Microfilaments and Tropomyosin of Cultured Mammalian Cells: Isolation and Characterization

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ABSTRACT Microfilaments were isolated from cultured mammalian cells, utilizing procedures similar to those for isolation of "native" thin filaments from muscle. Isolated microfilaments from rat embryo, baby hamster kidney (BHK-21), and Swiss mouse 3T3 cells appeared structurally similar to muscle thin filaments, exhibiting long, 6 nm Diam profiles with a beaded, helical substructure. An arrowhead pattern was observed after reaction of isolated microfilaments with rabbit skeletal muscle myosin subfragment 1. Under appropriate conditions, isolated microfilaments will aggregate into a form that resembles microfilament bundles seen *in situ* in cultured cells.

Isolated microfilaments represent a complex of proteins including actin. Some of these components have been tentatively identified, based on coelectrophoresis with purified proteins, as myosin, tropomyosin, and a high molecular weight actin-binding protein.

The tropomyosin components of isolated microfilaments were unexpected; polypeptides comigrated on SDS-polyacrylamide gels with both muscle and nonmuscle types of tropomyosin. In order to identify more specifically these subunits, we isolated and partially characterized tropomyosin from three cell types. BHK-21 cell tropomyosin was similar to other nonmuscle tropomyosins, as judged by several criteria. However, tropomyosin isolated from rat embryo and 3T3 cells contained subunits that comigrated with both skeletal muscle and nonmuscle types of myosin, whereas the BHK cell protein consistently contained a minor muscle-like subunit. The array of tropomyosin subunits present in a cell culture was reflected in the polypeptide chain pattern seen on SDS-polyacrylamide gels of microfilaments isolated from that culture. These studies provide a starting point for correlating changes in the ultrastructural organization of microfilaments with alterations in their protein composition.

Actin is the major protein of microfilaments in nonmuscle cells (1, 2). Other proteins similar to those found in muscle have been identified biochemically in these cells (2–5). The expectation that these might be associated with actin-containing microfilaments has been supported by immunofluorescence microscopy of cultured mammalian cells, utilizing antibodies against muscle or nonmuscle myosin (6–8), tropomyosin (9, 10), α-actinin (10–12), and filamin (13, 14). Thus, regions of the cell containing fibrous or diffuse staining for actin (2, 15) have corresponding fluorescence patterns with one or more of these antibodies (10, 14). Furthermore, in microfilament-enriched detergent-extracted cells (16–19) and isolated intestinal brush border (20–22), various combinations of proteins that comigrate with filamin, myosin, α-actinin, actin, and tropomyosin have been observed.

One of our goals in studying the role of microfilaments in cell motility has been to determine whether changes in the protein composition of microfilaments occur concomitantly with alterations in their supramolecular organization (e.g., contact-mediated conversion of microfilament meshworks to bundles, [23]). In this report, we describe experiments in which a population of microfilaments was isolated and characterized biochemically and morphologically. In addition to actin, several other proteins were present as determined by gel electro-

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phoresis. The initial fraction contained individual microfilaments. These tended to aggregate more readily than pure muscle F-actin into structures that resembled microfilament bundles, suggesting that the proteins bound to the actin might be, at least in part, responsible for the interaction.

During the course of this work, SDS-polyacrylamide gels of isolated microfilaments from some cell cultures displayed subunits that comigrated with both muscle and nonmuscle tropomyosins. This protein has been most extensively characterized, among the vertebrate nonmuscle systems, from blood platelets (24, 25), brain (25–28), and pancreas (25), in which cases the subunit molecular weights ranged from 26,000–30,000. Our observations on microfilaments prompted us to isolate tropomyosin from rat embryo cell cultures and from the continuous baby hamster (BHK-21) and Swiss mouse 3T3 cell lines. Evidence is presented for the expression of multiple forms of tropomyosin in these cells.

MATERIALS AND METHODS

Cell Culture

Baby hamster kidney (BHK-21/c13), Swiss mouse 3T3, and rat embryo cells were grown in Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% calf serum (Grand Island Biological Co.), and 50 μg of Gentamycin/ml (Schering Corp., Kenilworth, N. J.). BHK-21 cell cultures were also supplemented with 10% tryptose phosphate broth (Difco Laboratories, Detroit, Mich.). Stock cultures were maintained in plastic petri dishes (Lux Scientific Corp., Newbury, Park, Calif.) in a humidified 5% CO_2-95% air atmosphere at 37°C. Confluent cultures were removed from the dishes with trypsin-EDTA solution (0.05% trypsin, 0.54 mM EDTA, in phosphate-buffered saline: Dulbecco's phosphate-buffered saline [PBS] without Ca^{++} or Mg^{++} [29]) and replated. Roller cultures were maintained as previously described (8).

Rat embryo (RE) cell cultures were obtained by dissociating whole embryos with trypsin-EDTA. The cells were subcultured twice, frozen in medium with 5% glycerol, and stored in liquid nitrogen. Cells were thawed as needed and grown for several passages before use. For microfilament isolation experiments, extensively spread RE and 3T3 cells were selected by passing only those cells most tightly adherent to the culture dishes; the less well attached cells were removed by a preliminary, short-duration rinse with trypsin during the subculture procedure. BHK-21 cells were induced to spread by maintenance for two passages at low density in medium containing 0.25% calf serum.

