

DYNAMIC ASPECTS OF FILOPODIAL FORMATION BY REORGANIZATION OF MICROFILAMENTS

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ABSTRACT

The coelomocytes of the sea urchin, *Strongylocentrotus droebachiensis*, may be prevented from clotting with 50 mM ethylene glycol-bis(β -aminoethyl)-*N,N,N',N'*-tetraacetate, 50 mM Tris-HCl, pH 7.8 and subsequently separated into various cell types on sucrose gradients. One cell type, the petaloid coelomocyte, spontaneously undergoes a striking morphological transformation to a form exhibiting numerous, thin cytoplasmic projections (filopodia). Moreover, the transformation is reversible.

Ultrastructurally, the formation of the filopodia results from a progressive reorganization of actin-containing filaments into bundles that are radially oriented. The formation of the filament bundles is initiated at the cell's periphery and proceeds inward. Simultaneously, the cytoplasm in between the bundles is withdrawn, exposing finger-like filopodia. Ultimately, the filopodia can be extended by up to four times their original length.

Biochemically, actin is the most abundant protein in whole cell homogenates and is extractable in milligram quantities via acetone powders. An actomyosin complex may also be isolated from these cells and is presumed to be active in producing the various forms of motility observed.

Cellular morphogenesis and motility are two basic processes that most, if not all, eucaryotic cells undergo during their life histories. The development of anisometric cell shape by means of a slow contractile process (5, 22), through the explosive polymerization of monomers into polymers (28), or via amoeboid motion (25) has been firmly linked to the presence and action of microfilamentous systems.

In many cases, the microfilaments exist as discrete linear structures approx. 60–80 Å in diameter, and because they reversibly bind heavy meromyosin (HMM) from skeletal muscle, they have been called actinlike (12). The filaments are often seen in bundles or complex networks just beneath the cell membrane (10, 19, 23) and, in some instances, extending into cytoplasmic projections

either in conjunction with microtubules (27) or alone as bundles of microfilaments (28). In cells that have been virally transformed (17, 20, 31), the disappearance of microfilamentous stress fibers and the subsequent changes in cell shape and motility serve to emphasize the association of cell asymmetry with the existence of microfilament bundles in many cell types.

Knowledge of the formation of microfilament bundles is fundamental to understanding how cells change and ultimately maintain their shape in organisms ranging from protozoa to vertebrates. The study of the formation of filament bundles, while still incomplete, will be instrumental in dissecting the functions of these structures. It is the purpose of this paper to describe a unique system, echinoderm coelomocytes, in which the ultrastruc-

tural and biochemical aspects of filament bundle formation and concomitant filopodial formation and extension are examined.

MATERIALS AND METHODS

Collection of Cells

Strongylocentrotus droebachiensis were collected by the Supply Department of the Marine Biological Laboratory, Woods Hole, Massachusetts. The coelomic fluid was collected by cutting through the test of the animal with scissors. The coelomic fluid was then made 50 mM in both ethylene glycol-bis (β -aminoethyl)*N,N,N',N'*-tetraacetate (EGTA) and Tris-HCl, pH 7.8 and subsequently layered on top of a 0.8 M sucrose solution and centrifuged at 5,000 g for 5 min. Approx. 50% of the coelomic fluid cells collected at the interface of coelomic fluid and sucrose; they were purified petaloid and/or filopodial cells. All other cell types (see reference 13) were sedimented to the bottom of the tube. The collected cells were washed two times and resuspended in 0.5 M NaCl, 10 mM Tris-HCl, pH 7.8.

Light Microscopy

All light microscopy was performed with a Zeiss microscope equipped with Nomarski differential interference optics. Photographs (35 mm) were taken with a Nikon camera (Nikon, Inc., Instrument Div., Garden City, N. Y.) on Kodak Panatomic X film. Cine films (16 mm) were taken with a Sage time-lapse unit (Sage Instruments Div., Orion Research Inc., Cambridge, Mass.).

Transmission Electron Microscopy

Petaloid coelomocytes were collected as described above and fixed according to the procedure of Tilney et al. (28), embedded in Epon and thin-sectioned with a diamond knife. A Jeol 100 S electron microscope was used at an accelerating voltage of 80 kV.

Whole cells were negatively stained according to the method of Clarke et al. (6). In addition, cells were rinsed with 0.1% Triton X-100 (Calbiochem, San Diego, Calif.) after they had adhered to a polylysine-coated grid and before application of fixative and stain. This treatment left the intracellular morphology intact and greatly increased the obtainable resolution of such preparations.

The HMM decoration of filaments was accomplished by applying the HMM (1 mg/ml) after the attached cells had been lysed with detergent and before fixation and negative staining. Control experiments included rinsing the cells with 1 mM ATP after the HMM had been applied.

Scanning Electron Microscopy

Coelomic cells, unpurified and clotted, were allowed to settle on a polylysine-coated cover glass. The attached

cells were fixed in 2% glutaraldehyde, 50 mM cacodylate buffer, pH 7.0. Subsequently, the cells were dehydrated in acetone, critical-point dried, coated with gold, and viewed in a Jeol SM-35 scanning electron microscope.

Biochemistry

All chemicals were reagent grade. Whole cells (0.1 ml-packed vol) were homogenized in a glass homogenizer containing 1 ml of 0.1 M NaCl, 10 mM Tris-HCl, pH 7.0 with 50 μ l of 0.06% phenyl methyl sulfonyl fluoride (PMSF) (Calbiochem) added to inhibit serine protease activity (30). Samples of the homogenate were taken and dissolved in Fairbanks et al. sample buffer (8) and electrophoresed on 7.5% SDS-polyacrylamide gels according to standard procedures. The gels were stained with Fast green (Eastman Kodak Co., Rochester, N. Y.) and scanned at 650 nm on a Joyce-Loebl densitometer (Joyce, Loeb, and Co., Ltd., Gateshead-on-Tyne, England).

