THE DISTRIBUTION, ULTRASTRUCTURE, AND CHEMISTRY OF MICROFILAMENTS IN CULTURED CHICK EMBRYO FIBROBLASTS

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ABSTRACT

The distribution, ultrastructure, and chemistry of microfilaments in cultured chick embryo fibroblasts were studied by thin sectioning of flat-embedded untreated and glycerol-extracted cells, histochemical and immunological electron microscopic procedures, and the negative staining of cells cultured on electron microscopic grids. In these cultured cells, the microfilaments are arranged into thick bundles that are disposed longitudinally and in looser arrangements in the fusiform-shaped cells. In the latter case, they are concentrated along the margins of the flattened cell, on the dorsal surface, and particularly at the ends of the cell and its ventral surface, where contact is made with the plastic dish or with other cells. Extracellular filaments, presumably originating from within the cell, are found at these points of contact. The microfilaments are composed in part of an actin-like protein. These filaments are between 70 and 90 Å in diameter, they are stable in 50% glycerol, they have an endogenous ATPase (myosin-like?) associated with them, they bind rabbit muscle heavy meromyosin, and they specifically bind antibody directed against isolated actin-like protein. In the cultured chick embryo fibroblasts, the microfilaments are essential for the establishment and maintenance of form, and they are probably critical elements for adhesion and motility. The microfilaments might also serve as stabilizers of intramembranous particle fluidity.

INTRODUCTION

Much study is currently directed toward understanding the function and chemistry of cytoplasmic microfilaments. These cytoplasmic elements, reported to be 40-80 Å in diameter, are found in a wide variety of cells—from the motile amoebae (1-6) to the endothelial absorptive cells of the intestine, where they form the core of the microvilli (7). The presence of microfilaments has been correlated with numerous functions: inter- and intracellular movements (2, 5, 8), cytokinesis (9-12), the establishment and maintenance of form (7, 13-15), etc. Treatment of cells and tissues with cytochalasin B, a mold metabolite, results in a disruption of the microfilaments concomitant with the alteration of many cellular functions, such as endocytosis (16), motility (17), morphogenic development (15), and metabolite transport (18-20).

Proteins similar in chemical properties and morphology to skeletal muscle actin and actomyosin have been isolated or identified in cells that manifested contractile or shape-changing properties and contained cytoplasmic microfilaments—amoebae (21-23), slime molds (24-29), blood platelets (30), leukocytes (31, 32), brain (33), and the brush border of chicken intestine (34).
Recently, Yang and Perdue isolated an actin-like protein from cultured chick embryo fibroblasts (35). This protein was electrophoretically homogeneous in two acrylamide-gel systems—sodium dodecyl sulfate and urea. It was indistinguishable from rabbit skeletal muscle actin in its electrophoretic mobility, morphology, and combining properties with myosin. Circumstantial evidence indicates that the cytoplasmic microfilaments are the actin-like proteins.

A study was initiated to describe the chemistry, ultrastructure, and distribution of microfilaments in the cultured fibroblasts. These cells contain many microfilaments. They also round up when incubated in the presence of cytochalasin B (36) or when transformed by avian oncogenic RNA viruses (37). In both cases, the changes in cell shape are correlated with a decrease in the number and distribution of cytoplasmic microfilaments. By means of the techniques of histochemistry, immunology, and negative staining of whole cell mounts, it is concluded that the microfilament system is made up, in part, of an actin-like protein which has ATPase activity (myosin?). The microfilaments serve as the fibroblasts' principal structural elements. They are concentrated at the margin and tip of the cells and in membrane extensions (e.g., pseudopodia). These filaments appear to pass through the plasma membrane and to interact with the substrate. The externalization of filaments may provide points of attachment of the cell to the substrate and thereby aid in intercellular movements.

**MATERIALS AND METHODS**

**Culture Conditions**

Primary cultures of chick fibroblasts were prepared from 12-day-old chicken embryos and cultured by previously described methods (37).

**Culturing Cells for Electron Microscopy**

Chick embryo fibroblasts which were to be examined as thin sections with the electron microscope were grown on glass slides that had been cleaned, carbon-coated, and “stabilized” according to the procedures of Robbins and Gonatas (38). In certain experiments, the cells were grown on slides that had a carbon-“stabilized” coat and a layer of vacuum-evaporated palladium (39). The cells were in the log phase of growth when they were taken for electron microscopy.

**Extraction of Chick Fibroblasts with Glycerol**

Chick embryo fibroblasts were extracted with glycerol in a manner similar to that employed by Hoffmann-Berling (40) for fibroblasts and Simard-Duquesne and Couillard (41) for amebae. The glycerol-extracted cells were prepared for electron microscopy by overnight washing with 0.25 M sucrose (pH 7.5) or 0.01 M cacodylate (pH 7.5).

