ISOLATION AND CHARACTERIZATION OF TWO FORMS OF A CYTOSKELETON

KENNETH T. EDDS

From the Marine Biological Laboratory, Woods Hole, Massachusetts 02543. Dr. Edds' present address is the Department of Anatomical Sciences, S.U.N.Y.-Buffalo, Buffalo, N.Y.14214

ABSTRACT

Isolated petaloid coelomocytes from the sea urchin *Strongylocentrotus droebachiensis* transform to a filopodial morphology in hypotonic media. Electron micrographs of negatively stained Triton-insoluble cytoskeletons show that the petaloid form consists of a loose net of microfilaments while the filopodial form consists of paracrystalline bundles of microfilaments. Actin is the major protein of both forms of the cytoskeleton. Additional polypeptides have molecular weights of ~220,000, 64,000, 57,000, and 27,000 daltons. Relative to actin the filopodial cytoskeletons have an average of 2.5 times as much 57k polypeptide as the petaloid cytoskeletons. Treatment with 0.25 M NaCl dissociates the filament bundles into individual actin filaments free of the actin-associated polypeptides. Thus, one or more of these actin-associated polypeptides may be responsible for crosslinking the actin filaments into bundles and maintaining the three-dimensional nature of the cytoskeletons.

KEY WORDS cytoskeleton · actin · actin-associated proteins · electron microscopy · gel electrophoresis

Actin-containing microfilament bundles are dynamic structures that can change their form and distribution within a cell, depending on the activity or function of the particular cell type. They exist and function both as axially oriented structural supports for cytoplasmic extensions and as part of the actomyosin-based contractile apparatus found in many motile cells (4, 11, 17, 22). Ultrastructural analysis of these fibrillar structures has shown that the filaments will reversibly bind heavy meromyosin, indicating that they contain actin (1, 13), a fact confirmed and extended by light microscope studies utilizing fluorescent antibodies (12, 15). Actin-associated proteins have been hypothesized to be an integral part of these microfilament complexes; recently, detergent-resistant structures have been prepared for biochemical analysis, and some actin-associated components have been demonstrated (2, 18, 20, 23). The nonmuscle cell actin-associated proteins form a diverse group and include, among others, high molecular weight proteins (21), intermediate-sized polypeptides (14, 16), and relatively small, low molecular weight proteins (6). The role of each of these is currently being investigated.

The present report describes the isolation, from two morphologically distinct forms of the sea urchin coelomocyte, of a detergent-insoluble microfilamentous cytoskeleton. Although these two forms of the coelomocyte cytoskeleton have different structures, they differ in polypeptide composition only in the amount of a 57,000 mol wt polypeptide.

MATERIALS AND METHODS

Collection and Transformation of Coelomocytes

The petaloid coelomocytes from the sea urchin, *Stron-*

gylocentrotus droebachiensis, were collected and prevented from clotting by the addition of 30–50 mM EGTA (ethyleneglycol-bis(β-aminoethyl ether)N,N'tetra acetate) as previously described (9). The unclotted petaloid coelomocytes collected in ~100 ml of coelomic fluid were layered on top of 0.7 M sucrose dissolved in 0.5 M NaCl, 10 mM TES (N-Tris[hydroxymethyl]methyl-2 aminoethanesulfonic acid), pH 7.5. The petaloid coelomocytes will not penetrate the sucrose-salt solution when centrifuged at 5,500 rpm in a Sorvall HB-4 rotor (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.) for 5 min (g force = $5{,}000_{max.}$) and can be collected at the coelomic fluid/sucrose interface. These purified petaloid coelomocytes were washed and resuspended in 0.5 M NaCl, 5 mM TES, pH 7.5. The morphological transformation to the filopodial form was elicited by hypotonic shock, i.e., dilution to 0.3 M NaCl with 5 mM TES, pH 7.5.

