemination; taken 70 s after insemination. Arrowheads indicate fertilization cones. × 460.

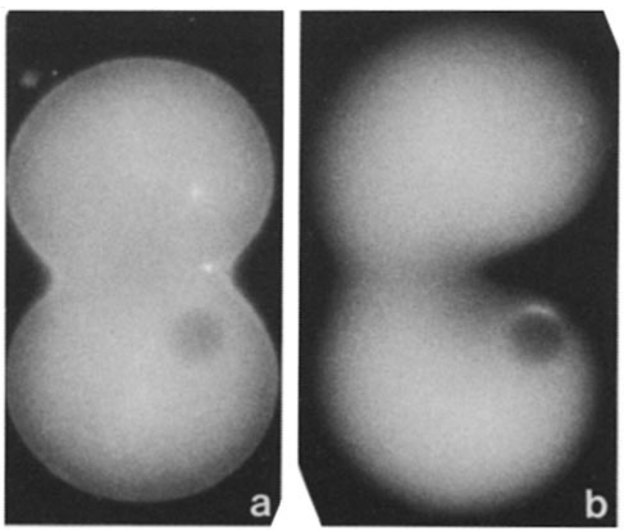

FIGURE 8 Distribution of the 95,000-mol-wt protein in the live egg at cleavage. (*a*) Rhodamine-labeled 95,000-mol-wt protein was injected into a fertilized *Hemicentrotus* egg 30 min prior to the first cleavage. (*b*) A similar injection was performed with rhodamine-labeled BSA 24 min before the cleavage. × 430.

(*a*) Both proteins co-migrated in an SDS gel showing an apparent subunit molecular weight of ~95,000. (*b*) Both buffers co-eluted from a Sephacryl S-400 column, yielding a Stokes radius of 7.7 nm. (*c*) Both proteins have very similar amino acid composition. (*d*) Both have a dumbbell-shape appearance of the same length (50 nm) when viewed with an electron microscope. (*e*) Both were able to cross-link actin filaments side by side to form loose actin bundles. (*f*) The molar ratio of these proteins to actin at a critical gelling point was similar. (*g*) The 95,000-mol-wt protein showed a weak cross-reactivity with anti-skeletal alpha-actinin antibodies. Furthermore, the 95,000-mol-wt protein did not show any actin filament-cutting or filament end-binding activity. We conclude that the 95,000-mol-wt protein from sea urchin eggs belongs to the alpha-actinin family, and we call this protein sea urchin egg alpha-actinin. That the actin filaments in the crude 0.05 M KCl precipitates were short may be due to the presence of Ca-insensitive capping proteins (reference 17 and S. Ishidate and I. Mabuchi, manuscript in preparation) in this fraction, which are known to be fractionated in the actin peak on the DEAE-cellulose column chromatography.

The sea urchin egg alpha-actinin increased the viscosity of F-actin solution in a Ca-sensitive manner. On the other hand, muscle alpha-actinin did not show any Ca sensitivity. Alpha-actinin-like proteins have already been isolated from some nonmuscle cells as mentioned in the beginning of this paper and some of them have been reported to be Ca sensitive (5, 6, 10, 20, 35, 38, 40). It is possible that the ancestor of this protein family was sensitive to calcium ions and that some of them lost this property during evolution.