Microfilament Isolation

The procedure was based on muscle native thin filament isolation methods (30, 31). While still attached to petri dishes, the cells were rinsed three times with PBS at room temperature. All steps from this point were carried out at 4°C. Cells were scraped with a rubber policeman into 1.5 ml of buffer A per 100-mm petri dish. Buffer A consisted of 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM potassium phosphate buffer, pH 7.4. The cells were broken using five strokes, by hand, in a Potter-Elvehjem (glass/Teflon) homogenizer. The homogenate was centrifuged for 15 min at 15,000 *g*. The pellet was resuspended in the same volume of buffer A, and centrifuged again. Then the pellet was suspended in buffer B (having the same composition as buffer A but at pH 6.0; 0.5 ml/10 petri dishes). ATP (adenosine triphosphate) was added from a 50 mM sodium ATP (Grade II; Sigma Chemical Co., St. Louis, Mo.) stock solution, pH 6.0, to a final concentration of 5 mM. After incubation for 3 min, the suspension was centrifuged for 20 min at 80,000 g_{max}. The supernate was collected. The pellet was reextracted with one-half of the original volume of buffer B and ATP, and centrifuged again. Supernates were combined. This sample contained numerous thin filaments and was designated as microfilament fraction I.

Microfilaments were further enriched from this fraction by dialysis against 50 mM NaCl, 10 mM Tris-Cl, pH 7.5, with 5–40 mM $MgCl_2$ for 12–48 hours. The precipitate that formed was collected by centrifugation for 20 min at 1,400 *g*, washed once with dialysis buffer, and prepared for electron microscopy or SDS-gel electrophoresis.

Retraction Fibers

The medium was withdrawn from RE or BHK-21 cells growing on a substrate and replaced with trypsin-EDTA solution. The culture was observed with an inverted microscope until many cells, in the process of rounding, possessed retraction fibers (32). The trypsin solution was withdrawn, and PBS was added to the cells, which were then scraped from the surface with a rubber policeman. The cell suspension was applied to carbon-Formvar-coated grids, negatively stained, and examined in the electron microscope.

Cultured Cell Tropomyosin

Tropomyosin was isolated from cultured cells by a modification of the methods employed for isolation of tropomyosin from platelets (24), brain (26), and rabbit muscle (33). Cells were harvested from roller bottles, rinsed with PBS, and extracted with 3 vol of ice-cold 95% ethanol, 1 mM DTT (dithiothreitol) by homogenization in a motor-driven glass-Teflon homogenizer. After 10 min, the suspension was centrifuged for 5 min at 1,600 *g*. Extraction of the pellet was repeated twice with ethanol/DTT and twice with ether/DTT. The resulting powder was allowed to dry overnight at 4°C and was then extracted for 18 h in 1 M KCl, 1 mM DTT adjusted to pH 7.0 (50 ml/g powder). The extract was clarified by centrifugation for 3 h at 40,000 *g*, immersed in 100°C water for 10 min, and clarified. The supernate was dialyzed against 4 vol of 1 mM DTT. Ammonium sulfate fractionation between 40 and 60% saturation was performed by gradual addition of the solid salt and continual adjustment to pH 7.0 with 1 N KOH. The pellet, obtained by centrifugation for 30 min at 12,000 *g*, was resuspended and dialyzed exhaustively against 1 M KCl, 1 mM DTT. The protein concentration was adjusted to 1 mg/ml, and isoelectric fractionation at pH 4.1 was performed by addition of 0.1 N HCl. Precipitated protein was collected by centrifugation for 60 min at 13,200 *g*.

At this stage, the sample exhibited a low A_{278}/A_{260} ratio, indicating nucleic acid contamination. Therefore, the pellet was suspended in and dialyzed against 0.3 M NaCl, 0.5 mM DTT, 10 mM Tris-Cl, pH 7.5, and run through a 1.5- × 5-cm column of DEAE-cellulose (Whatman DE-52) in the same buffer. A single, nucleic acid-free peak was collected. The sample was then dialyzed against 0.2 M KCl, 0.5 mM DTT, 10 mM Tris-Cl, pH 7.5, and adjusted to a protein concentration of 0.7 mg/ml. The protein precipitating between 53 and 60% saturation with $(NH_4)_2SO_4$ was collected and dialyzed into the solution desired. Tropomyosin was stored at −20°C in 0.1 M KCl, 0.5 mM DTT, 10 mM Tris-Cl, pH 7.5

Other Proteins

Rabbit skeletal muscle tropomyosin, actin, myosin, and heavy meromyosin subfragment 1, and BHK-21 cell high molecular weight (HMW) protein were prepared as described previously (33–35). Myosin was stored at −20°C in 50% glycerol (vol/vol), 0.6 M KCl, 10 mM Tris-Cl, pH 7.5.

Assays

Protein concentration was estimated by the microbiuret method (36). Unless otherwise stated, SDS-polyacrylamide gel electrophoresis was carried out according to Fairbanks et al. (37) or Laemmli (38), and gels were stained according to the former (37). In experiments in which conditions were modified from these methods, changes are specified in the figure legends. Densitometer traces of stained gels were obtained on a Zeiss PM6 spectrophotometer. Amino acid analysis was performed using a Durrum D-500 analyzer, after 24-h hydrolysis in 6 N HCl at 110°C *in vacuo*. Values obtained for threonine and serine were increased by 5 and 10%, respectively, to correct for hydrolysis. ATPase activity was assayed as described by Hartshorne and Mueller (33). Inorganic phosphate was determined by the method of Fiske and SubbaRow (39).