**Electron Microscopic Localization of ATPase Activity**

Chick fibroblasts grown on carbon-coated slides and, in some instances, extracted with 50% glycerol-0.01 M cacodylate were fixed at 0–4°C with 1% hydroxyadipaldehyde-0.01 M cacodylate (pH 7.5) containing sucrose to 250 mosM (42, 43). After fixation for 30 min, the cells were rinsed with 0.25 M sucrose (pH 7.5) and incubated at 38°C for 60 min in 6 ml of a phosphate-trapping system (44), containing 3 mM MgSO4, 3 mM adenosine triphosphate (ATP), 20 mM Bicine (N,N-bis[2-hydroxyethyl]glycine) buffer (pH 7.8), 1 mM Pb(N03)2, and sucrose to a final milliosmolar concentration of 250. Controls were similarly incubated with or without lead or ATP.

**Binding of Heavy Meromyosin**

Glycerinated fibroblasts were incubated with rabbit muscle heavy meromyosin (45) for 8 h at 4°C according to the procedure of Ishikawa et al. (46).

**Preparation of Immune Serum Against Fibroblast Actin-Like Protein and the Localization of Antigenic Activity**

Homogeneous fibroblast actin-like protein (35), approximately 1 mg, was injected, together with adjuvant, into a hybrid W/Fu × BN rat. At 2-wk intervals, the animal was given booster injections. At 6 wk, the animal was bled and serum prepared and frozen. Antiserum was also made against rabbit muscle actin. I am indebted to Dr. Robert Nowinski for the preparation of these sera. The titer of antibody directed against the fibroblast and rabbit muscle proteins was too low to give precipitin bands in the agar gel diffusion test. This failure to promote a major antibody response is probably due to the ubiquitous presence of similar proteins in all cells. A similar inability to produce high titers of antibody against chicken and fish skeletal muscle actins was observed by Wilson and Finck (47).

Antibodies against actin-like proteins were present in the serum, as evidenced by electron microscopy. Whole serum or a 40% ammonium sulfate-precipi-
tated fraction (gamma globulins) was placed on carbon-coated slides containing intact or glycerinated cells and incubated at 24°C for 30 min. The slides were washed three times with isotonic Tris, pH 7.4, and a drop of hybrid antibody containing sites directed against rat gamma globulin and ferritin was placed on the slide (48). After a 30-min incubation at 24°C, the slides were washed with isotonic Tris, and ferritin at a concentration of 0.5 mg/ml in culture medium was placed on the slides. Unbound ferritin was removed by washing. Controls with and without antiserum and hybrid antibody were also prepared.

Procedure for Flat Embedding

Chick fibroblasts grown on carbon-coated slides and, in some experiments, extracted with glycerol and/or incubated with Mg-ATP and lead, heavy meromyosin, or antisera, were fixed for 1 h at 0-4°C in 1% glutaraldehyde-0.05 M cacodylate (pH 7.4) and sucrose to a final milliosmolar concentration of 250 (42, 43). The fibroblasts were "postfixed" in 1% osmium-0.05 M cacodylate and sucrose to 250 milliosmolar concentration for 1 h at 0-4°C. The specimens were dehydrated at 0-4°C in ethanol and the microscope slide with adhering cells drained and infiltrated for 2 h with an epoxy medium consisting of EPON 812, dodecylsuccinic anhydride, nadic methyl anhydride, 2,4,6-tri(dimethylaminomethyl)-phenol, and dibutyl phthalate in the ratio of 50:25:25:4:1 (49). The slide was drained, and BEEM capsules containing embedding medium were inverted over representative cells. The medium was polymerized by incubation at 60°C. The polymerized block could usually be removed from the glass slide by pulling, but in some instances cooling with liquid nitrogen had to be employed. The flat embedded specimen was sectioned on a Sorvall MT-2 Porter-Blum ultramicrotome with a diamond knife and poststained with lead citrate (50). Vertical sections through the specimen and supporting film were obtained by reembedding the flat embedded specimen 90° from its original orientation.

Negative Staining of Whole Cells

Chick fibroblasts grown on nickel or titanium grids covered with a carbon-supported collodion film and, in some experiments, extracted with 50% glycerol were fixed for 30 min at 0-4°C in 1% glutaraldehyde containing sucrose and cacodylate buffer (pH 7.4) and rinsed in 0.01 M cacodylate. A drop of 1% phosphotungstic acid (pH 7.2) containing ferritin as a calibration marker was placed on the grid and maintained at 0-4°C for 2 min. The excess stain was removed with filter paper and the grid allowed to air dry.

Electron Microscopy of Fibroblast Specimens

The negatively stained and sectioned material was examined with a Hitachi HU 11-C electron microscope at an accelerating voltage of 75 or 100 KV and at an instrument magnification of between 2000 and 60,000. Images were recorded on Kodak Medium projector slide or Electron Image plates and developed in Kodak D-19 or HRP.