Acetone powders were made from 0.1 ml of cells by washing the coelomocytes with a graded series of cold acetone washes, ending with three changes of cold 100% acetone. These samples were then air dried. Extraction of the G-actin from the acetone powders was done according to the procedure of Spudich and Watt (24) except that, in the final stages, the polymerized filaments were not washed with high salt to remove ancillary proteins.

Crude actomyosin pellets were obtained from 0.1 ml of cells by the method of Pollard et al. (21).

Both actin and crude actomyosin pellets were dissolved in the Fairbanks et al. sample buffer and electrophoresed as described above.

RESULTS

The coelomic fluid of *Strongylocentrotus droebachiensis* contains a variety of cell types that form a cellular clot when collected outside of the organism. Prevention of clot formation is accomplished by making the freshly collected coelomic fluid 50 mM in both EGTA and Tris-HCl, pH 7.8. The coelomic fluid may be separated into its constituent cell types (see reference 13) on the basis of their intrinsic densities. Both petaloid and filopodial cells are obtainable in gram quantities.

Examination by light microscopy of the freshly collected and purified cells reveals a variable percentage (from 50% to 95%) in the petaloid form. Upon standing, however, all cells will become petaloid, i.e., filopodial cells will revert to the petaloid form, usually within 1 h. The reversibility of the transformation of the filopodial cells to petaloid is dependent on their remaining in suspension; they must not settle onto a substratum.

The petaloid coelomocytes display numerous cytoplasmic lobes radiating in all directions as they float freely in the coelomic fluid. Each cell is nucleated and averages 35 μm in diameter. Individual lobes appear, in optical section (Fig. 1), similar to the petals of a flower, hence the name petaloid. The arrangement and morphology of the petals is constantly changing and, when viewed with time-lapse cinemicrography at 20–30 times real time, the petals present a series of fluid, billowing undulations. The undulations appear to progress from the periphery of the cell back toward its center, but, unlike many migratory cells in culture, these cells undulate at all points on their circumference and do not move across the substratum.

The petals are, in essence, bladders of varying degrees of inflation. The walls of each bladder consist of two unit membranes separated by a variable thickness of cytoplasm and, in some cases, appear to open to the outside environment (Fig. 6).

The petaloid coelomocytes, when allowed to settle onto a glass slide, either remain as such and undulate, flatten to a thin, nonmotile disc shape, or undergo the morphological transformation depicted in Figs. 1–5. Those cells that undergo the transformation comprise approx. 10% of the population of cells that were petaloid when freshly collected. Why only a small percentage of cells transform is not yet fully understood. Those cells undergoing the transformation begin so by ceasing their undulatory activities and forming ridgelike thickenings in their cytoplasm (Fig. 2). These thickenings are interconnected by thin sheets of cytoplasm (Fig. 3) that are withdrawn gradually, leaving exposed the newly forming filopodia (Fig. 4). During the withdrawal, the individual filopodia are also lengthening by up to four times their original length to a maximum of approx. 40 μm . The outward extension phase of filopodial formation begins shortly after the thickenings become discrete structures at the light microscope level of examination, and are extended by an unknown mechanism. The resulting, fully transformed cell is highly filopodial with cytoplasmic strands connecting adjacent filopodia in a complex network. The filopodia are motile in that the cytoplasmic connections between them are repeatedly made and broken in a dynamic anastomosing network. These spontaneously transforming cells require up to 10 min to complete the process, although some

cells are fully transformed within 3–4 min at room temperature.

Ultrastructural Analysis

The ultrastructural counterparts to the light microscope observations were investigated with negatively stained, whole cells that had adhered to polylysine-coated electron microscope grids.

The plasma membranes of the whole cells were dissolved with the detergent Triton X-100 before glutaraldehyde fixation and negative staining, thus allowing the visualization of the arrangement of cytoplasmic linear elements. Both petaloid (Fig. 7) and filopodial (Fig. 9) forms of the coelomocyte can be studied at this level, as well as intermediate stages in the transformation process (Fig. 8).

In the petaloid stage, the individual petals are seen to contain linear elements which have a beaded substructure, are approx. 65 \AA in diameter, and are of indeterminate length (Fig. 10). They often occur in groups of two to several filaments. While many filaments are more or less radially oriented, many more are not (Figs. 7 and 10). Generally, there is no preferred filament orientation at this stage.

During the earliest stages of the transformation, these filaments are repositioned such that they have a definite radial orientation. The repositioning into bundles begins at the cell's periphery and progresses inward forming a branching network of filaments (Fig. 11). After the filaments are re-grouped into bundles, some of the bundles are bound together to form one larger unit (Fig. 8). Thus, filaments form small bundles and small bundles unite to form larger bundles. These newly formed bundles correspond to the ridgelike thickenings seen with the light microscope in Fig. 2. The formation of bundles continues into the perinuclear region (Fig. 9). When completed, the bundles will form the axial support for each filopodium. Each axial rod consists of a paracrystalline array of filaments with an average center-to-center spacing of 80 \AA (Fig. 12). The bundles taper to fewer filaments near their tips (Fig. 9). Each filament in the bundles reversibly binds HMM from rabbit skeletal muscle in the arrowhead array diagnostic of actin-containing filaments (Fig. 13). The filaments not yet arranged into bundles also bind HMM.

