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Identification of Actin-Binding Proteins from Sea Urchin Eggs by F-Actin Affinity Column Chromatography[1]

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Novel F-actin binding proteins of sea urchin eggs were searched for in order to study regulation of the actin cytoskeleton during fertilization and cell division. An extract of unfertilized eggs was analyzed by F-actin column chromatography. Several previously characterized F-actin-modulating proteins such as spectrin, myosin, and fascin bound to the column. The eluates from the column also contained proteins having apparent molecular weights of 225K, 150K, 70K, 60K, 45K, 40K, 38K, 36K, 34K, 20K, and 15K, which were thought to be novel cytoskeletal proteins judging from their molecular weights and non-reactivity to antibodies against previously characterized F-actin-modulating proteins. Most of the proteins in the F-actin column eluates co-sedimented with F-actin. Partial amino acid sequences of the peptides derived from the 45K and 40K proteins showed that these proteins are homologous to Arp3 and Arp2 subfamilies of actin-related proteins, respectively. The 150K protein seemed to be an unconventional myosin, that belongs to myosin VI subfamily. Amino acid sequences of two fragments from the 60K protein showed homology to that of coronin. The 150K protein was localized by immunofluorescence microscopy to the cleavage furrows in both whole cell sample and isolated cortex of dividing eggs. The 70K protein was uniformly localized in the cortical layer in the whole egg, but weak staining of the cleavage furrow region with the antiserum was observed in the isolated cortex. The 60K protein was localized to both the bulk cortical layer and the cleavage furrow, but the modes of localization were different.

Key words: actin-related proteins, coronin, F-actin affinity chromatography, myosin VI, sea urchin egg.

Actin plays important roles in motility and morphogenesis in eukaryotic cells, as a major constituent of the stress fibers, adherence junctions, lamellipodia, microvilli, contractile rings, and so on (*1*). Most of these structures are temporally formed, and their integrities are believed to be regulated by actin-modulating proteins and signal transduction pathways (*2*). Actin-modulating proteins have been classified into several groups on the basis of their actions on actin, such as F-actin-cross-linking proteins, F-actin-side-binding proteins, F-actin-capping proteins, F-actin-severing proteins, actin-depolymerizing proteins, G-actin-binding proteins, and motor proteins (*3*).

Sea urchin egg is a good material to investigate the regulatory system for the actin cytoskeleton. In unfertilized sea urchin eggs, actin filaments are hardly observed except in short microvilli on the surface (*4, 5*). Shortly after fertilization, the polymerization of actin occurs to form long microvilli and actin networks in the cortex (*6-9*). The contractile ring is formed during telophase through assembly and bundling of actin filaments at the equator and generates force for cytokinesis through actin-myosin II interaction (*10*).

Many actin-modulating proteins have been identified and purified from sea urchin or starfish eggs (*11, 12*). Among them, F-actin-cross-linking proteins found so far are as follows; 260K cross-linking protein (*13, 14*), spectrin (*15, 16*), villin (*17*), α-actinin (*18*), and fascin (*19*). Tropomyosin, which binds to the side groove of the actin filament helix, has also been found (*20*). These proteins are localized in the cortex or the microvilli formed in fertilized eggs and are considered to maintain the actin cytoskeleton. F-actin-severing or capping proteins such as villin (*17*), 100K severing/capping protein (*21*), 50K capping protein (*22*), and 45K severing/capping protein (*23, 24*) require Ca^{2+}

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Abbreviations: CBB, Coomassie Brilliant Blue R-250; DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; F-actin, filamentous actin; G-actin, globular actin; IEF, isoelectric focusing; 2-ME, 2-mercaptoethanol; Mops, 3-[*N*-morpholino]propanesulfonic acid; NEpHGE, nonequilibrium pH gradient electrophoresis; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylydene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TAME, *N*-*p*-tosyl-L-arginine methyl ester; TBS, Tris-buffered saline; TCA, trichloroacetic acid; Tween 20, polyoxyethylene sorbitan monolaurate.

ions for the activity and thereby have been thought to regulate the cytoskeletal structures through a Ca^{2+} signaling pathway. An F-actin-capping protein actolinkin is localized in the cell cortex of fertilized eggs and is suggested to be involved in the interaction between F-actin and the plasma membrane (*25*). Moesin, an ezrin-radixin-moesin family protein, localized in cortex of fertilized eggs is suggested to maintain actin filaments in the region (*26*). F-actin-depolymerizing protein depactin (*27*) and G-actin binding protein profilin (*28*) have been thought to sequester actin monomers in unfertilized eggs. Myosin II plays a key role in the force generation in cytokinesis (*29*).

Affinity column chromatography using F-actin as the ligand has been developed and used to search for F-actin-binding proteins in some eukaryotes. In a cellular slime mold *Dictyostelium discoideum*, ponticulin has been identified and suggested to participate in the actin-membrane interaction (*30*). SAC6, a homologue of fimbrin, and ABP1, which has an SH3 domain and a domain similar to cofilin, have been detected in budding yeast *Saccharomyces cerevisiae* (*31-33*). Miller *et al.* (*34*) have found many actin-binding proteins from *Drosophila melanogaster* embryos. Among them, a cDNA of a 140 kDa protein was cloned and it was revealed to be a novel member (group VI) of unconventional myosins (*35*). A 190 kDa protein named anillin has also been cloned and found to be a novel actin-bundling protein (*36*).

The existence of unidentified actin-binding proteins in sea urchin eggs has also been expected and these proteins would play important roles in the organization of the actin cytoskeleton during fertilization or cell division in the eggs. From this point of view, we analyzed an extract of unfertilized eggs by F-actin column chromatography. Many novel proteins were found in the F-actin-binding protein pools. Some of them were homologues of myosin VI, Arp3, Arp2, and coronin, which were identified in echinoderm eggs for the first time. Immunofluorescence microscopy with antibodies against three of the newly found proteins showed distinct staining patterns in fertilized eggs. Preliminary accounts of this study have been published in abstract forms (*37, 38*).

MATERIALS AND METHODS

Preparation of a Sea Urchin Egg Extract—Unfertilized eggs of sea urchin *Hemicentrotus pulcherrimus* or sand dollar *Clypeaster japonicus* were collected in natural seawater and washed twice with Ca^{2+}-free sea water. The eggs were dejellied in Ca^{2+}-free acidified sea water, washed with Ca^{2+}-free sea water and then with Na-K-EDTA (525 mM NaCl, 27.5 mM KCl, 2 mM EDTA, pH 7.0).