Materials

Fertilized chicken eggs were supplied by Sunny-side Hatchery, Oregon, Wis. The modified minimal essential medium (Eagle's) was supplied by Schwarz-Mann (Orangeburg, N. Y.) and the calf serum from Grand Island Biological Co. (Grand Island, N. Y.). The substrate adenosine-5'-triphosphate was purchased from Sigma Company as the sodium salt, the fixative 2-hydroxyadipaldehyde from Poly-science, Inc. (Rydel, Penn.), and ferritin (6 X crystallized) from Miles Laboratories, Inc. (Kankakee, Ill.).

RESULTS

The Morphology of Cells in Thin Sections

The morphology of the cultured chick embryo fibroblasts has been described by other investigators (51, 52). However, these observations were related to ultrastructural changes associated with viral infections. Cultured fibroblasts were flattened and fusiform in shape with a centrally positioned nucleus (Figs. 1 and 4 A). The cytoplasm contained numerous ribosomes, as large free polysomes or attached to a moderately developed endoplasmic reticulum (Fig. 4 B). Mitochondria and vacuoles were numerous in most of these cells (Fig. 1). Microfilaments, approximately 60–100 Å in diameter, were found in large numbers in the cultured fibroblasts; a description of the localization, ultrastructure, and chemical properties of these filaments was the objective of this study.

The Distribution of Microfilaments

Microfilaments coursed through the cytoplasm either as individual filaments (Fig. 5) or as filaments running parallel to one another and aggregated into fibrils or bundles (Figs. 1, 2, and 7). They were particularly abundant along the mar-
gins and the dorsal and ventral surfaces of the fibroblasts, where they lay just within the plasma membrane and were oriented in the longitudinal plane of the cell (Figs. 1, 4, 7, and 8).

The microfilaments were not oriented about the nucleus or golgi, as has been reported by DePetris et al. (53), Biberfeld et al. (54), and Franke (55), but they were frequently seen close to mitochondria, and in some instances they appeared to interact with those organelles.

Microfilaments were concentrated at the ends of the fusiform-shaped cells (Figs. 1, 2, 3, 4, 7, and 8) and passed into extensions of the plasma membrane, designated as pseudopodia (Figs. 2 and 3), where they probably serve as the principal element employed in maintaining the structural rigidity of these membranes' processes. Microtubules were not observed in these processes. The pseudopodia are 100-400 μm in diameter and fall within the range of membrane extensions designated as microspikes (36).

The microfilaments interact with the plasma membrane, and in some instances they appeared to have passed through the surface membranes at the ends and from the margins in the regions of the pointed ends of the cells (Figs. 1, 3, and 4). These extracellular filaments had a granular appearance, they were not as straight as intracellular filaments, they had a variable diameter of 65-100 Å (Fig. 3), and they interacted with the supporting layer (Figs. 3 and 4) or were found between and overlying the cultured fibroblasts.

Motility in fibroblasts is associated with membrane undulations at the leading edge and the ventral surface of the cells. The latter undulations involve the intermittent contact of the membrane with the supporting surface (57). The extracellular microfilaments observed in vertical section (Fig. 4 A and B) appeared to have their origin in the ends of the cells and lie between them and the carbon film (arrows). The intermittent contacts seen along the lower surface of the cell (Fig 4 C and D) contained a concentration of microfilaments.

The Diameter of Microfilaments

The diameter of the microfilaments in the cultured fibroblasts varied between 60 and 100 Å, with the majority having a diameter of 70-90 Å. These values are in agreement with the observations of Biberfeld et al. (54), Buckley and Porter (58), and Franks et al. (59). Filaments of a diameter of 20-30 Å, as described by Keyserlingk (60) in chick heart fibroblasts, were not observed. The presence of only one apparent type of filament is inconsistent with the contractile models proposed for striated (61) and smooth muscle (62). In this latter case, it has been demonstrated that two types of filaments exist. Kelly and Rice (62) described a thick filament among the 50-80 Å actin filaments in the smooth muscle of chicken gizzard. These authors found it necessary to fix the specimen below pH 6.6 in order to preserve what is believed to be the myosin filaments. Chick embryo fibroblasts were similarly fixed at pH 6.4 and examined for the presence of filaments with a diameter greater than 100 Å. Unlike the results of Kelly and Rice (62) for muscle, cultured fibroblasts contained only one morphological type of filament. Its diameter of 70-90 Å (Fig. 5) was identical with filaments fixed at pH 7.4.