Scanning Electron Microscopy

Normally, in the sea urchin, if the appropriate stimulus is present, some or all of the coelomic

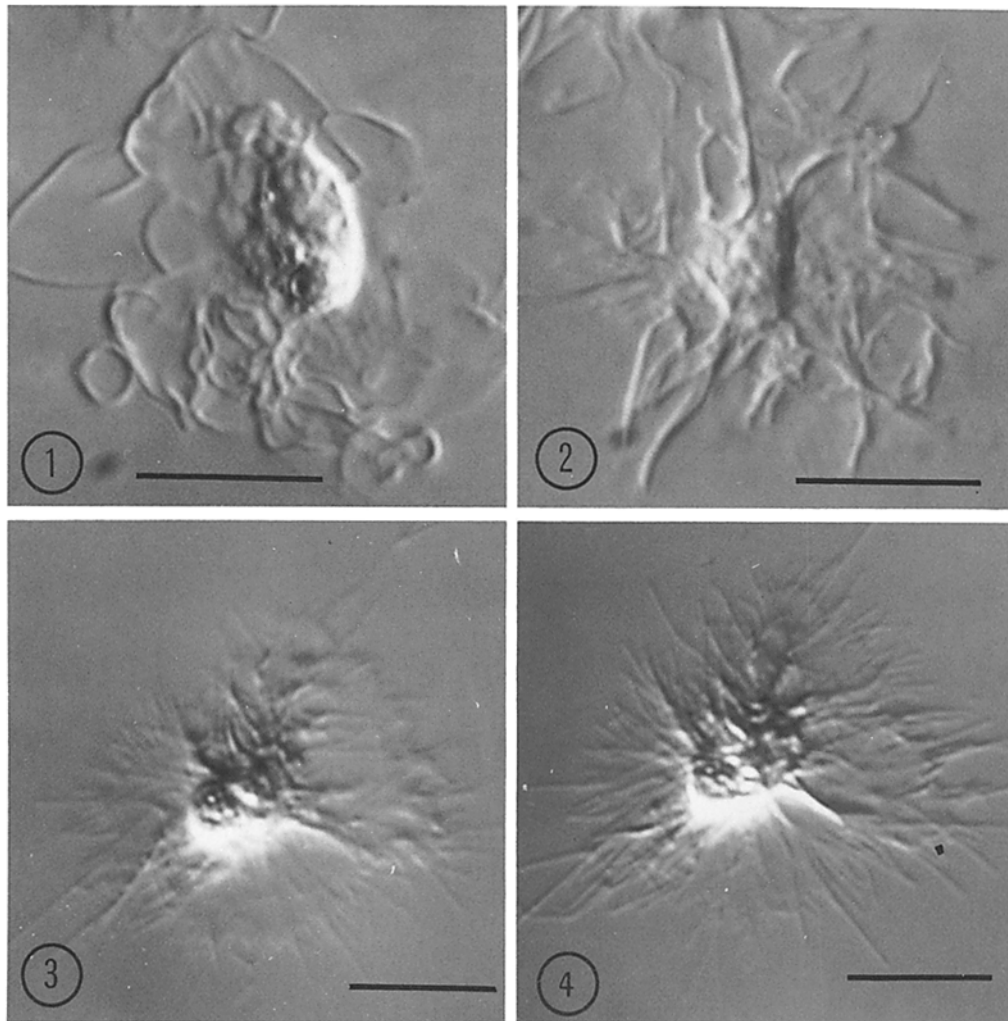


FIGURE 1 A typical petaloid coelomocyte seen in optical section. The central nucleus is surrounded radially by bladders (petals) that undulate slowly. Bar, $10\ \mu\text{m}$. $\times 2,500$.

FIGURE 2 A petaloid cell that has begun spontaneously to transform. The undulatory activity of the petals ceases, and ridgelike thickenings form in the cytoplasm. Bar, $10\ \mu\text{m}$. $\times 2,500$.

FIGURE 3 Cytoplasmic sheets in between the thickenings have begun to retract toward the cell body. Bar, $10\ \mu\text{m}$. $\times 1,900$.

FIGURE 4 Clearly distinguishable filopodia are apparent as the transformation continues, and the cytoplasmic sheets retract further. Note also that the individual filopodia are lengthening; compare cell diameter to that in Fig. 3. Bar, $10\ \mu\text{m}$. $\times 1,900$.

fluid will form a cellular clot as a lymphostatic mechanism. The role played by the filopodial coelomocyte in such a clot is illustrated in Fig. 14. The filopodia are extended radially in all directions, forming physical links with several of the neighboring cells. There is presumably an intercellular adhesive to hold the cells together and help

in maintaining the clot. The adhesiveness is divalent cation dependent, as EGTA abolishes its activity.

After the clot has formed, it begins to contract, presumably by shortening of the filopodia, resulting in the syneresis of coelomic fluid from the cellular mass.

Biochemical Components

The coelomocytes (0.1-ml packed vol) were dissolved in Fairbanks et al. sample buffer and electrophoresed according to standard procedures (see Materials and Methods). The whole cell homogenate gel is shown in Fig. 15 A. The most

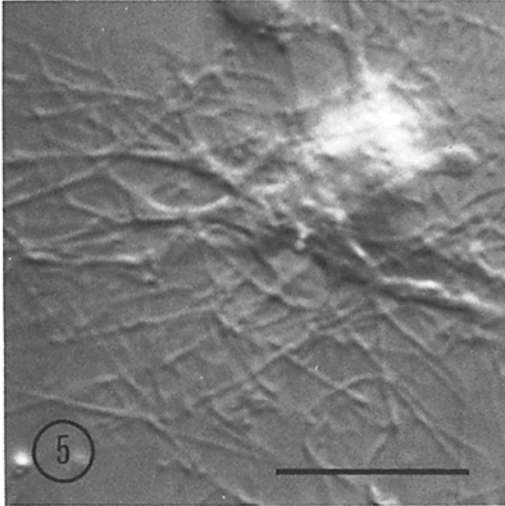


FIGURE 5 A fully transformed coelomocyte. The filopodia form a three-dimensional, anastomosing network of cytoplasmic strands. Individual filopodia may reach 40 μm into the surrounding fluid. Bar, 10 μm . $\times 2,500$.

abundant species of polypeptide comprises 14% of total coelomocyte protein, comigrates with actin in a skeletal muscle standard (Fig. 15 D), and has an apparent mol wt of 42,000 daltons. Furthermore, G-actin can be extracted from acetone powders of these cells. The G-actin, at 1 mg/ml, polymerizes under appropriate salt conditions and is sedimentable in a pure form essentially free of other proteins (Fig. 15 B).

In addition, a similar quantity of cells (0.1 ml) can yield, through standard procedures, a crude actomyosin pellet (Fig. 15 C). The coelomocyte myosin appears to have a heavy chain mol wt of 200,000 daltons, as it comigrates with the heavy chain from rabbit skeletal muscle myosin. No attempts were made to further purify the crude actomyosin.