Extracts of the eggs were prepared according to Fishkind *et al.* (*16*). The eggs were further washed with an extraction medium (0.9 M glycerol, 1 mM EGTA, 1 mM DTT, 1

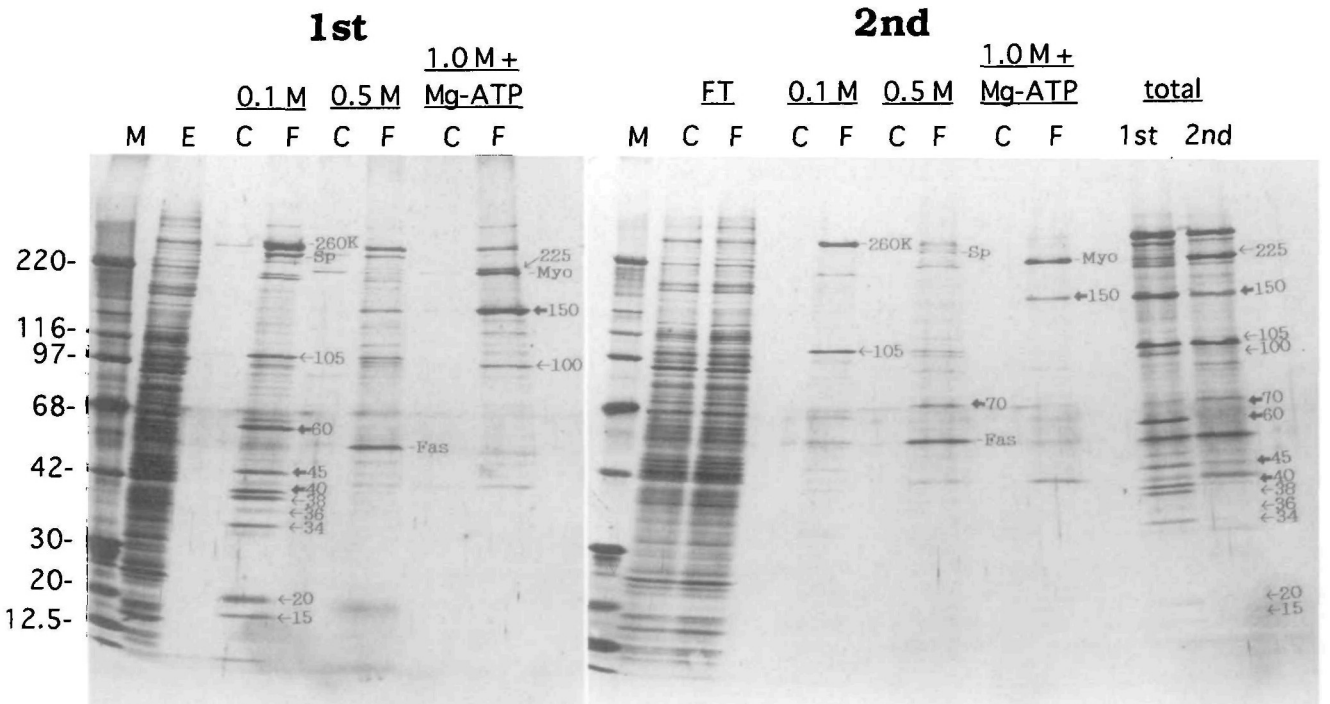

Fig. 1. **SDS-PAGE of the proteins eluted from columns that had been loaded with the extract of sea urchin *H. pulcherrimus* eggs.** The proteins eluted from F-actin and control BSA columns were run in parallel on the same 5-20% gradient gel. The gels were silver-stained. *Lanes are marked as follows:* M, *molecular weight markers;* E, extract; C, control column; F, F-actin column. FT, flow-through fractions from the first columns. These fractions were applied to the second columns. total, total proteins bound to the first and the second F-actin columns, respectively. Bars with abbreviated names in the picture indicate proteins detected by antibodies against previously characterized actin-binding proteins. 260K, 260K cross-linking protein; Sp, spectrin; Fas, fascin; Myo, myosin heavy chain. Thin arrows with apparent molecular weight $\times 10^3$ indicate proteins which were not recognized with the antibodies used in Fig. 2. Bold arrows indicate proteins to which antibodies were generated and/or whose amino acid sequences were analyzed in this study. Numbers on the left indicate the molecular weights $\times 10^3$ of marker proteins, chick gizzard myosin heavy chain, β-galactosidase, phosphorylase *b*, bovine serum albumin, rabbit muscle actin, carbonic anhydrase, and soybean trypsin inhibitor.

mM ATP, 2 mM TAME, 0.5 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.1 M Hepes-KOH, pH 7.5) and then re-suspended in four volumes of the extraction medium and homogenized at 4°C with a motor-driven teflon-glass homogenizer. The homogenate was centrifuged at 12,000 × g for 30 min. The supernatant was further centrifuged at 100,000 × g for 2 h and the final supernatant (10-15 mg protein/ml) was stored frozen at −80°C. The stored extract was diluted five times with A-buffer (50 mM Hepes-KOH, 2 mM DTT, 0.5 mM EDTA, 0.5 mM EGTA, 1.1 M glycerol, 0.05% NP-40, 2 mM TAME, 0.1 mM PMSF, 10 μg/ml leupeptin, pH 7.5) (*39*), and then centrifuged at 100,000 × g for 30 min. The resultant supernatant was applied to affinity columns.

Column Chromatography—Columns for affinity chromatography were prepared according to the method of Miller and Alberts (*39*). Rabbit back muscle actin was prepared by the method of Spudich and Watt (*40*) and further purified through a Sephadex G-100 (Pharmacia Biotech, Uppsala, Sweden) column. F-actin polymerized in 50 mM Hepes-KOH, pH 7.5, 0.1 M KCl, 3 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.2 mM ATP was stabilized with 10 μg/ml phalloidin, and then coupled to Affi-Gel 10 (Bio-Rad, Hercules, CA, USA) mixed with Sepharose CL-6B (Pharmacia Biotech). The coupling efficiency was about 0.5-1.0 mg/ml resin mixture. Coupling of BSA to Affi-Gel 10 was carried out in the same manner.

F-actin and BSA columns with equal bed volumes were equilibrated with A-buffer. An equal volume of the extract was applied to the each column at a flow rate of one column volume/h. The columns were rinsed with A-buffer at a flow rate of three column volumes/h. Then the columns were eluted successively with A-buffer containing 0.1 M KCl, A-buffer containing 0.5 M KCl and A-buffer containing 1.0 M KCl plus 1.0 mM ATP and 3 mM $MgCl_2$. Sometimes the columns were one-step eluted with A-buffer containing 1.0 M KCl plus 1.0 mM ATP and 3 mM $MgCl_2$. The frow-through fraction from each column was re-applied to the same column and eluted in the same manner.