Electron Microscopy

Samples for negative staining were applied to carbon-Formvar-coated 200-mesh copper grids. After 60 s, excess sample was removed with filter paper, and the grid was rinsed several times with buffer and several times with 1% aqueous uranyl acetate. The last uranyl acetate rinse was left for 30 s, the excess was removed with filter paper, and the grid was allowed to dry. Samples were observed with a Philips 201C electron microscope.

RESULTS

Isolated Microfilaments

Microfilaments isolated from homogenates of RE cells were morphologically indistinguishable from muscle thin filaments and were identified as actin-containing filaments by decoration (40) with myosin subfragment 1 (Fig. 1). Donut-shaped objects

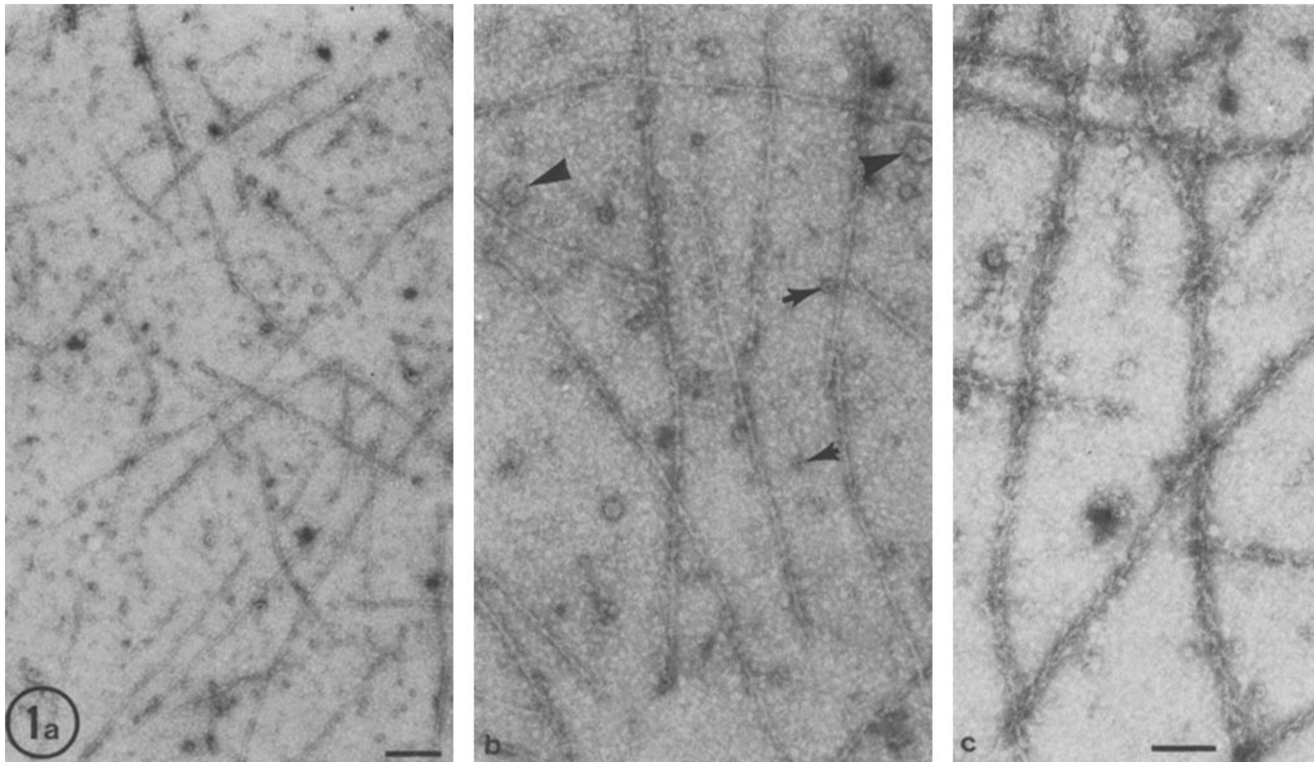

FIGURE 1 Microfilament fraction I from RE cells. The fraction contained numerous filaments whose size and substructure were similar to those of actin (*a* and *b*). The straight, smooth profile of the filaments suggests that tropomyosin may be present. Large and small donut-shaped objects (large and small arrows, respectively) were often observed in this fraction. Arrowhead complexes were formed upon addition of muscle myosin subfragment 1 to the fraction; the microfilaments had been attached to the grid before subfragment 1 decoration (*c*). Fraction I was applied to grids undiluted, and subfragment 1 was added at 0.2 mg/ml in buffer A. Electron micrographs of negative stains. (*a*) Bar, 0.2 μm, × 37,000. (*b* and *c*) Bar, 0.1 μm, × 87,500.

of various diameters were also seen. No other fibrous structures (intermediate filaments or microtubules) were observed in these preparations.