DISCUSSION

In the echinoids (sea urchins), there is little contractile body wall that could aid in closing a wound in the animal's exoskeleton and, therefore, any wound sealing defense mechanism must rely on cellular aggregation to form a "plug."

The coelomic fluid of echinoderms contains a variety of cell types, and their coagulative properties were first described in 1880 (9). The specific coelomocytes described here have been called active phagocytic leukocytes (15), bladder amoeb-

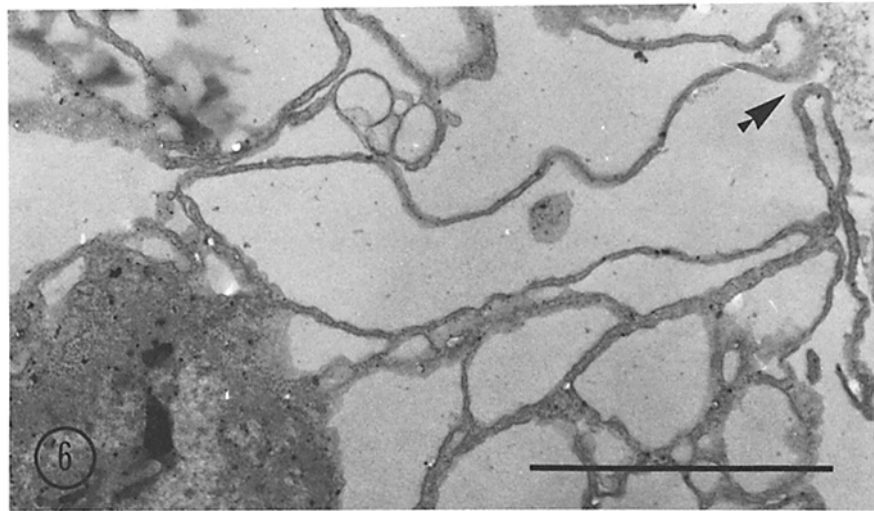


FIGURE 6 A petaloid coelomocyte in thin section. This image is the ultrastructural counterpart to Fig. 1. Note that the walls of each bladder are composed of two unit membranes separated by a variable thickness of cytoplasm. The considerable potential surface area in the walls of the individual compartments is available for use in forming the filopodia. The arrow indicates an apparent opening in a bladder to the outside fluid. Nucleus is in lower left corner. Bar, 5 μm . $\times 8,000$.

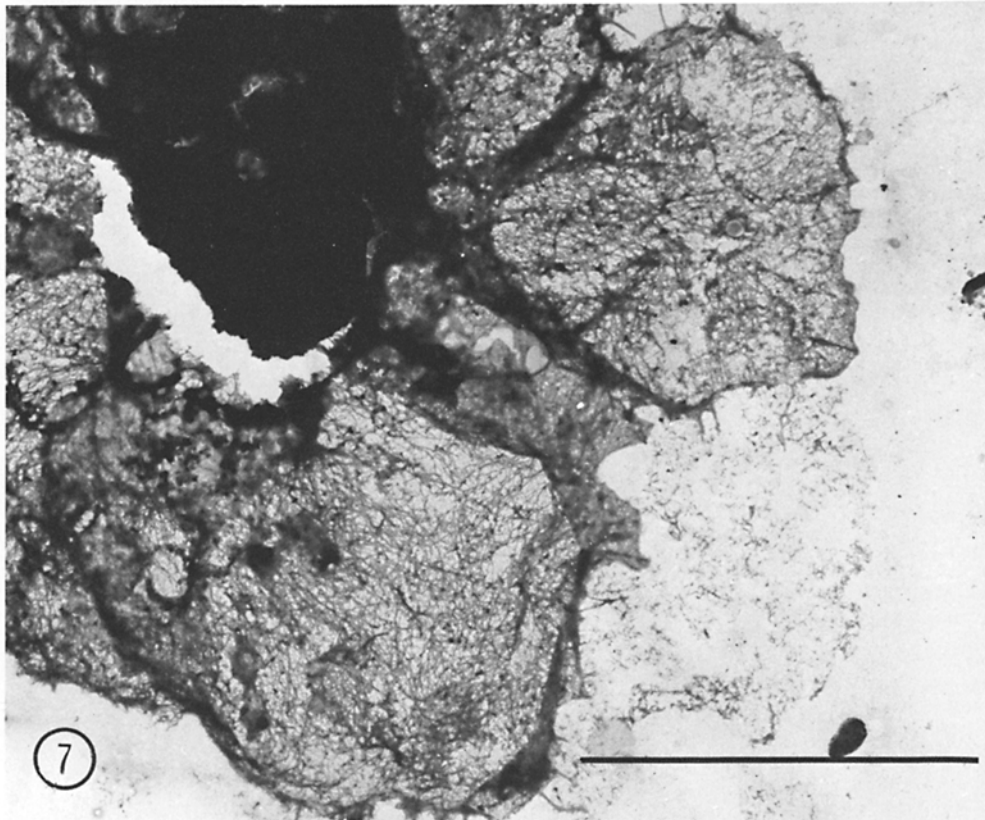


FIGURE 7 A petaloid coelomocyte that has adhered to a polylysine-coated EM grid, has been detergent treated, fixed, and negatively stained. The individual filaments are just discernible at this magnification. Bar, 10 μm , \times 5,200.

cytes (26), and petaloid coelomocytes (13), and all terms are accurate to differing degrees. However, they will be referred to herein as the petaloid coelomocytes.

The formation of an echinoderm coelomic fluid clot includes an initial rapid gelation of the fluid, extension of cytoplasmic projections (filopodia) by constituent cells, and a subsequent clot retraction via those same filopodia. The entire phenomenon is reminiscent of hemostasis in vertebrates, with the filopodial coelomocyte being analogous to the vertebrate platelet.