Histochemical Localization of ATPase Activity in Intact Fibroblasts

It had been reported by Hoffman-Berling (63) that a contractile-like protein could be extracted...
The leading ends of cultured fibroblasts have numerous membrane processes, 0.1–0.4 \( \mu \text{m} \) in diameter. Microfilaments in the region of the processes and at the cell margins exclude cytoplasmic components such as ribosomes (R), endoplasmic reticulum, mitochondria, etc. The microfilaments were the only cytoplasmic elements seen within the membrane processes. Filaments were frequently observed outside of the cell. The external filaments in Fig. 3 are in contact with the carbon-coated (C) substratum. Fig. 2, \( \times 22,500 \), Fig. 3, \( \times 30,000 \).

from cultured sarcomas which has conformation-changing ability and ATPase activity. To determine whether one of the enzymatic components of the microfilaments might be an ATPase, chick embryo fibroblasts grown on carbon-coated slides were incubated in the lead-phosphate-trapping system of Wachstein and Meisel (44) with Mg\(^{++}\) or Ca\(^{++}\) and ATP buffered at pH 7.8. The use of intact fibroblasts precluded the entrance of substrate into the cells; thus only enzyme sites on or external to the plasma membrane should show reaction products. A lead-phosphate precipitate that was an indication of enzyme activity was observed occasionally on the surface membranes, but most of the reaction product was associated with extracellular filamentous material (Figs. 7, 8, and 9). This precipitate was found most often at the ends of the cells and at the margins of these ends; filaments with precipitate were also observed along the sides of the fibroblasts. The microfilaments pictured in Figs. 7 and 9 appeared to pass through the plasma membrane and to emerge as filaments that had lead-phosphate adsorbed on them. One of the extracellular filaments (insert, Fig. 9) was approximately 75 \( \AA \) in diameter and had precipitate adsorbed on it with periodic spacings of about 400 \( \AA \).

The presence of precipitate on the extracellular
filaments could possibly be explained by postulating that sites on the filaments preferentially adsorbed granules of lead-phosphate that had been produced by enzymatic hydrolysis of ATP by components of the plasma membrane. To test this possibility, a control series of incubations was run by fixing the cultured fibroblasts with glutaraldehyde and osmium, followed by extensive washing with isotonic sucrose and (a) incubating the cells for 1 h with substrate, Ca$^{++}$ or Mg$^{++}$, and lead, or (b) incubating as in the first series, but with the addition of a very active ATPase.
FIGURE 5  Chick embryo fibroblasts that had been fixed at pH 6.4 had microfilaments with a diameter of between 70 and 90 Å. Thick filaments of a myosin type were not observed in this preparation. × 80,000.

FIGURE 6  Intracellular microfilaments were preserved in fibroblasts extracted in 50% glycerol. In the presence of Ca++ or Mg++ and ATP, the filaments appeared less aggregated, and there was some indication of a globular or subunit composition. In two of these filaments, there was a suggestion of a helix (arrowheads) with a partial strand separation (open arrow). × 120,000.
As evidenced by the deposition of lead phosphate, extracellular filamentous material had an ATPase activity associated with it. The extracellular filaments were frequently in line with intracellular bundles (B). In Fig. 9, microfilaments interacted with the tangentially sectioned plasma membrane. Extracellular filaments emanated from within this same region of the plasma membrane. The extracellular filament (inset, Fig. 9) was 75 Å in diameter and contained granules of lead phosphate that were spaced about every 400 Å. Fig. 7, X 13,500, Fig. 8, X 10,000, Fig. 9, X 55,000.

preparation of sonicated rabbit heart mitochondria. Under the two conditions, no precipitate was adsorbed to the extracellular filaments.

**Histochemical Localization of Phosphatase Activity in Glycerol-Extracted Fibroblasts**

The "muscle model" system developed by Szent-Gyorgyi (64) for studying contraction in 50% glycerol-extracted muscle has been employed in studies of amoebae (65), slime molds (66, 67), and fibroblasts (40, 60). These latter cells, when extracted in the same manner, were observed to contract when incubated in the presence of ATP.

Cultures of chick embryo fibroblasts were extracted in 50% glycerol for periods of up to 2 months. The glycerol was washed from the cells, and they were incubated in one of three isotonic media containing (a) Ca++ or Mg++ and lead, but no ATP; (b) ions, lead, and ATP; or (c) ions and ATP, but without lead. The glycerol extraction procedure removed soluble proteins and possibly solubilized some structural components, but it did not modify the shape of the cells or remove
FIGURE 10 The extraction of cultured fibroblasts with glycerol, up to a concentration of 50%, resulted in the removal of soluble components but preserved the shape of the cell, microfilaments, and some membranes, including the plasma membrane and the ribosome-containing endoplasmic reticulum. × 12,000.

FIGURES 11 and 12 Incubation of the glycerinated cells with 3 mM Mg++ or Ca++, 3 mM ATP, and 1 mM lead nitrate resulted in the deposition of lead phosphate on the microfilaments. Some membranes and especially the endoplasmic reticulum also hydrolyzed the ATP. Fig. 11, × 12,000; Fig. 12, × 40,000.
membranes and microfilaments (Fig. 10). The microfilaments were aggregated into fibrils, which branched and were present throughout the cytoplasm, but they were usually concentrated at the ends and margins of the cells.