SDS-polyacrylamide gel electrophoresis of microfilament fraction I (Fig. 2*d*) showed that it was enriched, relative to whole cell homogenates (Fig. 2*a*), for several proteins, including actin. In spite of this enrichment, the gel profile of fraction I was more complex than profiles obtained from various muscle thin filaments (30, 31). To purify microfilaments more extensively, fraction I was dialyzed for 12–18 h against a magnesium-containing solution. (Pure actin forms paracrystals in magnesium [see reference 41].) Under these conditions microfilaments became aggregated into small bundles (Fig. 3). In most cases, the lateral association of parallel microfilaments did not display an obvious superstructure, but in a few experiments a banding pattern characteristic of actin paracrystals was observed (Fig. 3*c–e*). Isolated microfilaments formed lateral aggregates in a variety of solutions containing phosphate or Tris buffers in a pH range of 7.5–8.0, with 0–50 mM NaCl and 5–60 mM $MgCl_2$. The aggregates formed in vitro were similar to microfilament bundles seen *in situ*. The latter were visualized within the retraction fibers left behind by cultured mammalian cells as they assumed a rounded morphology during trypsin treatment. For comparison, negatively stained retraction fibers are shown in Fig. 3*f*.

Extended dialysis (48 h) of microfilament fraction I against 50 mM NaCl, 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, resulted in a change in microfilament structure. Filaments were on average thinner, less straight, and were not laterally aggregated (Fig. 4*a*). In addition to donut-shaped objects, myosin-like filaments were observed in these precipitates.

The RE cells used for these experiments were large, flat, well-spread cells (34) that displayed prominent phase-dense fibers (microfilament bundles). BHK-21 cells are usually not as well spread, being instead spindle shaped (32). The morphology of 3T3 cells is intermediate between that of BHK-21 and RE cells (42). We were unsuccessful in initial attempts to isolate from BHK-21 or 3T3 cells a microfilament fraction similar to that obtained from RE cells. It was found, however, that after inducing the cells to adopt a well-spread morphology and coincident prominent phase-dense fibers (see Materials and Methods), such a fraction was isolatable. Ultrastructural examination of fraction I from BHK-21 or 3T3 cells revealed individual microfilaments. Short-term dialysis of this material against magnesium-containing solutions resulted in precipitation of laterally aggregated microfilaments. Extended dialysis as described for RE cell microfilaments yielded aggregates that contained myosin-like filaments, but not bundled microfilaments (Fig. 4*b*). Thus the procedure was applicable to continous as well as to secondary cell cultures.

The proteins present in magnesium solution precipitates of microfilament fraction I (short- and long-term dialysis) were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2*e* and *g*). Actin and several other proteins were enriched relative to fraction I (Fig. 2*d*). The molecular weights of these latter polypeptides were 250,000, 200,000, 37,000, 35,000, and 29,000. In order to estimate the ratios of these polypeptides to actin, quadruplicate microfilament samples of RE cell cultures were

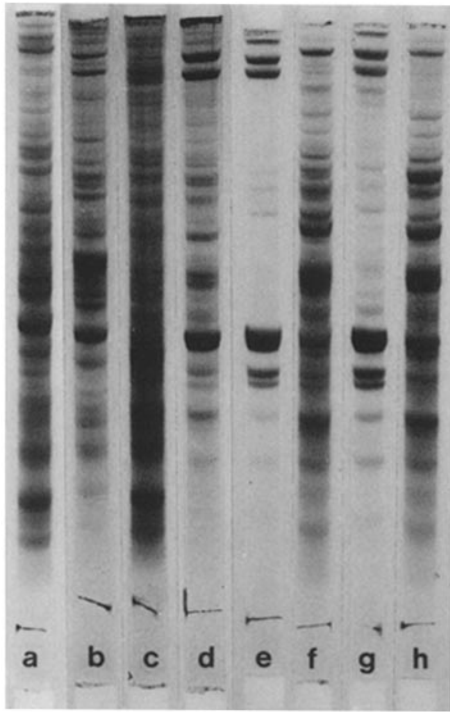

FIGURE 2 SDS-polyacrylamide gel (37) analysis of rat embryo cell microfilament isolation fractions. (*a*) Whole RE cell homogenate, 30 μg. (*b*) Proteins released into buffer A upon cell rupture, 15 μg. (*c*) Residual cellular material pelleted by 20-min 80,000 *g* centrifugation, 40 μg. (*d*) Microfilament fraction I, corresponding with Fig. 1, 20 μg. (*e* and *f*) Precipitate and supernate, respectively, after 12-h dialysis of fraction I against magnesium-containing solution and low-speed centrifugation. (*g* and *h*) As in *e* and *f*, except 48-h dialysis. (*e* and *g*) 20 μg. (*f* and *h*) 25 μg. In this and all electrophoresis figures, sample was applied to the top of the gel and migrated toward the anode.

isolated, and samples were dialyzed to obtain precipitates. These were analyzed by quantitative densitometry of Coomassie Blue-stained SDS-polyacrylamide gels, and the results were averaged. The mean molar ratios of actin per 250,000, 200,000, 37,000, 35,000, and 29,000 mol wt polypeptide chain were 39, 35, 5, 11, and 20, respectively.

Many less prominent protein bands were also present in these gels. They have not yet been identified, and some may result from proteolysis during microfilament isolation. However, they were consistently observed, and the inclusion of the protease inhibitor, phenylmethane sulfonylfluoride, during homogenization and dialysis did not alter their appearance.

Most of the proteins in the precipitates were also represented in the supernatant fractions (Fig. 2*f* and *h*). The precipitates had been collected by low-speed centrifugation, to avoid enriching for components not specifically associated with isolated microfilaments. Therefore, microfilaments that were not present in large aggregates would not be pelleted. Whereas the extract contained 8–10% of total cell protein, the precipitates yielded only 10–20% of this, or about 1% of the total. Thus, the procedures were not quantitative.