Individually, the petaloid coelomocytes display a fascinating, if not unique, form of motility. The motile activity commonly observed at the "front" of a migrating cell or cellular process (1) is similar to, but not identical with the movements displayed by the petaloid coelomocytes. The coelomocyte undulations progress from the edges of the cell, fold back on themselves, and appear to move

toward the cell's center. Unlike many migrating cells in culture, these cells undulate at all points on their periphery and do not traverse the substratum. The fact that the undulatory activity is present on all surfaces of the cell may not allow for any directed motion, but rather may be related to its known phagocytic capabilities (14). Whether the "folding back" and centripetal movements are optical illusions or real processes awaits further investigation.

The formation of the filopodia is a motile event that is not unique to these cells, but perhaps the reversibility and speed with which it happens (3–4 min) are worthy of note. The extension of the filopodia during their formation is similar to that occurring in neurite extension (4), and the similarity of phenomena suggests a common molecular mechanism.

The mechanism of transformation becomes clearer with the visualization of filaments forming

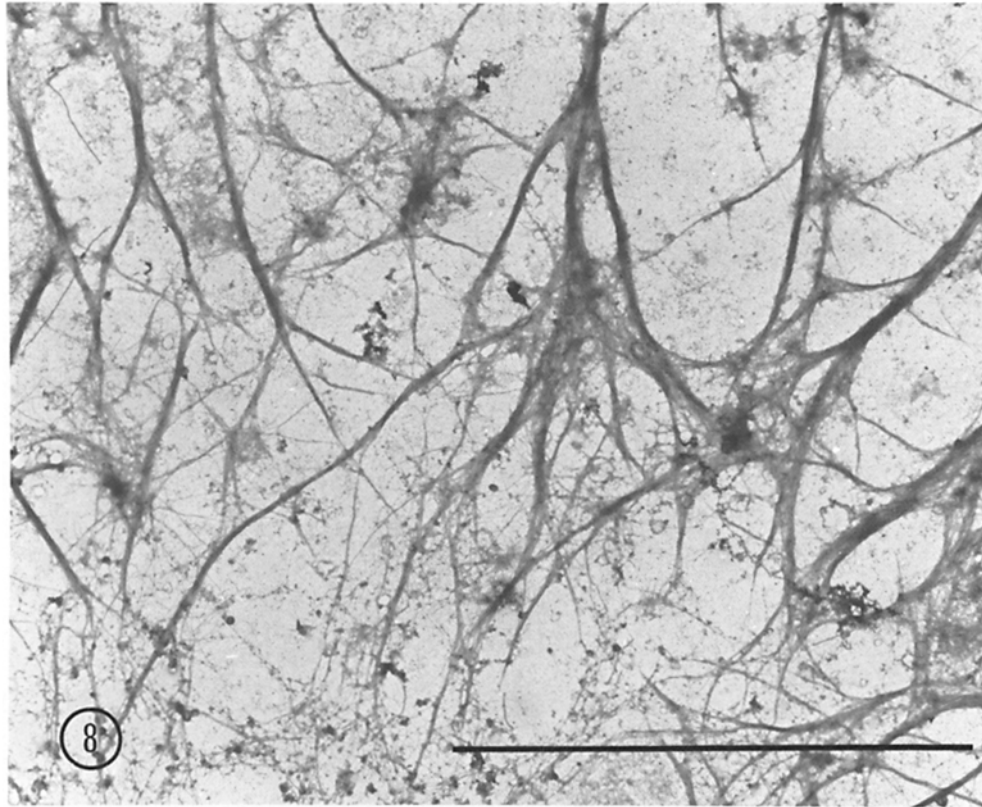


FIGURE 8 A low magnification view of a portion of partially transformed coelomocyte. The filament bundles in the peripheral regions of the cell (top of figure) are fusing to form still larger bundles, whereas in the innermost cytoplasm (bottom) some individual filaments are still apparent. A gradient of transformation exists, with the outermost regions being farthest along in the transformation. Bar, $10\ \mu\text{m}$. $\times 6,800$.

bundles at the ultrastructural level. However, an essential aspect of the transformation that remains obscure is the retraction of the interbundle cytoplasm. This restructuring of the cell's membranous surface occurs after the axial supports for each filopodium are partially formed and, in some cases, may be delayed considerably. This delay appears to be related to the cell's being in contact with a substrate. The retracting cytoplasm contains filaments that are presumed to be either incorporated into the bundle, involved with the retraction of the webbing, or both (Fig. 9). The origin of the filopodial limiting membrane may be the shrinking petals. Considering that each petal contains more membranous surfaces than just its outer "shell" (Fig. 6), the internal, membrane-limited compartments may provide enough surface to cover all filopodia. Alternatively, other sources may be utilized as well.

Equally intriguing is the formation of the bundle from existing filaments. It is unknown how the filaments are regrouped. One candidate for a mechanism of gathering the filaments into bundles involves the existence of postulated cytoplasmic myosin filaments at specific points that act as "fingers" that gather, through a cyclic action of the cross-bridges, the membrane-associated actin-containing filaments to those points. As the more peripheral segments of the bundles are completed, the myosin would move centripetally gathering those filaments into the bundle. Once the bundle is formed, there is presumably a "glue" that helps to maintain the structure and hold the filaments together, and, in this instance, may be similar to the 55,000 dalton protein (29) shown recently to aid in filament packing in sperm acrosomal processes and artificially induced sea urchin egg extracts (7).