Studies of the localization of proteins in the cell by microinjection of the fluorescently labeled proteins into living cells have a risk that the injected proteins bind to sites that are not functionally important even if the binding is specific. However, we think that this type of experiment will give results suggestive of the intracellular localization of cytoskeletal proteins especially in the case where the exchange of these proteins between cytoskeletal and cytoplasm is active or the organization of cytoskeletal changes so that the components are once dispersed and then reorganized. Rhodamine-labeled egg alpha-actinin localized in the fertilization cone and in the cell cortex upon fertilization. In the sea urchin egg, no cytoskeletal elements are observed before fertilization. Upon fertilization, however, dramatic changes occur in the cell cortex; that is, a fertilization cone appears at the position of sperm entry after the cortical exocytosis (51), microvilli elongate from the surface (4), and the stiffness of the cortical gel layer increases (15). All these changes may involve the appearance of actin filaments in these regions. Since the actin bundles in the microvilli may be formed through the interaction of actin with an actin-bundling protein, fascin, into a tight paracrystalline structures (7, 18, 28, 37, 45), and we did not detect migration of the fluorescently labeled egg alpha-actinin into microvilli, we have directed our attention concerning the function of alpha-actinin to the fertilization cone and cortical gel layer. The former is a cytoplasmic protrusion containing actin bundles (24, 50). These actin filaments seemed to originate in the plasma membrane and orient in a parallel fashion so that the pointed end of the filament points to the cytoplasm (50). Although the process of formation of the actin bundles in the microvilli and fertilization cone are apparently similar (50), the fertilization cone is a transient structure existing only for several minutes in contrast to the microvilli which may persist for hours. Thus it may be reasonable to consider that the organizations of the actin bundles in the cone are different from those in the microvilli. Considering that the interaction between egg alpha-actinin and the actin filaments is regulated by factors such as Ca ion concentration, pH, and tropomyosin, it is probable that alpha-actinin plays a role in bundling the actin filaments in the cone.

The cortical gel layer of the fertilized eggs does not seem to have a well-defined structure. Rootlets of the microvillar actin bundles protrude into this layer (2, 4, 27). These rootlets seem to be interconnected by the meshwork of the actin filaments, which can be observed when viewed in a tangential plane (44). The present observation that the fluorescently labeled egg alpha-actinin localizes in the cortical layer upon fertilization strongly suggests that this protein is involved in the actin meshwork formation in the cortical layer. The reported intracellular Ca ion release upon fertilization of *Lytechinus pictus* eggs (47) may not be inconsistent with the property of the cross-linking of actin filaments by this protein in vitro since the increase in the Ca ion concentration is transient. However, we need more information, including the time course and localization of the Ca ion increase in the *Hemicentrotus* eggs, in order to discuss this subject.

An interesting theory for the intracellular function of alpha-actinin in these cells is that this protein is involved in the formation of the contractile ring at the cleavage furrow. The contractile ring is a structure composed of actin filaments packed loosely in parallel (43). Filaments of two different polarities are contained in the ring (41), which makes it possible for each to slide over the other. Antibodies directed against chicken gizzard alpha-actinin have been localized in the cleavage furrow of cultured chick embryo cells by the immunofluorescence technique (12, 36), although the frequency of the cells that showed the staining in the furrow region was reported to be low (36). We observed a slightly thicker fluorescent layer at the furrow region as compared with the polar region of an egg microinjected with rhodamine-labeled egg alpha-actinin. This thickening might be due to the formation of the contractile ring. Considering that egg alpha-actinin cross-links actin filaments in vitro, it is possible that this protein plays an important role in the formation of the contractile ring by cross-linking the actin filament in this region.

We are grateful to Drs. Kazuyo Ohashi of the Chiba University and Kazuo Sutoh of the University of Tokyo for providing skeletal muscle alpha-actinin and tropomyosin, respectively, to Dr. Yoshiaki Nonomura of the University of Tokyo for the gift of antiserum against chicken skeletal muscle alpha-actinin and advice on immunoblotting, and to Dr. Vivianne T. Nachmias of the University of Pennsylvania for careful reading of the manuscript.

This research was supported partly by grants from the Ministry of Education, Science and Culture of Japan.

This paper is paper IV in the series "Actin-modulating proteins in the sea urchin egg." Paper III is reference 17.

Received for publication 6 June 1984, and in revised form 24 October 1984.