Co-Sedimentation of the F-Actin Column-Binding Proteins with F-Actin—Protein fractions eluted from the F-actin columns were dialyzed against polymerizing buffer (50 mM Hepes-KOH, 0.1 M KCl, 5 mM $MgCl_2$, 0.2 mM DTT, 0.2 mM ATP, 0.1 mM PMSF, pH 7.2). After centrifugation at 100,000 × g for 1 h, 141.7 μl each of the supernatants was mixed with 8.3 μl of 1.8 mg/ml G-actin (final concentration, 0.1 mg/ml). After polymerization for 1 h at room temperature, 50 μl of each solution was mixed with 4.4 μl of 5 M KCl or 4.4 μl of 60 mM ATP (final concentration, 0.5 M KCl or 5 mM ATP). The samples were then centrifuged at 100,000 × g for 1 h, and the supernatants and the pellets were analyzed by SDS-PAGE.

Antibodies—Antisera to three of the newly found F-actin-binding proteins were raised in rabbits. Proteins eluted from the columns were precipitated with 10% (w/v) TCA, electrophoresed in SDS-polyacrylamide gels, and the protein bands were cut out after staining with CBB. The gel strips were washed with distilled water and homogenized in PBS (0.15 M KCl, 20 mM Na-phosphate buffer, pH 7.2) and emulsified with an equal volume of Freund's incomplete adjuvant. About 30-50 μg of each protein was subcutaneously injected at 2-week intervals. The rabbits were bled on the 10th day after the last injection.

Antibodies against starfish oocyte myosin (*41*), sea urchin egg α-actinin (*18*), sea urchin egg fascin (Mabuchi, unpublished), sea urchin egg 45K capping/severing protein (Ohnuma, unpublished), sea urchin egg actin (Mabuchi, unpublished), sea urchin egg actolinkin (*25*), starfish oocyte depactin (*27*), sea urchin egg profilin (Mabuchi, unpublished), and 15K calcium-binding protein (*42*) were raised as already published or to be published. Antibodies against sea urchin egg 260K cross-linking protein (*13*), sea urchin egg spectrin (*43*), sea urchin lantern muscle tropomyosin (*20*), p57 (*44*), and *Acanthamoeba* Arp3 (*45*) and panclonal antiserum against fimbrin (*46*) were kind gifts from the authors.

SDS-PAGE, Two-Dimensional Gel Electrophoresis and Immunoblotting—SDS-PAGE was carried out according to Laemmli (*47*), using a 5% acrylamide or 5-20% acrylamide gradient gel. The gels were stained with CBB or Silver Stain Plus Kit (Bio-Rad).

Two-dimensional gel electrophoresis was carried out according to O'Farrell with slight modifications. Isoelectric focusing (*48*) was carried out at 300 V for 3 h and NEpHGE (*49*) was carried out at 200 V for 1 h in the first dimensional gels containing 2% Ampholine (Pharmacia Biotech) (pH 4-6.5 for isoelectric focusing, and pH 3.5-10 : pH 5.7 = 1 : 4 for NEpHGE). Samples were electrophoresed with or without isoelectric point markers (2-D SDS-PAGE standards; Bio-Rad).

Immunoblotting analysis was performed according to the procedure developed by Towbin *et al.* (*50*) with modifications. The electrophoresed proteins were transferred to PVDF membranes. The PVDF membranes were blocked with 1% skim milk in TBS (0.15 M NaCl, 20 mM Tris-HCl, pH 7.6), reacted with the first antibodies for 2 h at room

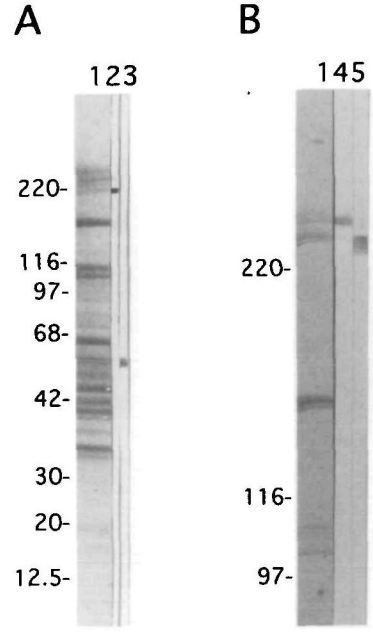

Fig. 2. **Immunoblot analysis of total binding proteins of the first F-actin column using antibodies against previously characterized actin-binding proteins.** (A) 5-20% gradient gel. (B) 5% gel. 1, CBB staining pattern; 2-5, immunoblots with antibodies against myosin (2), fascin (3), 260K cross-linking protein (4), and spectrin (5).

temperature, washed with TBS containing 0.05% Tween 20 and incubated with alkaline phosphatase-labeled second antibodies (Bio-Rad) for 1 h at room temperature. Development of enzyme activity on the PVDF membrane was carried out according to the manual of Bio-Rad.

Amino Acid Sequence Analysis and Homology Matching—The F-actin column fractions were subjected to SDS-PAGE. After brief staining with CBB, the bands were excised from the gels and treated with *Staphylococcus aureus* V8 protease (Sigma, St. Louis, MO, USA) or lysyl endopeptidase (Wako Pure Chemical Industries, Osaka) according to the method of Cleveland *et al.* (*51*). The proteolytic fragments of each protein were blotted onto PVDF membranes and run on a 473A gas phase protein sequencer equipped with an on-line PTH amino acid analysis system (Applied Biosystems, Norwalk, CT, USA). Database searches were done using BLAST and FASTA programs.

Immunofluorescence Microscopy—Indirect immunofluorescence microscopy was performed according to Mabuchi (*10*). For labeling whole eggs, eggs were attached onto a protamine (10 mg/ml)-coated glass slide and fixed for 15 min at room temperature with 5% (v/v) formalin dissolved in F-buffer (0.1 M KCl, 2 mM $MgCl_2$, 1 mM EGTA, 10 mM Mops-NaOH, pH 7.4) containing 0.8 M glucose (glucose-F-buffer). They were further incubated for 45 min with glucose-F-buffer containing 5% formalin and 0.5% NP-40. They were then washed with F-buffer containing 0.8 M glycerol (glycerol-F-buffer) and incubated with the primary antibodies dissolved in glycerol-F-buffer for 1 h. They were washed for 6 h in glycerol-F-buffer and then incubated with rhodamine-conjugated goat anti-rabbit IgG (Cooper Biomed., Malvern, PA, USA) for 1 h. After being washed overnight, the eggs were stained with 2 units/ml FITC-phalloidin (Molecular Probes, Junction City, OR, USA) and 0.2 μg/ml DAPI dissolved in glycerol-F-buffer and 0.1 M 2-ME in order to stain actin filaments and DNA, respectively.

Cortices of cleaving eggs were isolated on a protamine-coated glass surface and processed for immunofluorescence microscopy in the same manner as described above.