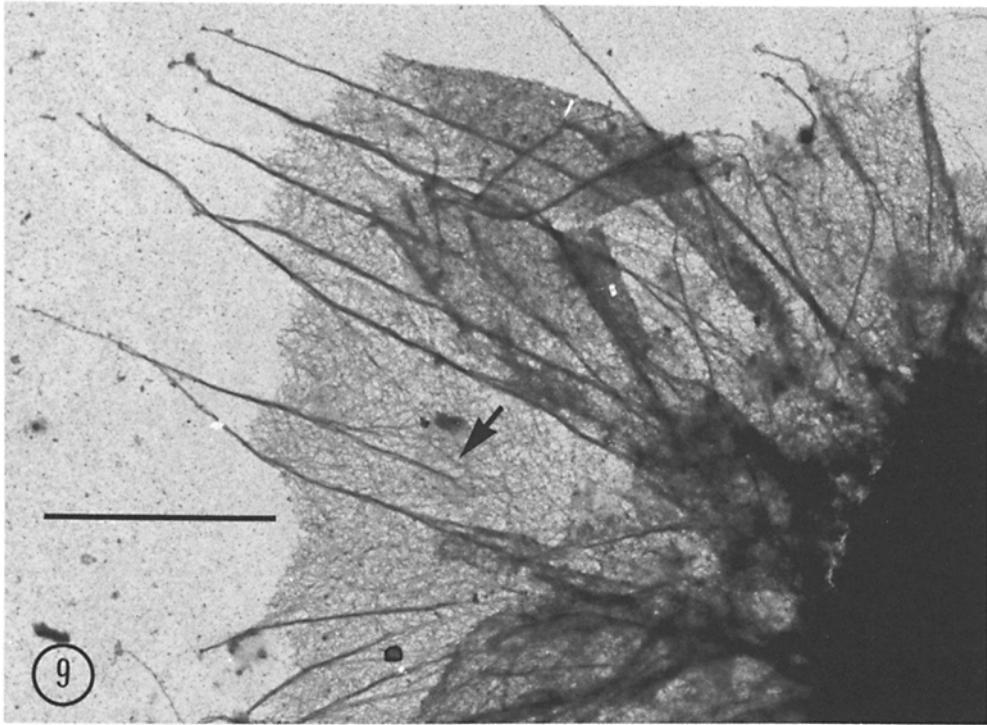


FIGURE 9 A negatively stained, transformed coelomocyte. Note the numerous filament bundles, some of which terminate at the time of fixation, in a relatively peripheral area of the cytoplasm (arrow). The cytoplasmic webbing in between filament bundles was still retracting when this cell was prepared. Individual filaments are still present in the retracting cytoplasm. Bar, $5 \mu\text{m}$. $\times 6,000$.

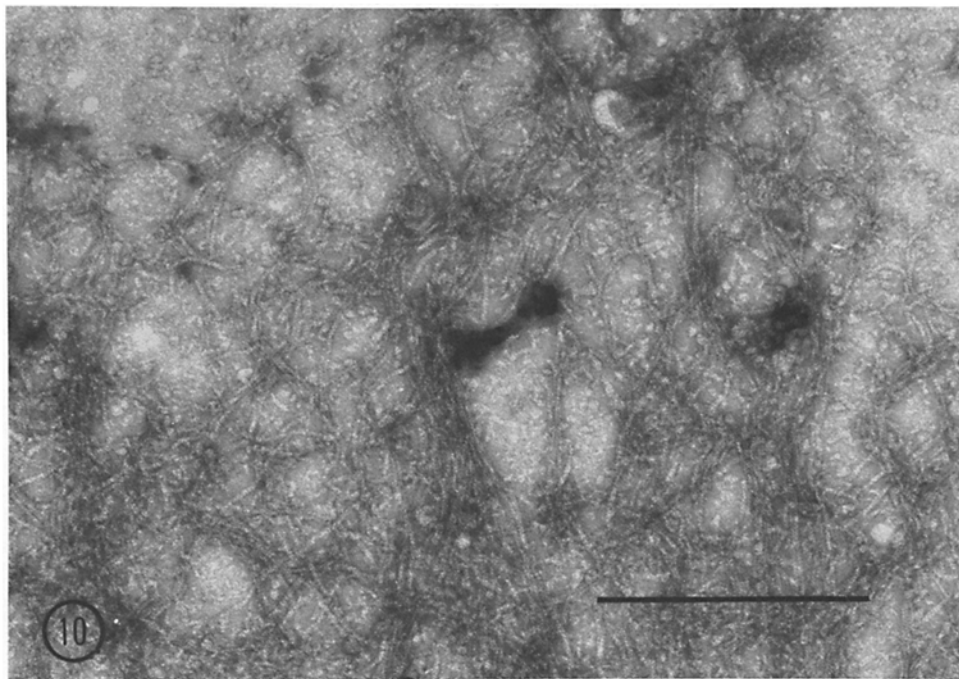


FIGURE 10 Higher magnification of the edge of a petal. Top of figure is outermost. Note the apparent random and loose arrangement of the filaments. Bar, $0.5 \mu\text{m}$. $\times 72,000$.