The polypeptides present in isolated microfilaments were tentatively identified by comparison of their mobilities on SDS-polyacrylamide gels with purified proteins (Fig. 5). The major microfilament proteins comigrated with subunits of (i) a BHK-21 cell high molecular weight protein (35) whose properties were similar to those of filamin and actin-binding protein, (ii) BHK-21 cell myosin heavy chain, (iii) actin, and (iv) cultured cell tropomyosin. With regard to this last protein, it was noted that in RE (Fig. 2*e* and *f*) and 3T3 cell microfilaments (Fig. 6*i*), there were proteins whose migrations corresponded to molecular weights of 29,000 and a doublet at 35,000–37,000. BHK-21 cell microfilaments (Fig. 6*b*), on the other hand, contained major 29,000 and minor 35,000 molecular weight subunits. In order to identify properly these proteins, a more careful investigation of tropomyosin from cultured cells was undertaken.

Isolation and Characterization of Cultured Cell Tropomyosin

BHK-21 CELL TROPOMYOSIN: BHK-21 cells were used for most experiments on tropomyosin because these cells could be grown easily in large quantities. Beginning with 30 ml of packed cells (from 60 roller bottles), 2–3 mg of tropomyosin was obtained. On SDS-polyacrylamide gels (Fig. 6*a* and *c*) the sample had a major band (65–75% of total protein) at 29,000 mol wt. This band was sometimes observed to run as a doublet, depending on the particular gel conditions (28). A minor 35,000 mol wt subunit (15–20% of total protein) was also present. Both BHK-21 cell tropomyosin subunits comigrated with proteins of isolated microfilaments (Fig. 6*a*–*d*). The characteristics of the purified tropomyosin that were studied included paracrystal formation, amino acid composition, actin-binding ratio, and its ability to function in a hybrid calcium-sensitive actomyosin ATPase system.

BHK-21 tropomyosin formed bipolar paracrystals when a 2–3 mg/ml sample of the protein was dialyzed against 50 mM $MgCl_2$ (Fig. 7). The paracrystal structure was similar to that of rabbit skeletal tropomyosin, but the period was considerably shorter. BHK-21 tropomyosin paracrystals displayed an axial repeat distance of 34.2 ± 0.08 nm (standard deviation, $n = 40$) compared with 38.8 ± 0.6 nm ($n = 37$) measured in skeletal muscle tropomyosin paracrystals. These values were in reasonable agreement with values of 34.3 ± 0.5 nm and 39.5 nm reported for platelet and skeletal tropomyosins, respectively (24). Two different BHK-21 preparations resulted in identical paracrystal structures.

The amino acid composition of BHK-21 tropomyosin (Table I) was within the range of published values for platelet and brain tropomyosins (24, 26). The presence of a few proline residues was probably evidence of minor contaminants seen in the gels.

Binding of BHK-21 tropomyosin to rabbit skeletal actin was tested. Actin was combined with an excess of skeletal muscle or BHK-21 cell tropomyosin. Under the assay conditions (26), tropomyosin was soluble; only bound tropomyosin would be pelleted with the actin. The mixtures were centrifuged and the pellets analyzed. From densitometer traces (Fig. 8) of fast green-stained gels (43), actin:tropomyosin molar ratios were determined. The values were 8.2 and 7.0 for the muscle and BHK-21 cell tropomyosins, respectively. If the muscle value is used as an internal control for our experiment (the accepted ratio for muscle tropomyosin binding to actin is 7.0 [44]), then each BHK-21 tropomyosin molecule would bind to six actin monomers. This result is consistent with the value predicted for the lower molecular weight tropomyosin species of shorter axial dimension (see Figs. 6*a* and 7, and reference 45).

BHK-21 cell tropomyosin in combination with muscle troponin formed a calcium-dependent complex that regulated skeletal muscle actomyosin Mg^{++}-ATPase activity (Table II).