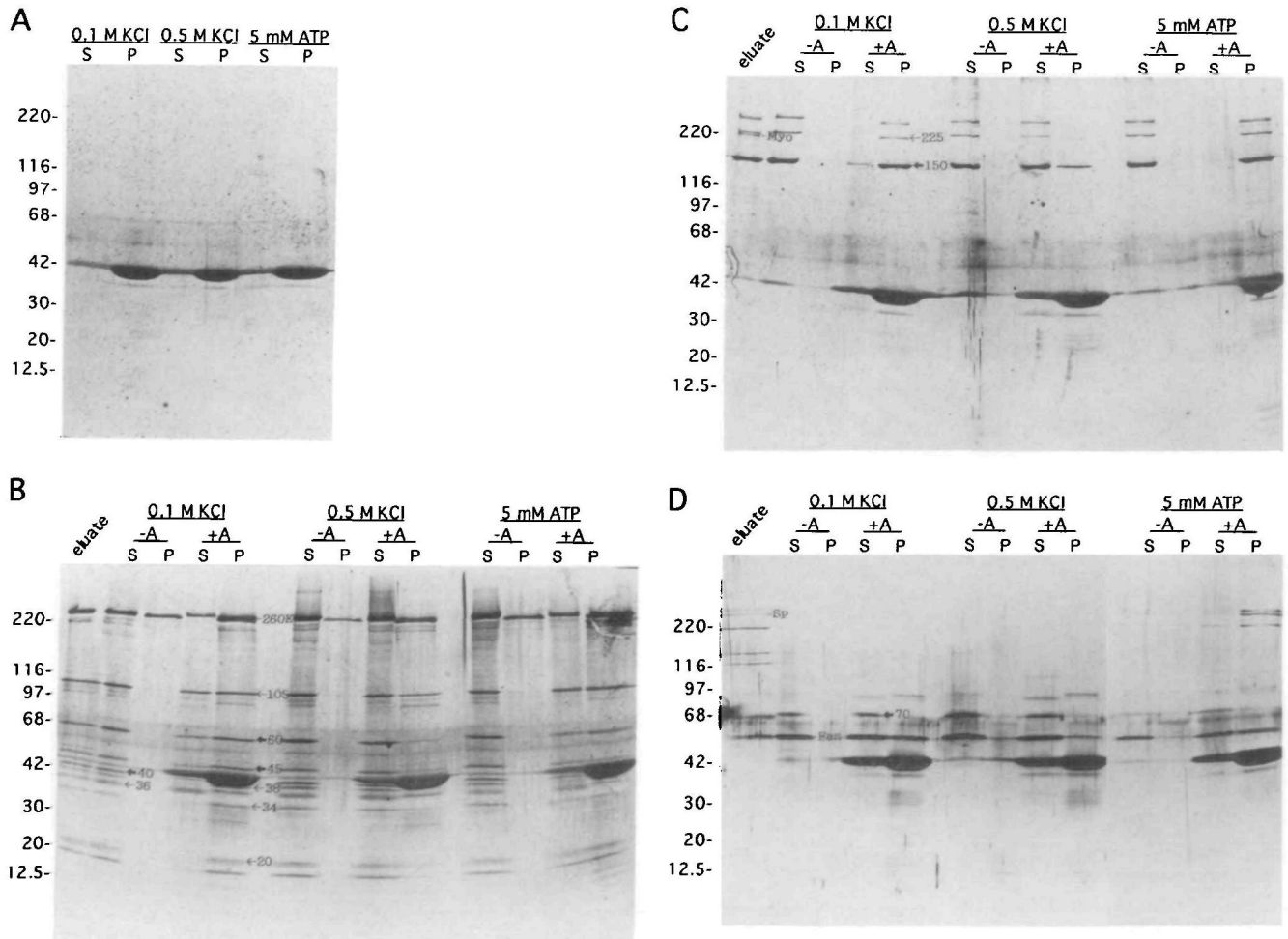

Fig. 3. **Co-precipitation of the F-actin column eluates with F-actin.** Actin was polymerized with each eluate which had been dialyzed against the polymerizing buffer, and then KCl or ATP was added to a final concentration of 0.5 M KCl or 5 mM ATP. Eluates without F-actin were also centrifuged. Ten microliters of the eluates before dialysis, the supernatants and the precipitates after centrifugation of 50 μl samples were electrophoresed. The samples were run on 5-20% gradient gels and silver-stained. (A) Actin alone, (B) 0.1 M KCl eluate from the first column, (C) Mg-ATP eluate from the first column, (D) 0.5 M KCl eluate from the second column. Lanes are marked as follows: eluate, eluates before dialysis; −A, eluates without actin; +A, eluates with actin; S, supernatants; P, pellets.

RESULTS

F-Actin Column-Binding Proteins from the Sea Urchin Egg Extract—The extract of unfertilized sea urchin (*H. pulcherrimus*) eggs was loaded in an amount exceeding the capacity of the F-actin column in order to detect minor proteins which have high affinity to F-actin. The flow-through fractions from the first column were re-applied to the second column to identify proteins which have lower affinity to F-actin than the proteins adsorbed to the first column.

Proteins adsorbed to the F-actin and BSA columns were eluted successively with 0.1 M KCl, 0.5 M KCl, and 1.0 M KCl plus Mg-ATP. Figure 1 shows SDS-PAGE of each eluate from the F-actin and control BSA columns. In case that 300 mg protein, that is the extract of about 7 ml packed eggs, was applied to each of the two 20 ml F-actin columns (0.5-0.8 mg F-actin/ml resin), more than 15 major proteins were found to bind specifically to the columns. In the first chromatography on the F-actin column, proteins of apparent molecular weights of 260K (260K in Fig. 1), 105K, 45K, 40K, 38K, 36K, 34K, 20K, and 15K were eluted with 0.1 M KCl (0.1 M KCl eluate), and proteins of apparent molecular weights of 250K (Sp in Fig. 1) and 55K (Fas in Fig. 1) were eluted with 0.5 M KCl (0.5 M KCl eluate). The final eluate with 1.0 M KCl plus Mg-ATP (Mg-ATP eluate) contained proteins of apparent molecular weights of 225K, 220K (Myo in Fig. 1), 150K, and 100K. In the second cycle of the F-actin column chromatography, the 225K, 45K, 40K, 38K, 20K, and 15K proteins seen in the first chromatography were scarcely detected on SDS-PAGE, and the amounts of the 60K and 150K proteins were less than those in the first chromatography. On the contrary, the amount the 220K protein in the Mg-ATP eluate increased and a protein of an apparent molecular weight of 70K, which was hardly stained with silver, appeared in the 0.5 M KCl eluate in the second chromatography. When the flow-through fraction from the second column was applied to the third F-actin column, the 260K, 220K, and 55K proteins were again detected in the eluates (data not shown). The major proteins seen in the F-actin column eluates were not significantly adsorbed to the control BSA column.