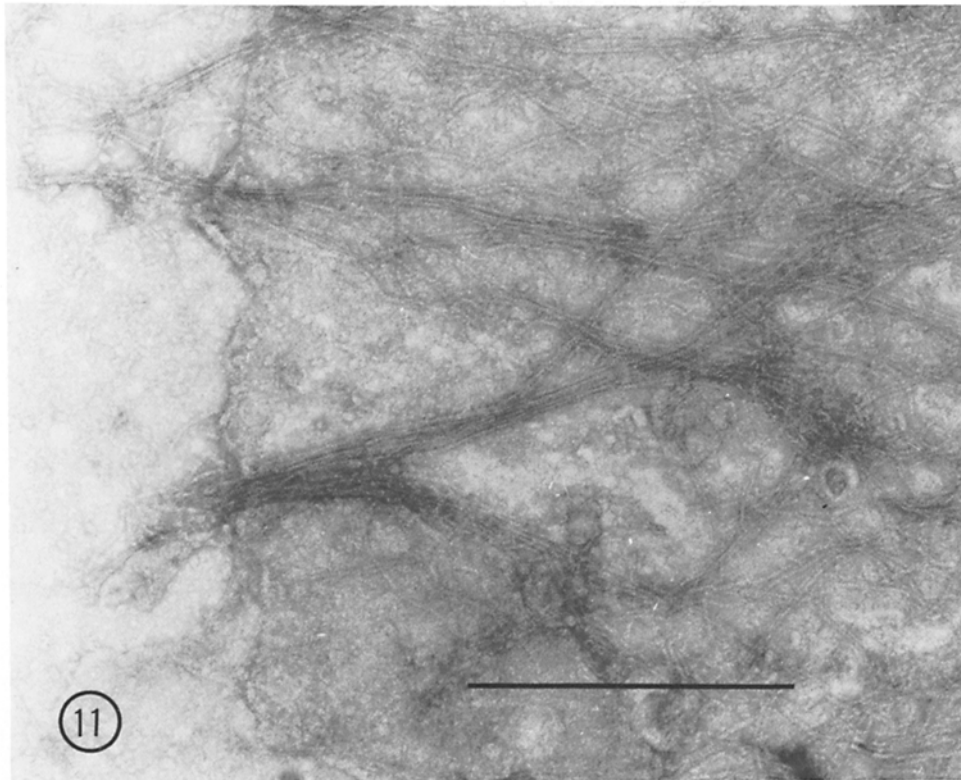


FIGURE 11 The filaments begin to coalesce at points on the periphery of the cell, forming a radially oriented network of filaments branching out perpendicular to the membrane. The filaments are progressively organized into bundles as the transformation to the filopodial form continues. The filament bundles ultimately form the axial support for each filopodium. Bar, $0.5 \mu\text{m}$. $\times 72,000$.

Assuming that an actomyosin system is involved in clot retraction, it may be necessary to first assemble and position the contractile machinery involved with motive force production, i.e. re-group the filaments into bundles and form the filopodia before clot retraction can occur. Thus, the transformation may be viewed as a necessary adjunct to performance of a function. Similarly, in the case of *Thyone* sperm, the acrosomal process, in essence a filopodium, must be extended before its function can be consummated. The extension of the *Thyone* acrosome results from the explosive polymerization of monomeric actin into filament bundles (28).

It is uncertain, as yet, how widespread these two differing methods of cytoplasmic extension are, but it is probable that they are universal, with many cells utilizing both the restructuring of existing filaments and the polymerization method.

The initiation of filament bundle formation in the peripheral regions of the coelomocyte is simi-

lar to that occurring in fibroblasts as recently reported by Bragina et al. (3). Those authors postulate that there is something unique about the newly formed peripheral cytoplasm in spreading fibroblasts that allows for initiation of microfilamentous bundles. Such uniqueness may involve the flux of ions (possibly Ca ions) across the membrane at specific points as part of the initiation process.

Biochemically, as well as ultrastructurally, there is evidence for large quantities of actin in the coelomocytes. The actin can be extracted in the G form via an acetone powder and polymerized under conditions that are identical for skeletal muscle actin polymerization. Furthermore, the actin can be extracted with a myosin-like molecule bound to it, and both are sedimented as a complex. The myosin-like protein comigrates, on SDS-polyacrylamide gels, with the heavy chain portion of myosin from rabbit skeletal muscle and therefore has a mol wt of 200,000 daltons. The

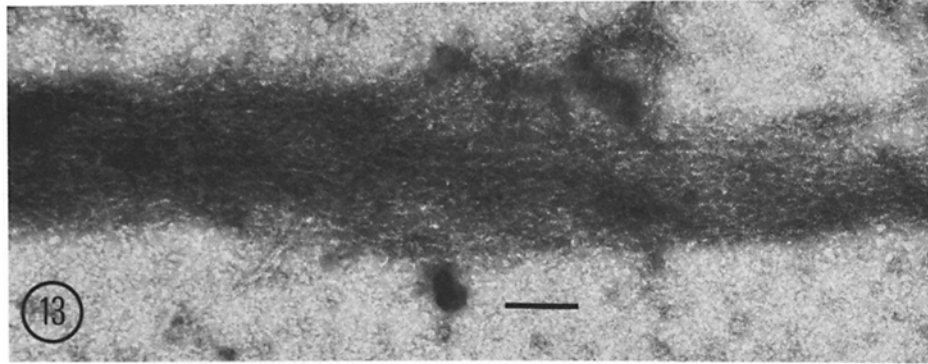
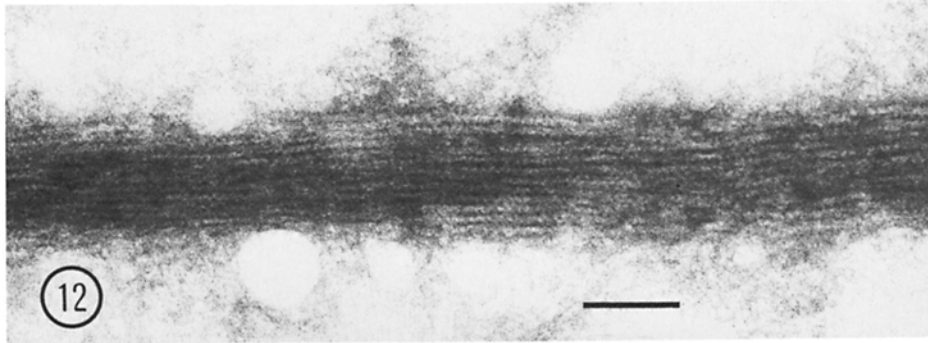


FIGURE 12 Higher magnification of a filament bundle formed by the inwardly directed reorganization of actin-containing filaments into tightly packed groups. Note the beaded substructure of some of the filaments as well as the paracrystalline packing of many filaments. Bar, $0.1 \mu\text{m}$. $\times 124,000$.

FIGURE 13 A disrupted filament bundle decorated with HMM in the distinctive arrowhead array that is diagnostic of actin-containing filaments. Bar, $0.1 \mu\text{m}$. $\times 90,000$.