REFERENCES

1. Bader, M.-F., and D. Aunis. 1983. The 97-kd α-actinin-like protein in chromaffin granule membranes from adrenal medulla. Evidence for localization on the cytoplasmic surface and for binding to actin filaments. *Neuroscience.* 8:165–181.
2. Begg, D. A., and L. I. Rebhun. 1979. pH regulates polymerization of actin in the sea urchin egg cortex. *J. Cell Biol.* 83:241–248.
3. Bretcher, A., and K. Weber. 1980. Villin is a major protein of the microvillus cytoskeleton which binds both G and F actin in a calcium-dependent manner. *Cell.* 20:839–847.
4. 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.
5. Burridge, K., and J. R. Feramisco. 1980. Non-muscle α-actinins are calcium-sensitive actin-binding buffers. *Nature (Lond.).* 294:565–567.
6. Condeelis, J., and M. Vahey. 1982. A calcium- and pH-regulated protein from *Dictyostelium descoideum* that cross-links actin filaments. *J. Cell Biol.* 94:466–471.
7. DeRosier, D., E. Mandelkow, A. Silliman, L. Tilney, and R. Kane. 1977. Structure of actin-containing filaments from two types of non-muscle cells. *J. Mol. Biol.* 113:679–695.
8. Ebashi, S., and F. Ebashi. 1965. α-Actinin, a new structural protein from striated muscle. I. Preparation and action of actomyosin-ATP interaction. *J. Biochem. (Tokyo).* 58:7–12.
9. Ebashi, S., A. Kodama, and F. Ebashi. 1968. Troponin. I. Preparation and physiological function. *J. Biochem. (Tokyo).* 64:465–477.
10. Fechheimer, M., J. Brier, M. Rockwell, E. J. Luna, and D. L. Taylor. 1982. A calcium- and pH-regulated actin binding protein from *D. descoideum. Cell Motility.* 2:287–308.
11. Feramisco, A. 1979. Microinjection of fluorescently labeled α-actinin into living fibroblasts. *Proc. Natl. Acad. Sci. USA.* 76:3967–3971.
12. Fujiwara, K., M. E. Porter, and T. D. Pollard. 1978. Alpha-actinin localization in the cleavage furrow during cytokinesis. *J. Cell Biol.* 79:268–275.
13. Goll, D. E., A. Suzuki, J. Temple, and G. R. Holmes. 1972. Studies on purified α-actinin. I. Effect of temperature and tropomyosin on the α-actinin/F-actin interaction. *J. Mol. Biol.* 67:469–488.
14. Hamaguchi, Y., and Y. Hiramoto. 1981. Activation of sea urchin eggs by microinjection of calcium buffers. *Exp. Cell Res.* 134:171–179.
15. Hiramoto, Y. 1970. Rheological properties of sea urchin eggs. *Biorheology.* 6:201–234.
16. Hiramoto, Y. 1974. A method of microinjection into sea urchin eggs. *Exp. Cell Res.* 87:403–406.
17. Hosoya, H., and I. Mabuchi. 1984. A 45,000-mol-wt protein-actin complex from unfertilized sea urchin egg affects assembly properties of actin. *J. Cell Biol.* 99:994–1001.
18. Kane, R. E., 1975. Preparation and purification of polymerized actin from sea urchin egg extracts. *J. Cell Biol.* 66:305–315.
19. Katayama, E., T. Wakabayashi, F. Reinach, T. Masaki, and D. A. Fischman. 1984. Proximity of reactive lysyl residue to the antigenic site in rabbit skeletal myosin against the monoclonal antibody (MF-18) generated to chicken skeletal myosin. *J. Biochem.* 96:721–727.
20. Kobayashi, R., and Y. Tashima. 1983. Purification and characterization of an α-actinin-like protein from porcine kidney. *Biochim. Biophys. Acta.* 745:209–216.
21. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
22. Laurent, T. C., and J. Killander. 1964. A theory of gel filtration and its experimental verification. *J. Chromatogr.* 14:317–330.
23. Lazarides, E., and K. Burridge. 1975. α-Actinin: immunofluorescent localization of a muscle structural protein in nonmuscle cells. *Cell.* 6:289–298.
24. Longo, F. J. 1980. Organization of microfilaments in sea urchin (*Arbacia punctulata*) eggs at fertilization: effects of cytochalasin B. *Dev. Biol.* 74:422–433.
25. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
26. Mabuchi, I. 1973. A myosin-like protein in the cortical layer of the sea urchin egg. *J. Cell Biol.* 59:542–547.
27. Mabuchi, I., H. Hosoya, and H. Sakai. 1980. Actin in the cortical layer of the sea urchin egg. Changes in its content during and after fertilization. *Biomed. Res.* 1:417–426.
28. Mabuchi, I., and Y. Nonomura. 1981. Formation of actin paracrystals from sea urchin egg extract under actin polymerizing conditions. *Biomed. Res.* 2:143–153.
29. Mabuchi, I., and J. A. Spudich. 1980. Purification and properties of soluble actin from sea urchin eggs. *J. Biochem.* 87:785–802.
30. MacLean-Fletcher, S. D., and T. D. Pollard. 1980. Viscometric analysis of the gelation of *Acanthamoeba* extracts and purification of two gelation factors. *J. Cell Biol.* 85:414–428.
31. Martell, A. E., and R. M. Smith. 1974. Critical Stability Constants, Vol. 1. Amino Acids. Plenum Publishing Corp., NY. 269.
32. Maruyama, K., and S. Ebashi. 1965. α-Actinin, a new structural protein from striated muscle. II. Action on actin. *J. Biochem. (Tokyo).* 58:13–19.
33. Masaki, T., M. Endo, and S. Ebashi. 1967. Localization of 6S component of α-actinin at Z-band. *J. Biochem. (Tokyo).* 62:630–632.
34. Masaki, T., and O. Takaiti. 1969. Some properties of chicken α-actinin. *J. Biochem.* 66:637–643.
35. Mimura, N., and A. Asano. 1979. Ca^{2+}-sensitive gelation of actin filaments by a new protein factor. *Nature (Lond.)* 282:44–48.
36. Nunnally, M. H., J. M. D'Angelo, and S. W. Craig. 1980. Filamin concentration in cleavage furrow and midbody region: frequency of occurrence compared with that of alpha-actinin and myosin. *J. Cell Biol.* 87:219–226.
37. Otto, J. J., R. E. Kane, and J. Bryan. 1979. Redistribution of actin and fascin in sea urchin eggs after fertilization. *Cell.* 17:285–293.
38. Pollard, T. D. 1981. Puficiation of a calcium-sensitive actin gelation protein from *Acanthamoeba. J. Biol. Chem.* 256:7666–7670.
39. Robson, R. M., and M. G. Zeece. 1973. Comparative studies of α-actinin from porcine