Immunoblot analysis of the proteins in the F-actin column eluates from the first column was performed using antibodies against actin-binding proteins of echinoderm eggs (Fig. 2). We identified the 260K protein in the 0.1 M KCl eluate as 260K cross-linking protein, the 55K protein in the 0.5 M KCl eluate as fascin, the 250K protein in the

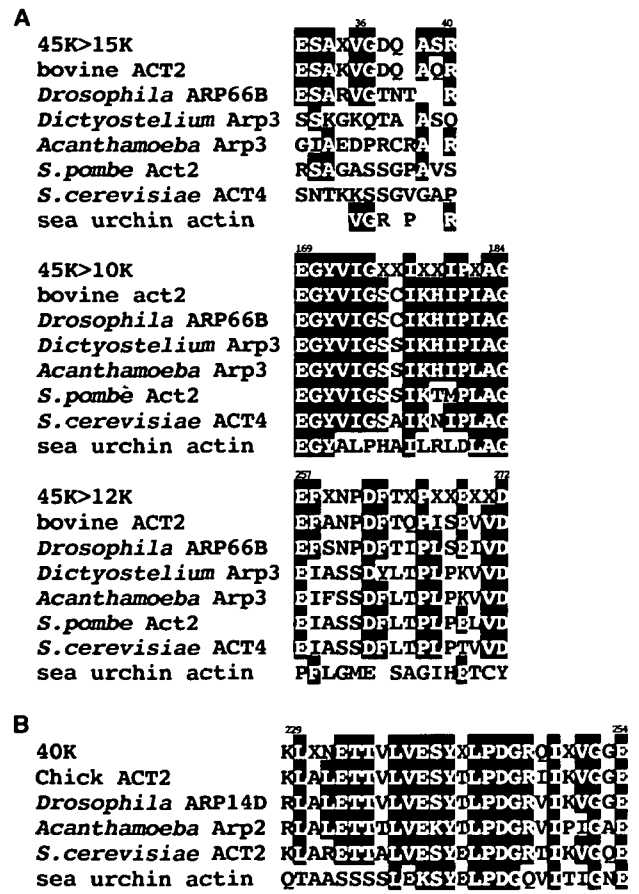

Fig. 4. **Partial amino acid sequences of the 45K and 40K proteins and their homology to actin-related proteins identified in other eukaryotes and to sea urchin conventional actin.** Numbers refer to residues of sea urchin conventional actin. (A) Sequences of three peptide fragments of the 45K protein aligned with homologous sequence of Arp3 subfamily. (B) A sequence overlapped by three independent peptides of the 40K protein aligned with homologous sequence of Arp2 subfamily. References: bovine ACT2 (*52*); *Drosophila* Arp66D and Arp55B (*53*); *Dictyostelium* Arp3 (*54*); *Acanthamoeba* Arp3 and Arp2 (*45*); *Saccharomyces cerevisiae* ACT4 (*55*); *Schizosaccharomyces pombe* Act2 (*56*); chicken ACT2 (*57*); *S. cerevisiae* ACT2 (*58*); and sea urchin conventional actin (*59*).

Fig. 5. **Partial amino acid sequences of two fragments of 150K protein and homology of the 70K fragment to myosin subfamilies.** Numbers refer to residues of chicken myosin II. References: porcine myosin VI (*60*); *Drosophila* myosin VI (*35*); bovine brush border myosin I (*76*); mouse dilute myosin V (*77*); and chicken myosin II (*78*).

Fig. 6. **Partial amino acid sequences of two fragments of 60K protein and their homology to *Dictyostelium* coronin and coronin-like proteins of vertebrates.** Numbers refer to residues of *Dictyostelium* coronin. References: *Dictyostelium* coronin (*61*); and coronin-like protein p57 (*44*).

0.5 M KCl eluate as spectrin and the 220K protein in the Mg-ATP eluate as myosin II heavy chain. The 34K protein band in the 0.1 M KCl eluate was found to contain tropomyosin. However, the major 34K component after two-dimensional electrophoresis was not recognized with the anti-tropomyosin antibodies (data not shown). The anti-α-actinin antibodies reacted with a minor material of 100K mol. wt. in the 0.1 M KCl eluate and the anti-fimbrin antibodies recognized a minor protein of 65K mol. wt. in the 0.1 M KCl eluate (data not shown). Antibodies against 45K severing/capping protein, actolinkin, depactin, profilin, and 15K calcium-binding protein did not significantly react with any proteins eluted from the column (data not shown).

In summary, the proteins that were not recognized by the antibodies described in "MATERIALS AND METHODS" (Fig. 1, arrows) were as follows: the 105K, 60K, 45K, 40K, 38K, 36K, 34K, 20K, and 15K proteins seen in the 0.1 M KCl eluate from the first column, the 225K, 150K, and 100K proteins in the Mg-ATP eluate from the first column, and the 70K protein in the 0.5 M KCl eluate from the second column. The isoelectric points of the 70K, 60K, 45K, 40K, 38K, 36K, 34K, 20K, and 15K proteins were estimated to be 4.5, 6.4, 5.8, 6.0, 9.0, 6.3, 8.0, 8.6, and 5.3, respectively.

The extract of unfertilized eggs of sand dollar *C. japonicus* were applied to the columns in the same manner as the extract of *H. pulcherrimus* eggs, and similar elution patterns were obtained except for the mobility of a protein that reacted with the antibodies against 260K cross-linking protein. The antibodies recognized the 260K band *H. pulcherrimus* while they only recognized a 400K material in the 0.1 M KCl eluate of *C. japonicus*. No polypeptide with mobility of 260 kDa was observed in the eluates of the sand dollar eggs (data not shown).

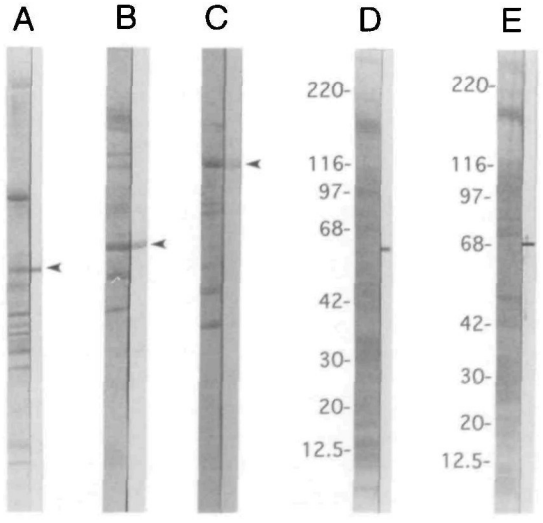

Fig. 7. **Reactivity of the antibodies against the 60K, 70K, and 150K proteins of sea urchin eggs.** Samples were run in 5-20% gradient gels. (A) 0.1 M KCl eluate from the first column reacted with anti-60K protein antibodies, (B) 0.5 M KCl eluate from the second column reacted with anti-70K protein antibodies, (C) Mg-ATP eluate from the first column reacted with anti-150K protein antibodies, (D) whole protein of unfertilized eggs reacted with anti-60K protein antibodies, (E) whole protein of unfertilized eggs reacted with anti-70K protein antibodies. Left strips, CBB-staining pattern; right strips, immunoblots with antibodies. Arrowheads in A, B, and C indicate the materials in the eluates reacted with the antibodies.