Fibroblasts which had been incubated in the presence of Mg$^{2+}$ or Ca$^{2+}$, ATP, and lead had a fine precipitate of lead-phosphate adsorbed to their microfilaments (Figs. 11 and 12). This ATPase activity was not limited to microfilaments in that membranes, and particularly membranes with ribosomes, had a substantial amount of lead-phosphate adsorbed on them. The presence of an ATPase with the microfilaments of Physarum polycephalum has previously been observed by Wohlfarth-Bottermann (67) and by Somogyi, Sotonyi, and Bujdoso (68) in rat muscle, using a similar histochemical and electron microscopic approach.

Chick fibroblasts were also incubated with ions and ATP, but without lead. The microfilaments in these cells were much less clumped than was observed in the control cells (Fig. 6), and individual filaments could be followed with ease. The ability of ATP to disaggregate filaments in fibroblasts is not consistent with the observations of Nagai and Kamiya (69) and Keyserlingk (60), who reported that this substrate enhanced the clumping of microfilaments in glycerol-extracted slime molds and heart fibroblasts. The filaments were straighter than had been observed in the control cells and were moderately long. Because of the apparent overlap of the filaments (Fig. 6), it was not possible to assign a specific length to them, although filaments up to 0.5 μ in length have been observed. The microfilaments have a granular or subunit organization, but no obvious periodicity was evident.

The Binding of Heavy Meromyosin to Microfilaments

Glycerinated fibroblasts bound heavy meromyosin, and this binding was associated with microfilaments (Fig. 13). The quantity of bound heavy meromyosin was frequently so great that it became difficult to discern individual "decorated" filaments displaying the characteristic arrowhead pattern. With this technique, it was apparent that there also was a population of microfilaments, 70–100 Å in diameter, which did not bind heavy meromyosin.

The Localization of Fibroblast Actin-Like Protein

The antiserum prepared against electrophoretically homogeneous actin-like protein reacted with components in cultured fibroblasts. In glycerinated fibroblasts, the antibody made against fibroblast actin bound to microfilaments (Fig. 14). The degree of binding of the antibody to the microfilaments was variable, thus attesting to the weak antigenicity of this protein. Antibody was not associated with detached fragments of plasma membrane, but there was some antigenic activity associated with amorphous regions in the cell. These were not mitochondria or obvious membranes; they could be depolymerized and aggregated actin-like protein. The addition of 8 μg of homogeneous actin-like protein to 50 μl of antiserum markedly reduced the binding of ferritin to the microfilaments. Antiserum prepared against purified rabbit muscle actin also bound to microfilaments. However, the degree of this binding was much less than that obtained with the fibroblast protein.

In intact fibroblasts, antigenic activity was observed at the ends of the cells, within extracellular filaments, and along the ventral surface of attached cells (Fig. 15), where the fibroblasts interacted with the growing surface and other cells. Some of this antigen remained associated with the carbon substrate. In control experiments—incubations without antiserum against the actin-like protein, or incubations without hybrid antibody—ferritin was only rarely found to be adsorbed to the cell membrane, and never as clumps of many ferritin particles.

The Morphology of Chick Embryo Fibroblasts Examined In Toto

Suspensions of fibroblasts, when plated into culture dishes containing nickel or platinum grids covered with a carbon-stabilized collodion film, attached to the grids and grew normally as evidenced by their morphological appearance in the light microscope and their ability to divide.

These cells put out membrane processes which were 0.1–0.8 μ in diameter (Fig. 16). These smaller processes, although falling within Taylor's classification as micropiles (56), and the larger ones will be termed pseudopodia. The pseudopodia were limited to the ends of the cells or to points of interaction between cells. Only rarely
FIGURE 13 The microfilaments from glycerinated fibroblasts bound rabbit muscle heavy meromyosin to form "decorated" filaments. The quantity of bound meromyosin was so great as to almost obscure the arrowhead-like pattern (arrows). With this technique, it was apparent that there exists a second filament system within the decorated one that does not bind the meromyosin. The diameter of these filaments was no greater than 100 Å. × 110,000.