Collection of Cytoskeletons

Separate populations of petaloid or filopodial coelomocytes were lysed with 0.5% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) in 0.12 M NaCl, 2 mM $MgCl_2$, 5 mM TES, pH 7.5 for 15 min. at 4°C. The unlysed nuclei were pelleted by centrifugation at 1,500 *g* for 10 min. The resulting supernate was highly enriched in cytoskeletons and could be concentrated by low-speed centrifugation (30,000 *g* for 10 min.). Alternatively, "petals" and filopodia can be sheared from their respective cell types by rapidly pipetting the cells into and out of a 5-ml pipette (Finnpipette, Variable Volumetrics, Woburn, Mass.). The cellular fragments were separated from the cell bodies containing the nuclei by pelleting the latter at 1,500 *g* for 10 min. The fragments were then lysed as described above. The ionic strength of the buffers was varied by addition of NaCl while other parameters (pH, divalent cations) were kept constant. Two identical samples, one control and one treated with 0.25 M NaCl, 2 mM $MgCl_2$, 5 mM TES, pH 7.5 for 15 min on ice, were initially centrifuged at low speed (30,000 *g* for 10 min.), and the resulting pellets of material were collected. The supernates from each sample were spun again at high speed (100,000 *g* for 3 h), and those pellets were collected. Finally, any nonsedimentable material was collected from the high-speed supernate by addition of cold trichloroacetic acid to a final concentration of 5%. All samples were treated identically with the pellets or precipitates dissolved in equal volumes of electrophoresis sample buffer, electrophoresed, and quantitated by densitometry.

Egg and Coelomocyte Extracts

The procedure of Kane (14) was followed for the preparation of egg and coelomocyte extracts. Eggs were collected from *S. droebachiensis* and *S. purpuratus* by KCl injection and washed in an isotonic medium containing 2 mM EGTA before homogenization. The coelomic cells were collected as described above and also washed in the EGTA-containing buffer. Extracts were made using 2 ml of packed, dejellied eggs or coelomocytes. The egg extracts were pelleted by centrifugation at 25,000 *g* for 15 min, and the coelomocyte extracts were spun at 100,000 *g* for 3 h. The contents of the coelomocyte extracts were precipitated by addition of cold trichloroacetic acid (see Fig. 7 *c*)

Gel Electrophoresis and Electron Microscopy

Gel samples were prepared by standard procedures, with care being taken to insure equivalent loadings on all gels. The samples were electrophoresed on 5% Tris-acetate gels and stained with Fast green (10). The gels were scanned at 650 nm, and the areas under the peaks were quantitated. Weight ratios of the cytoskeletal components were calculated by dividing the area under each peak by the molecular weight of the protein represented by that peak and comparing the values. The values reported in the Results section are the average of six preparations, with Fig. 3 being a tracing of a set of scans with near average amounts of each component. Molecular weights were estimated by coelectrophoresis according to standard procedures (24). Sheared cytoskeletons were applied to Formvar-coated EM grids, fixed in 1% glutaraldehyde, stained with 1% uranyl acetate, and examined with a Philips 300 electron microscope operated at 80 kV.

RESULTS

Isolation of Cytoskeletons

The Triton-insoluble cytoskeletons obtained from both whole coelomocytes and sheared petals and filopodia were similar. However, whole coelomocytes were routinely used for the biochemical experiments because the yield of material was greater. On the other hand, sheared cellular fragments were used in the ultrastructural preparations because the negatively stained images appeared cleaner with less background material obscuring the image (Figs. 1 and 2).

Ultrastructure of Cytoskeletons

Coelomocyte cytoskeletons from petaloid and filopodial cells are distinctive and similar in morphology to the cells from which they were obtained. As judged from negative staining, the petaloid cytoskeleton is composed mainly of 65 Å filaments. Most of these filaments are in loose networks but a few are aggregated into small bundles (Fig. 1 *b*). In contrast, the filopodial cytoskeletons are composed almost entirely of large, 10-μm or longer, bundles of 65-Å filaments (Fig.