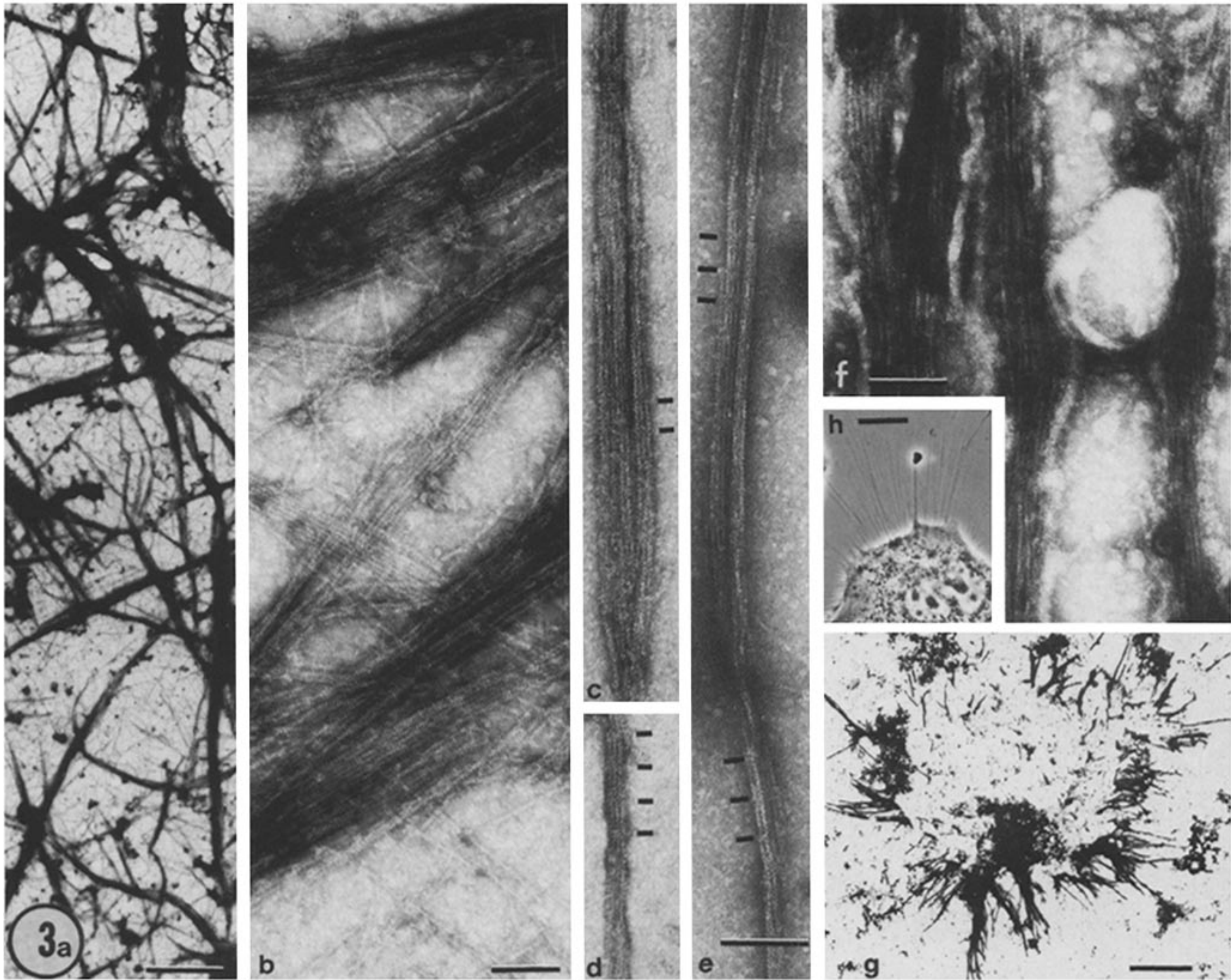

FIGURE 3 Isolated RE cell microfilament aggregates formed by 12-h dialysis against magnesium-containing solution, compared with *in situ* retraction fibers. At low magnification (*a*), clusters of dense aggregates were interspersed with a few individual microfilaments. Bar, 1 μm. × 11,000. At higher magnification (*b*), the dense areas were seen to consist of laterally aggregated microfilaments. Bar, 0.1 μm. × 92,000. In some cases (*c–e*) the crossover points of the actin helices were aligned across the aggregates (marked by dashes), forming paracrystalline structures. Bar, 0.1 μm. × 124,000. Retraction fibers (*f*) from RE cells consisted of membrane-bound microfilament bundles. Bar, 0.1 μm. × 107,000. At lower magnification (*g*), the remnants of the retracted cell were seen. In this sample, the cell body broke away during rinsing, leaving only the retraction fibers. Bar, 4 μm. × 2,250. Phase-contrast micrograph (*h*) of a BHK-21 cell in the process of rounding, leaving retraction fibers on the substrate. Bar, 20 μm. × 350. (*a–g*) Electron micrographs of negatively stained samples.

When the cell protein was added to pure rabbit muscle actin and myosin, the activated ATPase rate was insensitive to the calcium concentration. However, when muscle troponin was added, a calcium-regulated response was obtained. Thus, BHK-21 (nonmuscle) tropomyosin had binding sites similar to muscle tropomyosin for both actin and troponin.

RE AND 3T3 CELL TROPOMYOSINS: Because of the unusual pattern (relative to other reports on vertebrate nonmuscle tropomyosins [9, 24–28, 48]) of tropomyosin bands on the RE and 3T3 cell microfilament gels, we isolated the protein from these cells. Both cell types grew more slowly and to lower final density in roller culture than did BHK-21 cells. Nevertheless, sufficient protein was obtained to permit partial characterization of these proteins and comparison with the microfilament subunits.

RE cell tropomyosin was composed of three subunits (Fig. 6*e*) with molecular weights of 29,000, 35,000, and 37,000. The fastest migrating band (40–45% of the protein) comigrated with the major BHK-21 cell tropomyosin subunit, and the slower-migrating doublet (45–50% of total protein) comigrated with rabbit skeletal muscle tropomyosin on this gel system (Fig. 6*f*). All three bands comigrated with proteins of microfilaments isolated from RE cells (Fig. 6*g*). In the latter, the decreased intensity of the 29,000 molecular weight band relative to the 35,000–37,000 doublet (compare Figs. 2*d–h* and 6*e* and *g*) may be a function of different affinities of the tropomyosin species for actin (cf., reference 26).