Co-Sedimentation of Column Eluates with F-Actin—Actin was polymerized with 0.1 M KCl in the presence of the 0.1 M KCl eluate from the first column, the Mg-ATP eluate from the first column, or the 0.5 M KCl eluate from the second column. Then KCl or ATP was further added when necessary and the samples were centrifuged. These conditions did not affect the amount of polymerized actin (Fig. 3A).

Some proteins in the eluates, especially myosin II, were precipitated after dialysis against polymerizing buffer (see Fig. 3C). All the above proteins eluted from the F-actin columns co-sedimented with F-actin in the presence of 0.1 M KCl, except the 36K protein (Fig. 3B). The 70K protein eluted with 0.5 M KCl did not co-sediment with F-actin (Fig. 3D), but the 70K protein obtained on one-step elution with 1.0 M KCl plus Mg-ATP from the second column did co-sediment (data not shown). 260K cross-linking protein sedimented alone (Fig. 3B). The presence of 5 mM ATP in the co-sedimentation experiment did not affect the result for any eluate.

The 45K and the 40K Proteins Are Homologous to Actin-Related Proteins—Peptides derived from the 45K protein after digestion with V8 protease (45K>15K,

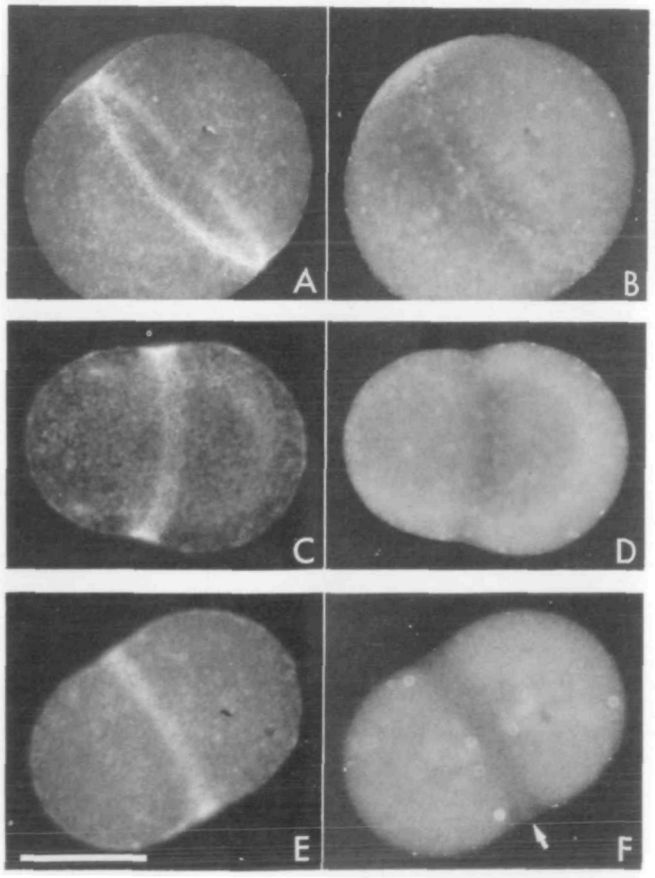

Fig. 8. **Localization of three antigens in fertilized sea urchin eggs by immunofluorescence microscopy.** (A) and (B), stained with anti-150K protein antibodies; (C) and (D), stained with anti-70K protein antibodies; (E) and (F), stained with anti-60K protein antibodies; (A), (C), and (E), fluorescence of FITC-phalloidin; (B), (D), and (F), fluorescence of rhodamine-second antibodies. Arrow indicates the cleavage furrow region stained with the anti-60K protein antibodies. Bar, 50 μm.

45K>12K, and 45K>10K), and those derived from the 40K protein after digestion with V8 protease (40K>15K and 40K>12K) and lysyl endopeptidase (40K>18K) were sequenced (Fig. 4). The sequence of the each of three fragments derived from the 45K protein was similar to the predicted amino acid sequences of actin-related protein Arp3 subfamily bovine ACT2 (*52*), *Drosophila* Arp66D (*53*), *Dictyostelium* Arp3 (*54*), *Acanthamoeba* Arp3 (*45*), *Saccharomyces cerevisiae* ACT4 (*55*), and *Schizosaccharomyces pombe* Act2 (*56*). The sequences of the three independent fragments derived from the 40K protein overlapped so the 26 amino acid-sequence became apparent. This sequence was found to be similar to the predicted amino acid sequences of Arp2 subfamily chicken ACT2 (*57*), *Drosophila* Arp55B (*53*), *Acanthamoeba* Arp2 (*45*), and *S. cerevisiae* ACT2 (*58*). All of the sequences showed less homology to the predicted sequence of conventional actins identified in several species of sea urchin (*59*). Neither of the proteins reacted with the anti-sea urchin egg actin antibodies, but the 45K protein reacted with anti-serum against *Acanthamoeba* Arp3 (data not shown).

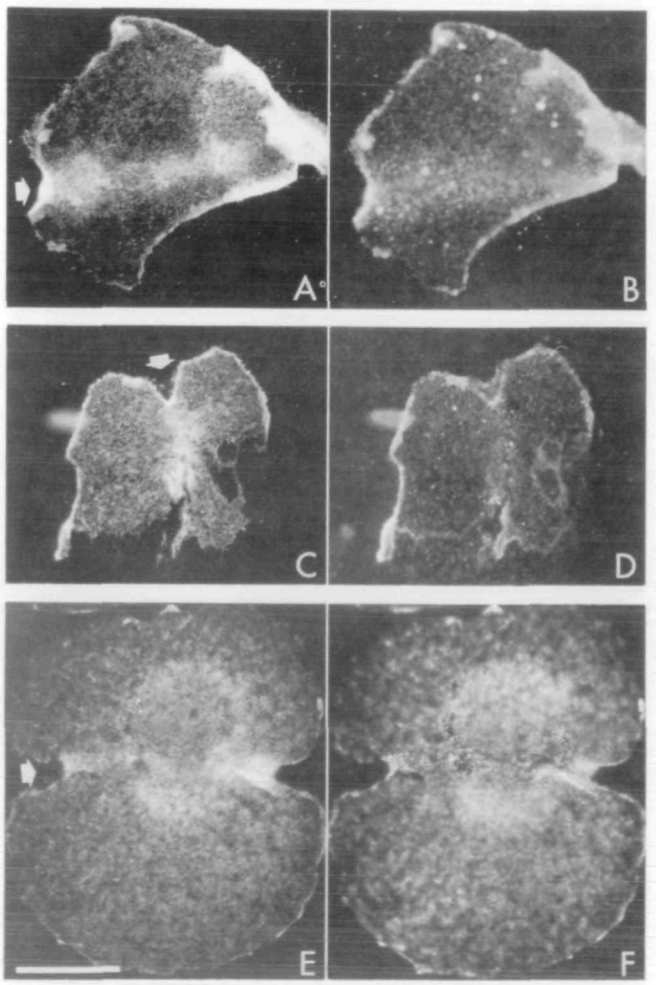

Fig. 9. **Immunofluorescence microscopy of isolated cortices of fertilized sea urchin eggs.** (A) and (B), stained with anti-150K protein antibodies; (C) and (D), stained with anti-70K protein antibodies; (E) and (F), stained with anti-60K protein antibodies. (A), (C), and (E), fluorescence of FITC-phalloidin; (B), (D), and (F), fluorescence of rhodamine-second antibodies. Arrows indicate the cleavage furrow region. Bar, 50 μm.