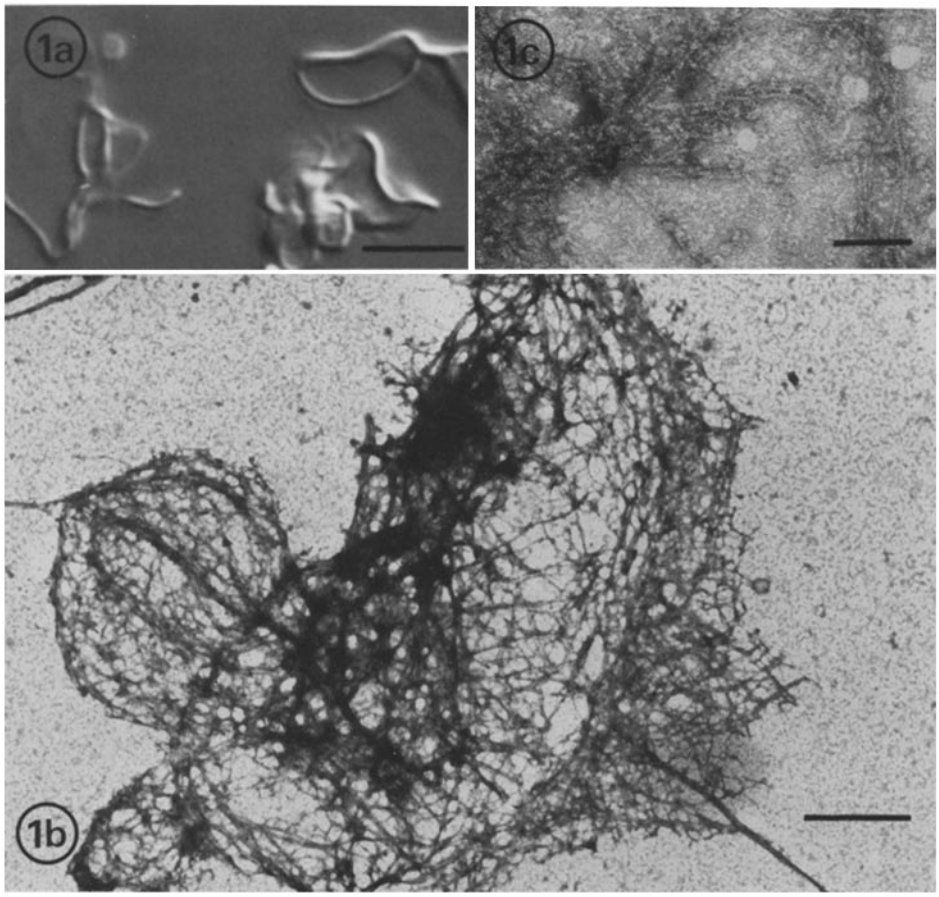

FIGURE 1 (*a*) Nomarski differential interference optics produce an optical section of sheared, isolated petals. Bar, 5 μm. × 2,800. (*b*) A petaloid cytoskeleton after detergent lysis. Negatively stained images show a netlike arrangement of microfilaments with a variable amount of bundling of filaments. Bar, 1 μm. × 14,000. (*c*) Higher magnification of a petaloid cytoskeleton demonstrating that unbundled, loosely organized filaments are its salient feature. Bar, 0.1 μm. × 110,000.

2*b*). Within each bundle the 65-Å filaments may be packed in one of three different ways: (*a*) many form a paracrystalline array with filaments spaced 90 Å apart center to center; (*b*) many are in loose bundles; and (*c*) rarely there are irregular bundles with a 13-nm longitudinal spacing (8).

Biochemistry of Cytoskeletons

Cytoskeletons washed in a buffer consisting of 0.12 M NaCl, 2 mM $MgCl_2$, and 5 mM TES, pH 7.5 are composed primarily of actin with four actin-associated polypeptides with molecular weights of ~220,000, 64,000, 57,000, and 27,000 daltons. Repeated washing of the cytoskeletons does not alter their composition but rather results in a diminution of all components. By quantitative densitometry of Fast green-stained sodium dodecyl sulfate (SDS) gels, actin is found to comprise 80–85% of the total cytoskeletal protein. The two types of cytoskeletons have similar compositions except that the filopodial forms are enriched in the 57,000-dalton component (Fig. 3). In the petaloid form, the weight ratio of 57,000 to actin ranged in six preparations from 1:30 to 1:20 and averaged 1:26; in the filopodial form, that ratio varied much less (between 1:9 and 1:11) and averaged 1:10. The other actin-associated proteins did not vary appreciably between the two forms. The weight ratios of those components to actin were: 220k:actin = 1:90, 64k:actin = 1:20, and 27k:actin = 1:5.