FIGURE 14 Antiserum prepared against fibroblast actin-like protein (35) bound to microfilaments in glycerinated cells. The degree of binding was variable. There were stretches of filaments that were devoid of ferritin-associated antiserum. The antiserum did not bind to plasma membrane or intracellular organelles, but it was associated with amorphous material (arrows) that could be partially depolymerized filaments. × 60,000.
In unstained sections cut perpendicular to the flattened cell, ferritin-associated antisera directed against fibroblast actin-like protein bound material at the leading end of the cells (A and B) and at points along the ventral surface of the cell where it interacted with the supporting layer (A). Ferritin-associated antibody was also observed bound between overlapping cell processes (A) and as residual clumps along the carbon-coated supporting layer (A). X 40,000.

were microvilli-like processes observed on the dorsal surfaces of the cells. The fibroblast pictured in Fig. 16 (arrow) had pseudopodia that interacted with small processes from an adjacent cell. Extracellular filaments (EF) were occasionally observed on the supporting film. The pseudopodia had fine striae lying within the membrane that apparently extended the full length of the processes. These striae were approximately 75 Å in diameter and comparable to microfilaments seen within the pseudopodia of sectioned material (Fig. 2).

When fibroblasts which had been grown on grids were extracted for up to 2 months with 50% glycerol, striae were still present within the cytoplasmic and marginal regions of these cells and could be followed into pseudopodia (Fig. 17). The striae were about 70–90 Å in diameter. The term striae has been used for the structural elements observed with negative staining; however, in view of their dimensions and insolubility in 50% glycerol, it is highly probable that the striae are the microfilaments observed in thin sections of Epon-embedded fibroblasts.

**DISCUSSION**

In cultured fibroblasts (e.g., chicken [17, 60], rat embryo [58], 3T3 [70], and BHK/21 [71]), microfilaments and microtubules are the structural elements that constitute the cells' cytoskeleton and establish and maintain their shape. In the cultured chick cells, the dissociation of the polymerized microfilaments leads to a retraction of the membrane processes and a rounding up of the
FIGURES 16 and 17 Negatively stained whole mounts of cultured fibroblasts established the presence of pseudopodia that were localized predominantly at the leading ends of the cells. These processes extended great distances and frequently interacted with adjacent cells (arrow, Fig. 16). Filamentous material that was extracellular (EF) was found on the plastic support. Fine striations of what were probably microfilaments, 70–90 Å in diameter (Fig. 17), were found in the cell processes. These filaments were not extracted by glycerol. Fig. 16, X 10,000, Fig. 17, X 120,000.

flat, fusiform-shaped cells. These effects have been observed in fibroblasts treated with cytochalasin A and B (36) as well as in EDTA- and trypsin-released ones and in those incubated for 6 h with iodoacetic acid. Microtubules, although present in these fibroblasts, are not essential for maintaining an asymmetric shape. Cells treated with 10 µg/ml colchicine did not assume a round shape and newly dissociated fibroblasts, plated into medium containing colchicine, spread out into a normal fusiform morphology.1

1 Perdue and Springer. Unpublished observations. 1972.
Transformation of cultured fibroblasts with oncogenic viruses results in an alteration in cell shape and changes in the number and distribution of microfilaments. Chick embryo fibroblasts, infected with the avian RNA virus Bratislava 77, assumed a round morphology after transformation, with cells piled up upon one another (72). Concomitant with this alteration in shape, there was a decrease in the number of microfilaments (73). McNutt, Culp, and Black (70 and 74) have recently examined 3T3 cells, SV40 virus-transformed 3T3 cells, and fluorodeoxyuridine and concanavalin A “flat revertants” of the transformed cells. Both 3T3 and the revertant cell lines stopped dividing when they grew to a confluent monolayer, and they were flatter and contained more microfilaments than the smaller and rounder SV40-transformed cells. The essentiality of a cytoskeleton of microfilaments and microtubules for the establishment of a fusiform shape in cells has also been implicated in the work of Johnson et al. (75) and Hsie et al. (76). Both groups have observed that increasing the effective concentration of cyclic AMP in many cell lines resulted in the cells' assuming a fusiform shape.

In the chick embryo fibroblasts, the microfilaments are arranged in thick bundles that run lengthwise in the fusiform-shaped cell, as previously described by Buckley and Porter (58), Goldman and Follett (71), and Spooner et al. (17), who related them to the “stress fibers” observed with light microscopy. The filaments, 80 Å in diameter, are also more loosely arranged and are concentrated along the margins of the flattened cell on the dorsal surface, particularly at the ends of the cell and its ventral surface, where it makes contact with the plastic dish and other cells. A similar distribution of these elements has been reported for cultured rat embryo cells (58), BHK-21 fibroblasts (71), 3T3 cells (70 and 74), glial cells (17), and chick heart fibroblasts (60, 77, 78). At the tip of the cell, these points of adhesion are similar to the “plaques” described by Abercrombie et al. (78) in the leading lamella of cultured chick heart fibroblasts. The points of interaction with the ventral surface which are more internal (Fig. 4) may correspond to the regions of increased refractility observed by Ambrose (57) in motile fibroblasts and to the attachment devices of cultured human glia-like cells (79).