The rat embryo cell culture was not a cloned, continuous cell line. It was, therefore, possible that the low and higher molecular weight tropomyosins resulted from an nonhomogeneous cell culture. We addressed this problem by isolating the protein from 3T3 cells, a cloned, established line. A similar pattern of subunits was obtained (Fig. 6*h*). This observation agreed with the presence of three bands on the polyacrylamide gel of 3T3 cell-isolated microfilaments (Fig. 6*i*). Thus, whereas the presence of multiple tropomyosins in a single cell has not

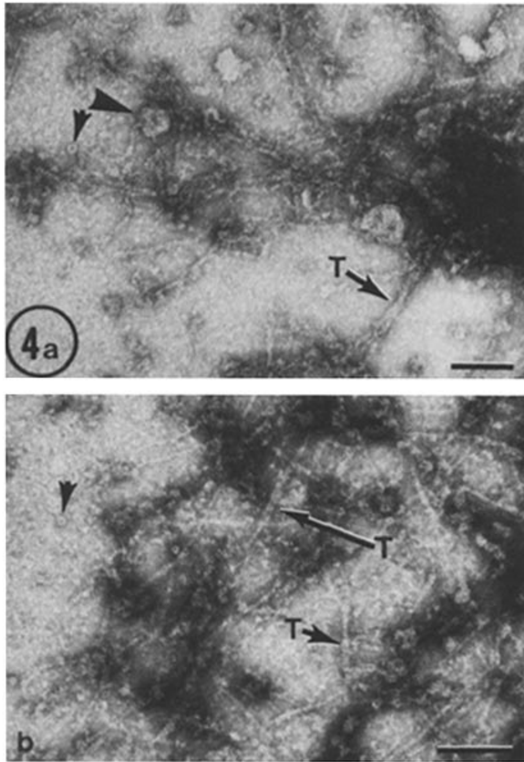

FIGURE 4 Isolated microfilament aggregates after 48-h dialysis. Individual, tangled microfilaments, which may have been partially depolymerized, were observed. Thicker filamentous material (*T*) that may represent myosin aggregates was also observed. Note large (large arrowhead) and small (small arrowheads) donut-shaped objects (as in Fig. 1). (*a*) From RE cells. Bar, 0.1 μm. × 83,000. (*b*) From BHK-21 cells. Bar, 0.1 μm. × 96,000. Electron micrographs of negative stains.

been rigorously demonstrated, the presence of all three subunits in 3T3 tropomyosin showed that their simultaneous presence in a culture did not necessarily arise as a result of the mixed tissue origin of the culture.

The tropomyosin purification scheme is quite selective; the final product is a heat- and organic solvent-stable protein, that is "salted-out" of solution within a specific, narrow range of $(NH_4)_2SO_4$ concentrations, and that precipitates at a particular isoelectric point. However, in order to demonstrate further that all three RE cell subunits that coisolated through this procedure were, in fact, tropomyosin, additional criteria were examined. The RE cell preparation and rabbit skeletal muscle tropomyosin coeluted from hydroxylapatite in a phosphate gradient (49). The amino acid composition of putative RE cell tropomyosin was similar to that of the BHK-21 cell, platelet, and brain proteins (Table I). In particular, the absence of proline was strong evidence that troponin, the most likely candidate for coisolation with tropomyosin in this protocol, was not present. Finally, Fig. 9 shows that all three RE cell subunits are tropomyosin by virtue of their anomolous migration in urea/SDS (50), and that the putative tropomyosin bands in the RE cell microfilament fraction behave similarly under these conditions, as well as being heat stable (51). A sample of a RE cell microfilament preparation was made 1 M in KCl, incubated for 10 min at 100°C, and then centrifuged for 3 h at 100,000 *g*. The supernatant (heat-stable) and pellet fractions were applied to polyacrylamide gels containing SDS (38) and to similar gels with 6 M urea and SDS. In these gel systems (Fig. 9), the RE cell tropomyosin subunits comigrated with heat-stable polypeptides of the microfilament extract, and all putative tropomyosin bands migrated more slowly than actin in the presence of urea/SDS. Polypeptides of the RE cell isolated tropomyosin fraction thus display characteristics consistent with their identification as tropomyosin. The possibility that one or more of the putative tropomyosin chains represent proteolytic fragments of native tropomyosin may eventually be addressed (*a*) by physically separating these chains and conducting assays for their biological activity, such as those described above for BHK-21 tropomyosin, or (*b*) by characterizing the genes that encode various tropomyosins.

DISCUSSION

Fractionation of rat embryo, BHK-21, and 3T3 cells by a gentle, rapid method resulted in a preparation of filaments that morphologically resembled muscle thin filaments. Decoration with muscle subfragment 1 demonstrated that the filaments contained actin. Increasing the magnesium concentration of the microfilament fraction by dialysis resulted in aggregation of the filaments into structures that resembled microfilament bundles.

Although results of immunofluorescence studies have indicated the presence of various proteins in association with filamentous structures in cells, it is important to establish the presence and to quantitate the amounts of these proteins by independent biochemical criteria. Our results provide a potential means to this end by demonstrating that a cell fraction, morphologically consisting nearly exclusively of individual microfilaments, was enriched, as determined by gel electrophoresis, for HMW protein, myosin, and tropomyosin, and that

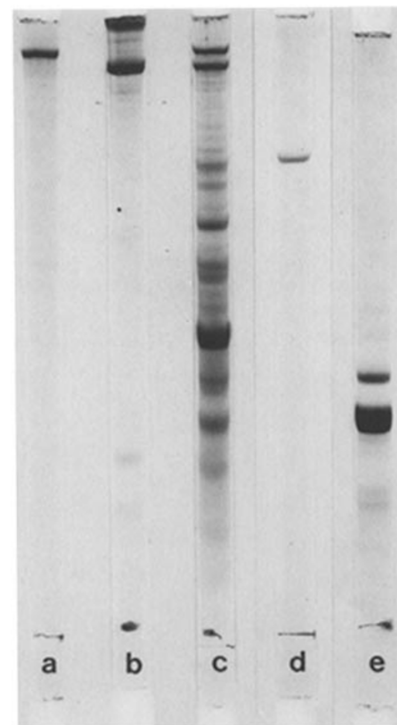

FIGURE 5 Identification of major microfilament proteins by comigration with purified proteins on SDS-polyacrylamide gels (37): (*a*) BHK-21 cell HMW protein, (*b*) BHK-21 cell myosin, (*c*) RE cell microfilament fraction I, (*d*) smooth muscle α-actinin (included as a marker only), and (*e*) BHK-21 cell tropomyosin. Compare also Figs. 6 and 9.