The 150K Protein Is Homologous to Myosin VI—The 150K protein treated with V8 protease gave two major fragments (150K>70K and 150K>60K). The sequence of the N-terminal region of the 70K fragment (150K>70K) was similar to the predicted amino acid sequences of porcine myosin VI (res. 500-514)(*60*) and *Drosophila* myosin VI (res. 499-513) (*35*) (Fig. 5). It showed less homology to other classes of myosins. The amino acid sequence of the N-terminal of the 60K fragment (150K>60K) showed no homology to known protein including above two myosin VIs.

The 60K Protein Is Homologous to Coronin—The N-terminal amino acid sequences of the two fragments derived from the 60K protein treated with V8 protease (60K>35K and 60K>15K) were analyzed (Fig. 6). Both of them showed homology to those of *Dictyostelium* coronin (*61*) and coronin-like protein p57 of mammals (*44*). The peptide antibodies against p57 weakly reacted with the sea urchin egg 60K protein (data not shown).

Reactivity of Antibodies against the 150K, 70K, and 60K Proteins—The specificities of the antisera against the 150K, 70K, and 60K proteins were examined by immunoblotting. The antibodies noticeably reacted with the corresponding antigens in the 0.1 M KCl eluate from the first column, the 0.5 M KCl eluate from the second column, and the Mg-ATP eluate from the first column, respectively (Fig. 7, A, B, and C, arrowheads). On blots of the whole proteins of unfertilized eggs, the anti-60K and anti-70K protein antibodies recognized each antigen (Fig. 7, D and E), while the anti-150K protein antibodies failed to detect the 150K protein band (data not shown).

Immunofluorescence Microscopy—Fertilized eggs were double-stained with the antisera and FITC-phalloidin. Actin filaments were concentrated in the equatorial region forming the contractile ring at telophase (Fig. 8, A, C, and E). Staining with the antibodies against the 60K, 70K, and 150K proteins showed that these antigens were localized in the cortical layer, although the staining patterns were distinct from each other. The anti-150K protein antibodies also stained the cleavage furrow (Fig. 8B). The anti-70K antibodies stained the cortical layer uniformly, but no obvious staining in the cleavage furrow was observed (Fig. 8D). The anti-60K protein antibodies stained both the bulk cortical layer and the cleavage furrow region. The latter staining was usually dim, but distinct from the staining of the bulk cortex (Fig. 8F, arrow). Punctuate staining with the anti-60K protein antibodies was sometimes observed at the periphery of the contractile ring (not shown). Each preimmune serum did not specifically stain any region of the eggs (data not shown).

Cortices of cleaving eggs adhered on glass slides were double-stained with the antisera and FITC-phalloidin. The contractile ring was intensely stained with FITC-phalloidin (Fig. 9, A, C, and E). The anti-150K protein antibodies stained the cleavage furrow region more strongly than other part of the cortex (Fig. 9B). The anti-70K protein antibodies also stained the contractile ring, but less obviously (Fig. 9D). The anti-60K protein antibodies stained both the cortex and the contractile ring. The cortical staining showed a network pattern like that of F-actin, while in the contractile ring region dotty stainings was observed (Fig. 9F).

DISCUSSION

Known F-Actin-Binding Proteins Bound Specifically to the F-Actin Columns—In this study, F-actin affinity column chromatography was used to detect the F-actin-binding proteins in extracts of sea urchin and sand dollar eggs. We used extracts of unfertilized eggs as starting materials because these eggs before fertilization have few actin filaments and most of the actin-binding proteins are thought to exist in the cytoplasm. These proteins may form pools from which the proteins would be supplied when the actin cytoskeletons are formed. For example, α-actinin and moesin move from the cytoplasm to the cortex during fertilization (*18, 26, 62*). In addition, the extraction medium used was chosen so that even membrane skeleton proteins such as spectrin, which would be present in the cortex of the unfertilized eggs (*63*), could be solubilized.

The finding that previously characterized F-actin binding proteins, namely 260K cross-linking protein, spectrin, myosin, and fascin, were detected in the eluates on immunoblotting indicates that an F-actin column is a good tool for searching for F-actin-binding proteins in sea urchin eggs as well as other cells. On the other hand, 45K severing/capping protein, actolinkin, profilin, and depactin were not detected in the eluates suggesting that an F-actin column is not useful to detect F-actin-severing/capping proteins or G-actin-binding proteins. Some of them might shear the ligand F-actin and flow through the column because gelsolin severs phalloidin-stabilized actin filaments (*64*). The reason why only a small amount of α-actinin was detected in the column eluates is not clear. The ability of α-actinin to bind to F-actin might be weaker than those of other F-actin-binding proteins in the extract or the solution used for the column (A-buffer, which contains both EGTA and EDTA) might have affected the ability.

Efficacy of the F-Actin Column Chromatography in Identification of F-Actin-Binding Proteins from Sea Urchin Eggs—Loading of an egg extract onto the F-actin column in excess of the maximum capacity of the column is effective to detect proteins of low contents but of high affinity to F-actin. The 225K, 45K, 40K, 38K, 20K, and 15K proteins may be such proteins, since these proteins were found only in the first column eluates when excess extract was applied to the column, while they could not be detected when a lower volume of the extract than that described in "MATERIALS AND METHODS" was applied. On the other hand, the 70K protein was detected only in the second column suggesting that it may have a lower affinity to the column. Therefore, the use of the second F-actin column is effective for detecting such proteins. The contents of 260K cross-linking protein, myosin II, and fascin may be much higher than those of the proteins newly identified in this study because these proteins were detected throughout the first to the third column chromatographies.

Analysis of unfertilized sand dollar *C. japonicus* eggs gave similar results. Therefore, most of the proteins newly found from sea urchin eggs in this study are thought to be ubiquitous in echinoid eggs. We consider that the 225K, 150K, 70K, 60K, 45K, 40K, 38K, 36K, 34K, 20K, and 15K proteins of sea urchin eggs found by the F-actin column chromatography would be novel proteins based on their mobilities on SDS-PAGE and non-reactivity to antibodies against previously characterized proteins. Since the amounts of the 45K, 40K, 38K, 36K, 34K, 20K, and 15K proteins in the eluates did not increase on standing for several days (data not shown), so they would not be proteolytic fragments of proteins of higher molecular mass in the eluates. The 105K and 100K proteins in the column eluates might be 100K severing protein (*21*), villin (*17*), or myosin I (*65*) which has been identified in sea urchin coelomocytes, but we could not confirm this because antibodies against these proteins are not available.