At these points of attachment, the actin-like protein has the appearance of being external. This was indicated by the presence of an ATPase activity on these filaments in intact cells and by the binding of antibody directed against the actin-like protein to these microfilaments. The presence of these external microfilaments may denote an essential function of these components, such as providing sites of cell attachment to the substratum. Abercrombie et al. (78) and others (17, 77, 80) believe some form of attachment of the
cells to its substrate is essential for the locomotion of the fibroblasts.

It can be argued that the presence of microfilaments external to the plasma membrane is an artifact that is unrelated to the implementation of cell adhesion or motility. What appear as external filaments originating inside the cell could be explained by the presence of debris lying within the plane of sectioning. This was the explanation given by Goldberg and Green (81) to account for the appearance of external microfilaments in cultured mouse cells. There is cellular debris (membrane fragments, extracellular filamentous and aggregated materials, etc.) on the substratum, and this might appear in longitudinal sections of cells 500-800 Å in thickness. However, in my opinion, this argument is less applicable to sections of fibroblasts cut perpendicular to the substratum. The ferritin-labeled antibody to fibroblast actin-like protein was bound to the tips of the cells in Fig. 15. This binding was 0.3-0.7 µm from the substratum and was very likely not derived from it.

A more plausible explanation for the presence of external microfilaments could be that they are the remains of cytoskeletal elements that have been exposed during the contraction of the cell’s cytoplasm. The cytoplasm could be contracting and pulling away from the bundles of filaments because of environmental trauma occurring during treatment (e.g., incubation with antibody, brief fixation, temperature changes, etc.) or during the movement of the cells. Isotonic conditions were generally employed during the respective treatments, but slight changes in the contractility of the cytoplasm could explain the external microfilaments pictured in Fig. 7. However, a much greater change in cytoplasm contractility would have to occur in order to account for the large quantity of external microfilaments pictured in Fig. 8. The discarding of actin-like protein by a retreating cell may be an explanation for these observations.

The extracellular filaments that are observed on the substratum by negative staining and embedding and sectioning procedures appear different from internal filaments. They are highly aggregated with an ill-defined diameter, and they have extracellular material associated along their lengths. These filaments are not collagen. Collagen was observed infrequently in these cultured fibroblasts, a condition possibly resulting from a lack of ascorbate in the medium (82). However, some of the extracellular filaments may be "beaded filaments"—a type of protofibril implicated in collagen synthesis (83). Filaments of this type were observed in negatively stained preparations.

The microfilaments in the chick embryo fibroblasts are composed, in part, of an actin-like protein. In thin sections, the microfilaments have a diameter of 70-90 Å; negatively stained filaments that were formed from fibroblast actin-like protein have a diameter of 80 ± 5 Å (35). In glycerinated cells, the microfilaments bind heavy meromyosin; polymerized actin-like protein also binds heavy meromyosin with a periodicity of 366 ± 43 Å (35). The microfilaments from intact as well as glycerol-extracted fibroblasts have an ATPase associated with them; fibroblast actin-like protein binds and activates a rabbit muscle myosin ATPase (35). Finally, globulin made in the rat and directed against homogeneous fibroblast actin-like proteins binds to microfilaments in the glycerinated cells.

The microfilaments may also contain a tropomyosin-like protein. Tropomyosin is a component of the thin filaments from muscle, and a protein having a subunit mol wt 35,000 and a mobility in SDS-acrylamide gels identical to that of the muscle protein was present in partially purified fibroblast actin-like protein (35).

There is present in the fibroblasts a second filament system which was detected by its failure to bind heavy meromyosin. The diameter of this filament was between 70 and 100 Å, not unlike that of the other microfilaments, and it appeared to be intertwined with the filaments that bound heavy meromyosin. Ishikawa et al. (46) had observed similar filaments in cultured cells. Goldmann and Follett (71) had previously found that BHK-21 cells contained two populations of filaments 40-60 Å and 80-100 Å in diameter. Many of these filaments were insensitive to treatment with cytochalasin Β (84). Glial cells also have microfilaments which are insensitive to this drug (17). Similarly, McNutt et al. (70) described a system of microfilaments, the β-filaments, which were 100 Å in diameter and localized in a random manner within the endoplasm of cultured 3T3 cells.

The myosin-like proteins which have been isolated from the amoeboid slime molds (27, 28, 85) have solubility properties that are very similar to smooth muscle myosin (86) and different from skeletal muscle myosin. Both proteins are soluble at KCl concentrations of about 0.05 M and require...
the presence of Mg\textsuperscript{++} or Ca\textsuperscript{++} for thick filament formation in vitro (86, 87). The physical shape of the slime mold proteins does not appear to differ from that of skeletal muscle myosin (87), but the ratio of the myosin-like protein to actin or actin-like proteins differs markedly, dependent upon the source. Adelman and Taylor (28) estimated that the ratio of slime mold myosin to actin is from 1:1 to 1:3; skeletal muscle has a ratio of these two components of 3:1.