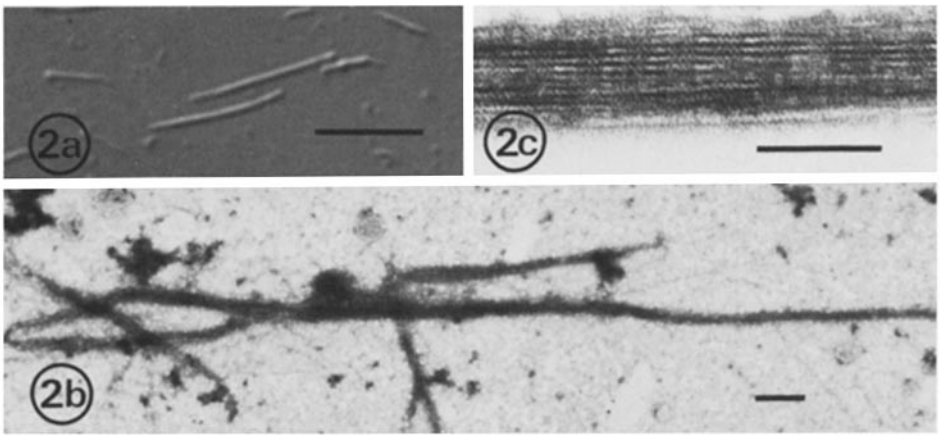

FIGURE 2 (*a*) Sheared filopodia. Note the absence of contaminating nuclei. Bar, 5 μm. × 2,800. (*b*) Detergent-lysed filopodia demonstrating the axial core of bundled microfilaments. Bar, 1 μm. × 6,500. (*c*) Some bundles exhibit highly ordered, crosslinked arrays of filaments that are spaced 90 Å apart center to center. Other bundles are not so well ordered, cf. Fig. 5*a*. Bar, 0.1 μm. × 165,000.

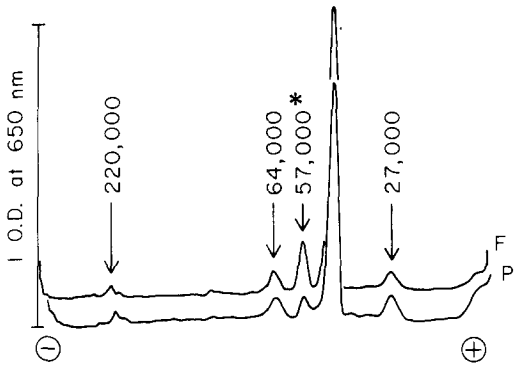

FIGURE 3 Preparations of petaloid (*P*) and filopodial (*F*) cytoskeletons were electrophoresed, stained with Fast green, and quantitated by scanning densitometrically at 650 nm. This tracing of a pair of scans represents an average preparation although the average values expressed in the text are those calculated from six preparations. Both cytoskeletons have similar components except that the filopodial preparations are enriched in the 57,000 component.

The filopodial cytoskeletons were dissociated into their components by treatment with 0.25 M NaCl for 15 min. at 4°C. By increasing the salt concentration to 0.25 M, the amount of cytoskeletal material that is pelleted during a low-speed spin is reduced (Fig. 4). This is accompanied by the appearance of individual filaments in the supernate (Fig. 5). These filaments comprise the majority (64%) of the actin in the 0.25 M NaCl sample while the actin-associated components, most notably the 57k and 64k proteins, are

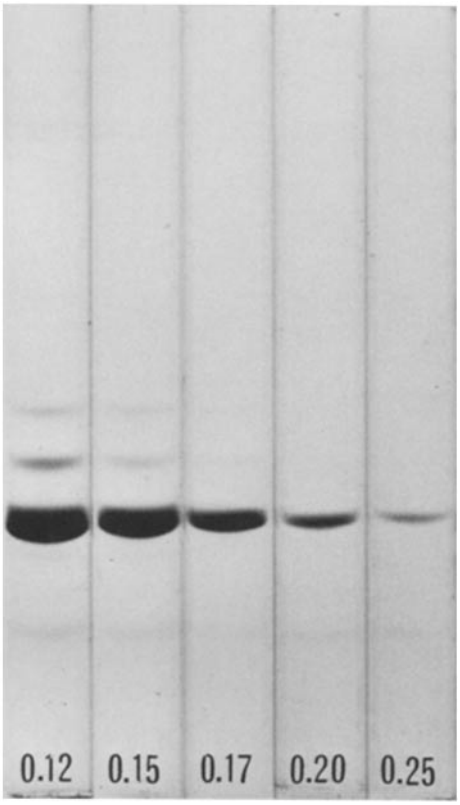

FIGURE 4 Tris-glycine-SDS gels (5%) of the low-speed (30,000 *g* for 10 min) pellets obtained from salt-extracted filopodial cytoskeletons. Note that as the salt concentration approaches 0.25 M NaCl, there is less and less material pelleted.