The 225K, 150K, 60K, 45K, 40K, 38K, 34K, 20K, and 15K proteins co-sedimented with F-actin, indicating that these proteins are likely to have the ability to bind directly or indirectly to F-actin. The 70K protein co-sedimented only on elution with 1.0 M KCl plus Mg-ATP in one-step manner. It might have bound indirectly to the column through other binding proteins. The binding of these proteins to F-actin is not likely to be non-specific, since the isoelectric points of these proteins are not extremely basic or acidic.

The Eluates Contained Actin-Related Proteins—The amino acid sequences of the 45K and 40K proteins in the 0.1 M KCl eluate from the F-actin column showed significant homology to actin-related proteins (Arps), Arp3 and Arp2, respectively. Their isoelectric points agreed well with those of Arps of other eukaryotes predicted from the amino acid sequences (*53, 57*). Furthermore, the 45K protein reacted with antibodies against *Acanthamoeba* Arp3. These two proteins from sea urchin eggs showed less homology to sea urchin conventional actins, which are known to be very conservative (*59*). The anti-sea urchin egg actin antibodies did not react with these proteins. Thus, it is not likely that the 45K and 40K proteins are conventional actins although sequence of conventional actin of *H. pulcherrimus* eggs are not yet known. All the results indicate that the 45K and 40K proteins are likely to be Arp3 and Arp2, respectively.

Arp2 and Arp3 have been suggested to be essential for the growth of yeast *S. cerevisiae* (*58*) and *S. pombe* (*56*), respectively, by genetical approach. Both *S. cerevisiae* ACT2 (Arp2) (*66*) and *S. pombe* Act2 (Arp3) (*67, 68*) colocalizes with F-actin as patchy structures in the cortex suggesting that these proteins interact with actin filaments *in vivo*, respectively.

Machesky *et al.* (*69*) have isolated a complex that contains Arp2, Arp3, and several novel proteins from an *Acanthamoeba* extract by a profilin-agarose column. The structure, subunit topology, and actin-binding activity of the Arp3/Arp2 complex have recently been reported (*70*). Interactions between Arp3, actin, profilin, and a novel protein Sop2p have also been reported (*67, 71*) in *S. pombe* by genetical and biochemical approaches. Also recently, a complex containing the Arp2-Arp3 heterodimer similar to that of *Acanthamoeba* was purified from human platelets as a factor that induces actin polymerization on bacterial surface (*72*). These observations suggest that Arp2 and Arp3 may play some regulatory roles in the assembly of the actin cytoskeleton.

Our finding that both the 45K and 40K proteins of sea urchin eggs are present in the same 0.1 M KCl eluate from the F-actin column suggests the possibility that these proteins form a complex that can bind to F-actin. The 38K, 34K, 20K, and 15K proteins in the 0.1 M KCl eluate could

be components of the complex because their mobilities on SDS-PAGE and their relative amounts as to the 45K and 40K proteins are similar to those of the proteins identified in the Arp complexes of *Acanthamoeba* and human platelets. Furthermore, profilin was not detected in the 0.1 M KCl eluate. Thus, it is not likely that these actin-related proteins have bound to the F-actin column *via* profilin. They might bind directly or indirectly to the column through other protein(s) in the 0.1 M KCl eluate.

Both the 45K and 40K proteins co-sedimented with F-actin, but dissociated from it in the presence of 0.5 M KCl. This indicates that these proteins do not co-polymerize with conventional actin, but may bind to the sides or ends of the actin filament as demonstrated in *Acanthamoeba* Arp3/Arp2 complex (*70*).

The Eluates Also Contained Myosin VI—One of the two peptide fragments of the 150K protein in the Mg-ATP eluate from the first column showed significant homology to myosin VI subfamily (*35, 60*).

Both the behavior of the 150K protein which was eluted from the F-actin column with 1.0 M KCl and Mg-ATP and its mobility on SDS-PAGE are also similar to *Drosophila* myosin VI (*34*). The content of the 150K protein in the egg extract seemed to be far lower than myosin II, but its affinity to F-actin seemed to be stronger because it bound to the first column while myosin II mainly bound to the second column. The reason why the 150K protein did not move into the supernatant after the addition of ATP in the co-precipitation assay with F-actin is no clear at present.

The 60K Protein May Be Coronin—The peptides derived from the 60K protein showed homology to coronin, which has been identified in *Dictyostelium*. Coronin is an F-actin-binding protein and has domains similar to the β subunit of trimeric G proteins (*61*). The roles of coronin in cell motility including cytokinesis in *Dictyostelium* amoebae have been suggested (*73*). Coronin-like proteins have recently been reported in mammals (*44*). The 60K protein was reacted with antibodies against these proteins. Therefore, it is likely that the 60K is a coronin-like one.

Localization of Newly Identified F-Actin-Binding Proteins in Fertilized Sea Urchin Eggs—The actin filaments in the furrow region may be regulated in a dynamic fashion. At the beginning of the telophase, actin filaments are concentrated and bundled together to form the contractile ring in the equatorial cortex (*10*), and during contraction the filaments are thought to be continuously destroyed (*74*). It is important to know what kind of proteins are localized in the cleavage furrow region in order to understand the dynamics of the contractile ring.

Antibodies against the 150K protein, probably myosin VI, stained the cleavage furrow. *Drosophila* myosin VI has been localized to cortical particles in interphase and to pseudocleavage furrows in mitosis (*35, 75*). These observations suggest a possibility that myosin VI plays roles in constructing the contractile ring, transporting particles which are necessary for cytokinesis (*75*) or force generation in cleavage.

Coronin has been reported to be essential for cytokinesis on analysis of *Dictyostelium cor*$^-$ mutant. However, it was not detected in the cleavage furrow region with anti-coronin antibodies (*73*). We found that the coronin-like 60K protein is localized in the cortical layer as well as the cleavage furrow region of sea urchin eggs, although the concentration in the cleavage furrow is not high by examining whole cell specimen. The staining patterns were different between the cleavage furrow region and the other parts of the isolated cortex although the reason for this difference is not clear at present. It could be that sea urchin coronin plays some role in the contractile ring distinct from that in the bulk cortex.

The 70K protein which showed localization throughout the cortex in the whole egg and a weak concentration in the furrow region of isolated cortex, may be associated with some accessory structures of the actin cytoskeleton, since the 70K protein may bind to F-actin indirectly.

Further analysis of the primary structure as well as biochemical characterization would be necessary to elucidate the function of these proteins.

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