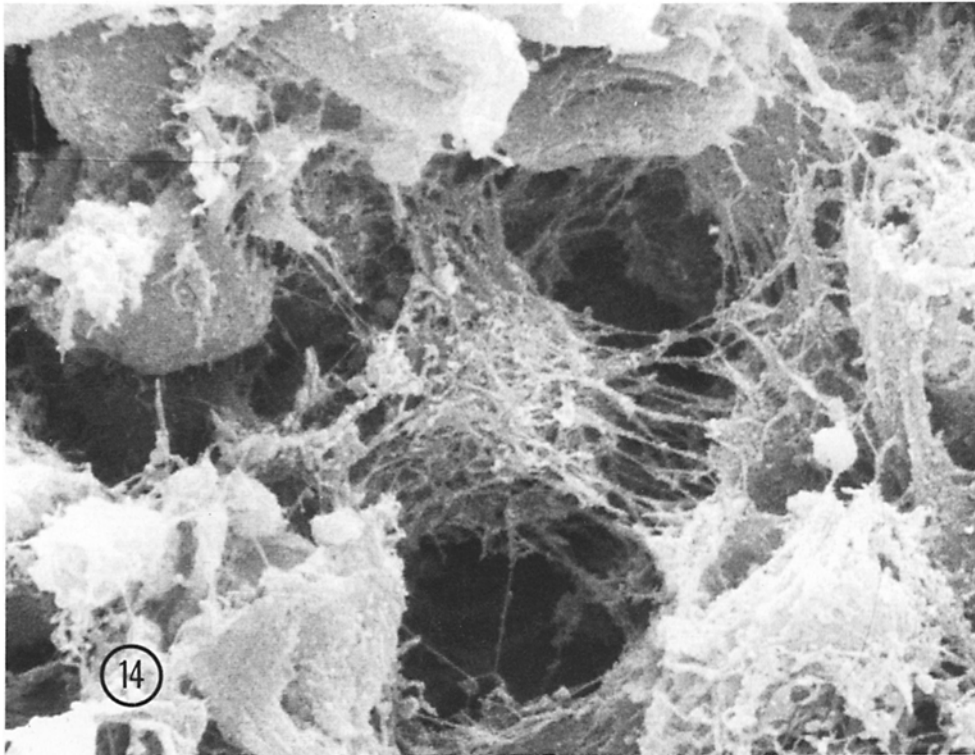


FIGURE 14 The filopodial form of the coelomocyte is essential for coelomic fluid clot formation and subsequent clot retraction. The filopodia serve to link neighboring cells in the clot and play a major role in clot retraction. $\times 5,000$.

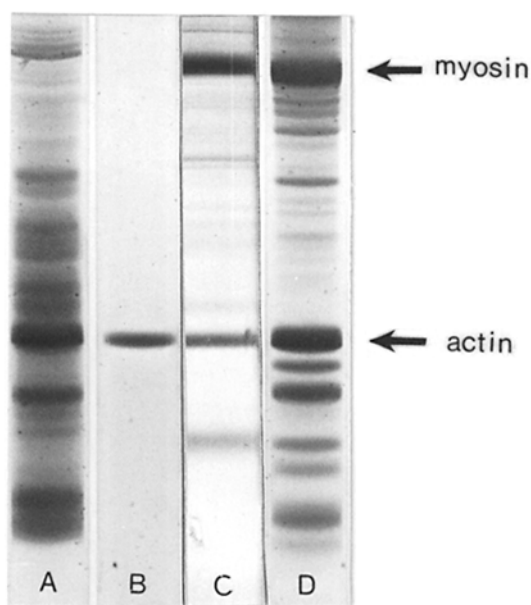


FIGURE 15 SDS-polyacrylamide gels (7.5%) stained with fast green of: (A) whole cell homogenate; (B) coelomocyte actin extracted from an acetone powder; (C) a crude actomyosin pellet prepared from petaloid coelomocytes; and (D) rabbit skeletal muscle standard for molecular weight comparison, actin mol wt = 42,000 daltons, myosin molecular weight (heavy chain) = 200,000 daltons. Gels A, B, and D were run at the same time, gel C at a different time.

apparent molecular weight of the myosin-like protein as well as its ability to complex with coelomocyte actin in the absence of Mg-ATP strongly suggests that an actomyosin complex is present in these cells and almost certainly is involved in the various forms of motility that these cells exhibit. The localization and characterization of the proposed myosin is being investigated.

One might expect, in light of hypotheses currently in vogue, that proteins or glycoproteins may be present in this system that allow for the attachment of the actin-containing filaments to the inner side of the plasma membrane. Spectrinlike proteins have been found in some primitive motile systems and are known to complex with actin, possibly in such a manner as to allow the storage of that actin until it is needed for cellular events, e.g., contractile ring formation. In addition, spectrin or spectrinlike proteins have been postulated to function in providing an elastic component to cell membranes (16). In fact, based on comigration criteria, there is a spectrinlike protein in this

system as well. The functioning of that spectrinlike molecule could range from attaching actin to the inner plasma membrane surface to providing an elastic but rigid component to the membrane that forms the limiting surface of each filopodium. Furthermore, other high molecular weight proteins are present in the whole cell homogenate (Fig. 15 A) that may be related, at least in molecular weight and perhaps function, to the actin-binding protein found in macrophages (11) and to alpha actinin which, in addition to being present in skeletal muscle, has been shown to be present in microvilli (18).

A most amazing aspect of this cellular transformation is its reversibility. In surveying the differing types of echinoderm coelomic fluid clot formations, Boolootian and Giese (2) noted that some cell types can reverse position and migrate from the clot once it is formed. It is unknown whether those migrating cells are similar to those described here. Regardless, the reversibility of these cells implies at least a potential reutilization of cells for similar or varied purposes. While the mechanisms and controlling factors involved with this transformation are currently unknown, a subsequent report will deal with the induction of transformation.

In summary, the petaloid coelomocytes can reversibly transform to a filopodial form by reorganization of randomly arrayed actin-containing filaments into filament bundles of paracrystalline nature. These bundles are initiated at foci on the cell's periphery and their formation progresses inward perpendicular to the membrane. The bundle forms the axial support for a filopodium. Each filopodium is membrane limited, with the membrane source presumed to be the shrinking petals. In addition, an actomyosin complex is present that plays a major role in the retraction of the whole coelomic fluid clot.

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