cardiac and skeletal muscle. *Biochim. Biophys. Acta.* 295:208–224.
40. Rosenberg, S., A. Stracher, and K. Burridge. 1981. Isolation and characterization of a calcium-sensitive α-actinin-like protein from human platelet cytoskeletons. *J. Biol. Chem.* 256:12986–12991.
41. Sanger, J. M., and J. W. Sanger. 1980. Banding and polarity of actin filaments in interphase and cleaving cells. *J. Cell Biol.* 86:568–575.
42. Schook, W., C. Ores, and S. Puszkin. 1978. Isolation and properties of brain α-actinin. *Biochem. J.* 175:63–72.
43. Schroeder, T. E. 1975. Dynamics of the contractile ring. *In* Molecules and Cell Movement. S. Inoué and R. E. Stephens, editors. Raven Pres, NY. 305–334.
44. 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.
45. 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.
46. 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.
47. Steinhardt, R. R. Zucker, and G. Schatten. 1977. Intracellular calcium release at fertilization in the sea urchin egg. *Dev. Biol.* 58:185–196.
48. Suzuki, A., D. E. Goll, I. Singh, R. M. Robson, and M. H. Stromer. 1976. Some properties of purified skeletal muscle α-actinin *J. Biol. Chem.* 251:6860–6870.
49. Suzuki, A., D. E. Goll, M. H. Stromer, I. Singh, and J. Temple. 1973. α-actinin from red and white porcine muscle. *Biochim. Biophys. Acta.* 295:188–207.
50. Tilney, L. G., and L. A. Jaffe. 1980. Actin, microvilli, and the fertilization cone of sea urchins eggs. *J. Cell Biol.* 87:771–782.
51. Tyler, A. 1965. The biology and chemistry of fertilization. *Am. Nat.* 99:309–334.
52. Tyler, J., and D. Branton. 1980. Rotary shadowing of extended molecules dried from glycerol. *J. Ultrastruct. Res.* 71:95–102.
53. Yeltman, D. R., G. Jung, and K. L. Carraway. 1981. Isolation of α-actinin from sarcoma 180 ascites cell plasma membranes and comparison with smooth muscle α-actinin. *Biochim. Biophys. Acta.* 668:201–208.
54. Yin, H. L., K. S. Zaner, and T. P. Stossel. 1980. Ca^{2+} control of actin gelation. Interaction of gelsolin with actin filaments and regulation of actin gelation. *J. Biol. Chem.* 255:9494–9500.