It has been suggested by Shoenberg (86), Adelman and Taylor (28), and Nachmias (87) that this smooth muscle-like protein from the slime mold may be present as dimers, and it might polymerize to form morphologically discrete thick filaments only under conditions of induced tensions or altered composition. Such a population of thick filaments was not observed in the cultured chick embryo fibroblasts. Moreover, if the histochemical localization of ATPase activity is a reasonable approximation of the in situ state, and this activity is myosin-like, then it would appear that the myosin is bound to the microfilaments as single units or small aggregates. Furthermore, not all microfilaments have this enzymatic activity associated with them.

The microfilament system does have the capacity to exert tension—an essential requirement for amoeboid movement and adhesion (2, 6, 13, 17, 78). James and Taylor (88) measured the stress developed by sheets of living chick fibroblasts and found it to be about \( \frac{3}{100} \) that of rabbit skeletal muscle—\( 3.4 \times 10^4 \) dynes/cm\(^2\) versus \( 3.9 \times 10^6 \). This tension-generating capacity is a property of the cells' filament system. Hoffmann-Berling (63) prepared a strip of glycerinated fibroblasts, under conditions in which the microfilaments remain intact, and demonstrated a shortening of these cells when they were incubated with ATP. The addition of rabbit muscle sarcoplasmic reticulum caused a relaxation of the induced tension. It is generally accepted that the reticulum removes Ca\textsuperscript{++} and implicates this ion in the control of contractility in the cultured fibroblasts. Perdue (89) has recently shown that the plasma membrane of the cultured fibroblasts has a specific ATP-dependent Ca\textsuperscript{++}-transporting function.

Knowledge of the mechanism for the shape-changing property of the filament system is wanting. In the unique case of the contractile ring—an organized system of microfilaments which divides cells—Schroeder (12) has documented the changes in the volume of this specialized structure during cell cleavage. He found that the volume of the contractile ring decreases as cleavage progresses. This decrease in volume is inconsistent with a sliding filament model of contractility and indicates that there was a depolymerization of the filament system. What is not known, however, is whether the tension exerted during cytoplasmic cleavage is the result of depolymerization of the microfilament system or is brought about by a sliding of adjacent filaments with their subsequent depolymerization.

Assembly-disassembly of the actin-like protein can by itself impose form on a cellular system. Tilney and Cardell (7), utilizing hydrostatic pressure, produced a breakdown of microfilaments in the terminal web and microvilli of the small intestine of the salamander. These effects were reversible; that is, the microfilaments reassembled and the microvilli reformer. The microfilaments in the microvilli were unidirectional and appeared to insert into dense, cell membrane-associated material that may function as sites of organization similar to that of the Z-line material in muscle. Like the observations of Schroeder (12) in the formation of the contractile ring, the reestablishment of the terminal web occurs at the cell membrane. Cytoplasmic organelles are excluded as the filamentous layer becomes thicker. These observations infer a form-controlling function of the cell membrane; it regulates the sites of assembly-disassembly of cytoskeletal elements, one of them being an actin-like protein.

A form-establishing and membrane-stabilizing role of microfilaments was proposed by Buckley and Porter (58) and received confirmation from Puck et al. (90). These authors demonstrated that the addition of dibutyryl cyclic 3',5'-AMP to epithelial-like cells stabilized membrane activity and caused the treated cells to assume a fusiform morphology. These changes were reversed by treating the cells with cytochalasin B.

Plasma membrane stabilization may be the microfilaments' most important function. In this study and those of others (7, 12, 17, 58, 70, 71, 78), attention has been drawn to the close apposition of a network of filaments with the plasma membrane and the subsequent exclusion of cytoplasmic organelles. These areas of membrane-filament association could provide stability to the cell membrane and thereby influence the functions of this membrane system. This association could be a direct linking of the microfilaments to intra-

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membranous particles, as was observed by Staehelin et al. (91) in the lumenal plasma membrane of urinary bladder epithelial cells. The cytoskeleton of microfilaments could impart membrane stability by inhibiting endocytosis, microvilli development, or the interaction of this membrane with intracellular organelles or adjacent cells. Finally, membrane stability could result from an increase in the viscosity of the cytoplasm immediately adjacent to the cell surface by the assembly of highly asymmetric microfilaments. Sol-gel transformations are implicit in many models of cell motility (67), and Pollard and Ito (92) have demonstrated that filament assembly is responsible for the increases in the viscosity of motile cytoplasmic extracts of Amoeba proteus.

The presence of microfilaments in cells as evolutionally distinctive as amoebae and higher vertebrates points up the need for a detailed examination of the chemistry of the actin-like protein, of the mechanism of microfilament assembly-disassembly, of the regulation of this process, and of the functions of these components in cells.

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