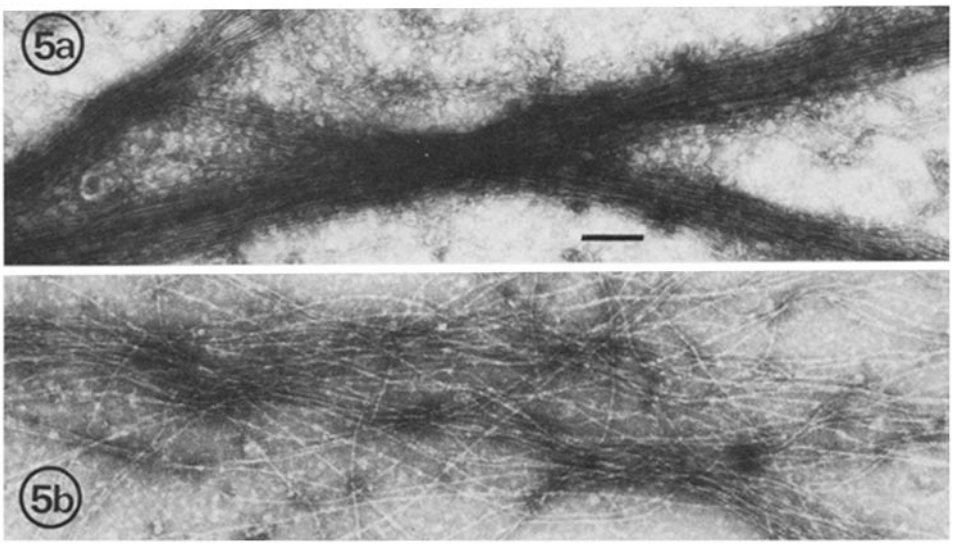

FIGURE 5 The ultrastructural correlates of Fig. 4 are shown in negatively stained preparations. (*a*) Control filopodial cytoskeletons incubated in 0.12 M NaCl. (*b*) By increasing the salt concentration to 0.25 M, the bundles are dissociated into individual filaments. (*a* and *b*) Bar, 0.1 μm. × 80,000.

stripped from the filaments and found in the supernate. In the control sample, more than half (60%) of the actin and most (83%) of the actin-associated proteins are pelleted in the first low-speed spin (see Fig. 6). Thus, the bundles are disrupted into actin filaments and monomer by brief treatment with moderate salt concentrations, and the actin-associated components are solubilized into the supernate.

Comparative Studies

Following the procedures outlined by Kane (14), extracts were made from both sea urchin eggs and petaloid coelomocytes. Extracts of dejellied eggs from either *S. droebachiensis* or *S. purpuratus* would not form a solid gel upon addition of KCl and ATP and/or warming to 40°C for 1 h. Birefringent fibrils were present but sparse in these extracts and could be pelleted at 25,000 *g* for 15 min and analyzed by SDS-polyacrylamide gel electrophoresis. They contain numerous proteins (Fig. 7 *b*); while coelectrophoresis of cytoskeletons vs. extract-derived fibrils show proteins of similar size in both preparations (Fig. 7 *a* and *b*), the fibrils were too complex to allow further comparisons. The petaloid coelomocyte extracts also did not form a gel or any birefringent fibrils. A very small amount of actin was pelleted from the extracts (100,000 *g* for 3 h) indicating that there was little actin polymerization. However, actin and a 57k component were extracted and could be precipitated with cold trichloroacetic acid from the supernate of the high-speed spin (Fig. 7 *c*).

DISCUSSION

The study of the formation, ultrastructure, and composition of cytoskeletons from a variety of cell types is an important step toward understanding how these macromolecular complexes function within cells. Sea urchin coelomocytes are ideal candidates for these studies because they possess the remarkable ability to transform morphologically from a petaloid to filopodial form and provide access to two morphologically unique forms of the same cell. The underlying cytoskeletons of each form are ultrastructurally and biochemically distinct.

Structurally, the isolated cytoskeletons differ in the degree of organization of their constituent microfilaments, and in that regard are indistinguishable from those observed in substrate-attached cells (9) and are therefore suitable for biochemical analysis.

Biochemically, the cytoskeletons contain actin and four actin-associated proteins (Fig. 3). While the greatest differences in amount of 57k protein are observed between the two forms of the cytoskeleton, the wider range of 57k protein to actin ratios observed among the petaloid preparations may be important as well. The 57k protein to actin

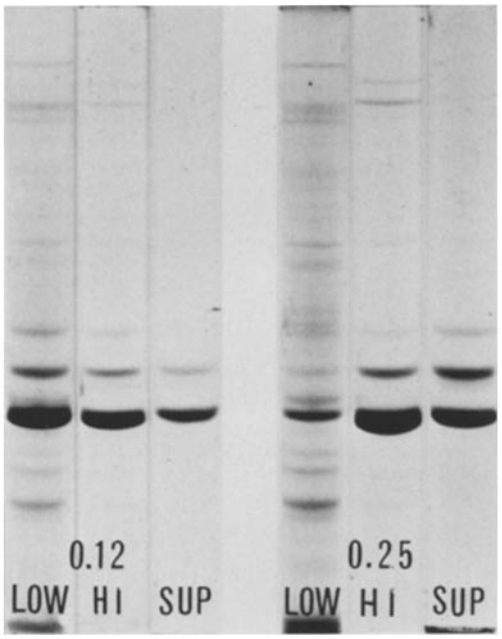

FIGURE 6 The salt-extracted filopodial cytoskeletons are dissociated into filaments that are largely stripped of their actin-associated proteins. Two identical samples of filopodial cytoskeletons were incubated and first centrifuged at low speed (30,000 *g* for 10 min) and then at high speed (100,000 *g* for 3 h); the pellets from each spin were collected. Finally, the supernates from the high-speed runs were precipitated and all six samples were electrophoresed. In the control sample on the left which was incubated in 0.12 M NaCl, most (60%) of the actin and associated proteins (83%) are pelleted in the initial low-speed spin (low) as might be expected because the bundles are still intact (see Fig. 5 *a*). After 15 min of 0.25 M NaCl, the bundles are dispersed into filaments which are pelleted in high-speed spin and are largely stripped of their associated components. These pelletable filaments comprise 64% of the actin in this sample. Most of the associated components have been solubilized and were precipitated from the high-speed supernate (0.25, *sup*).

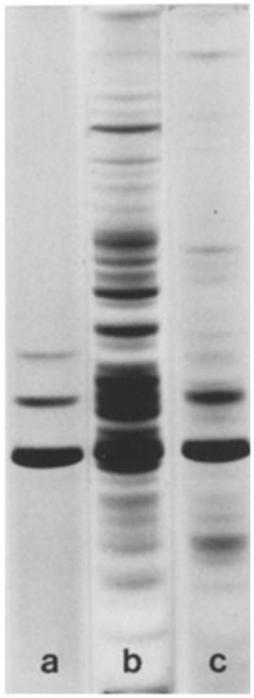

FIGURE 7 SDS-PAGE showing a comparison of the components in (*a*) filopodial cytoskeletons, (*b*) sea urchin egg extracts, and (*c*) coelomocyte extracts. While neither the egg extract (*b*) nor the coelomocyte extract (*c*) formed a solid gel after addition of ATP and warming, the electrophoretic patterns represent the components of each extract which were collected by centrifugation (*b*) or precipitation (*c*) (see text).

ratios varied from 30:1 to 20:1 among several petaloid preparations which may reveal a greater degree of cytoskeletal transformation in some populations of coelomocytes. For example, Fig. 1 *b* represents an average petaloid cytoskeleton with a small amount of bundle formation. If the 57k protein is directly involved in bundle formation, then the variations in its amount may be reflected in the variations in extent of bundle formation among cytoskeletons. The 57k protein may be acting as a crosslinking agent in this system, as a protein of similar size and solubility characteristics does in egg extracts (3). Furthermore, some of the filament bundles show periodic striations along their length (8) which are similar to those observed in microvilli of sea urchin eggs (5) and in gelled extracts of eggs (7). The egg actin gels exhibit the most pronounced striations after days of storage (14). The extent of the crosslinking in egg extracts can be altered, as observed in negatively stained preparations, by limiting the amount of 58k protein (3). Interestingly, when the 58k protein to actin weight ratio equals 1:10, i.e., the same as is observed in filopodial coelomocyte preparations, the striations are not so apparent and the bundles are more loosely organized than when the weight ratio is 1:5. Further strength is given to this purported identity by the recent work of Otto et al. (19) in which a fluorescently tagged antibody against the egg 58k protein is presumed to interact specifically with a similar protein in echinoderm coelomocytes and is seen to concentrate, during

the morphological transformation, in the actin filament bundles.

While direct comparisons of the cytoskeletal 57k component and the 58k protein from egg extracts were not feasible, it is worthy of note that the relatively simple, three-component actin gel is not readily prepared in all species of echinoderms, a fact that has been corroborated by other workers (D. Begg, personal communication). The simple cytoskeletal isolation and dissolution technique coupled with the ease of handling and transforming the system as outlined here will allow for the further characterization of these and other cytoskeletal components.

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