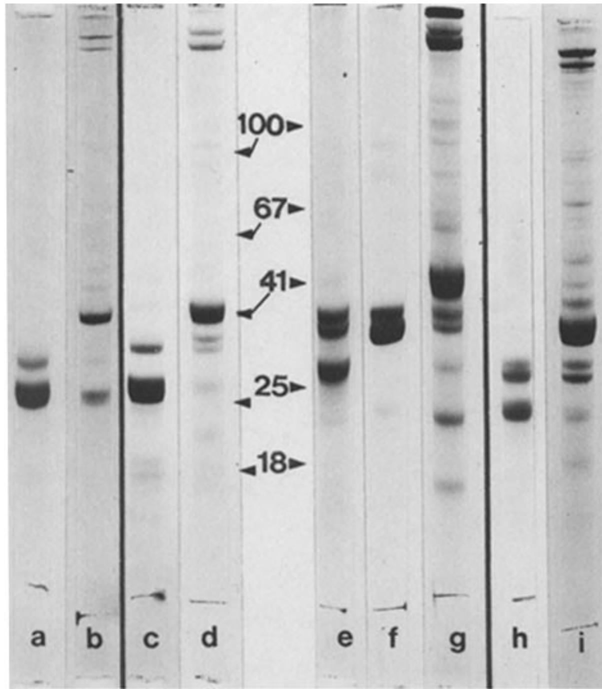

FIGURE 6 SDS-polyacrylamide gel electrophoresis (37) of isolated microfilaments and tropomyosin. BHK-21 cell tropomyosin (*a* and *c*) comigrated with polypeptides in microfilaments isolated from BHK-21 cells (*b*) and RE cells (*d*). RE cell tropomyosin (*e*) contained a major doublet that comigrated with skeletal muscle tropomyosin (*f*), as well as a major 29,000 mol wt chain. All three RE cell subunits comigrated with RE cell isolated microfilament subunits (*g*). 3T3 cell tropomyosin (*h*) was similar to the RE cell protein and comigrated with subunits present in microfilaments isolated from 3T3 cells (*i*). Molecular weight standards (from top to bottom): paramyosin, bovine serum albumin, actin, chymotrypsinogen A, and myoglobin. In comparing gels, note that R_f of polypeptides in gels *e–g* differs from those of other gels.

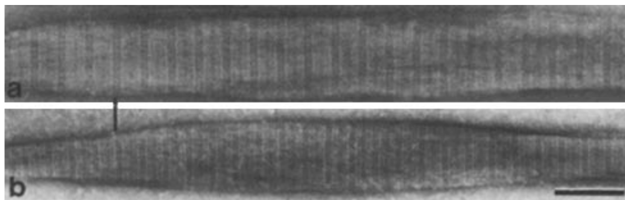

FIGURE 7 Paracrystals of BHK-21 cell and rabbit skeletal muscle tropomyosin. The paracrystals were formed by dialysis of 2-3 mg/ml samples of purified protein against 50 mM Tris-Cl, pH 8.0, followed by dialysis against 50 mM $MgCl_2$ in the same buffer (24). The structures are similar, but a vernier relationship exists between the muscle tropomyosin paracrystal (*a*) and the shorter axial period of the nonmuscle paracrystal (*b*). Electron micrographs of negative stains. Bar, 0.1 μm. × 92,000.

these proteins were further enriched when magnesium-induced aggregates of the microfilaments were separated from the remainder of the fraction.

The HMW protein, which is known to cross-link F-actin (35), may play a role in this aggregation. Isolated microfilaments containing HMW protein were aggregated in solutions containing as little as 5 mM $MgCl_2$, whereas in a parallel experiment a minimum of 20 mM $MgCl_2$ was required to form even small aggregates of purified skeletal muscle F-actin (not shown). Lateral aggregation was not highly dependent on specific conditions of salt, pH, or divalent cation concentration. Finally, we observed small donut-shaped objects similar to those seen in other preparations enriched for actin-binding proteins (52, 53). These structures were fairly abundant in negative stains of preparations in which microfilaments were not laterally aggregated, but were rarely seen among aggregated microfilaments. Perhaps the donut-shaped objects are a noninteracting form of actin-binding protein, and when they combine with actin to cross-link filaments, they assume a different morphology.

TABLE I

Amino Acid Compositions of Nonmuscle Cell Tropomyosins

	BHK-21 cell	RE cell	Human platelet*	Chick brain‡
Asp	88	83	88	84
Thr	30	28	26	30
Ser	41	52	25	38
Glu	245	241	256	224
Pro	6	0	0	5
Gly	37	28	33	32
Ala	94	109	105	84
Val	36	30	36	40
Met	12	19	20	15
Ile	30	29	37	33
Leu	98	96	106	72
Tyr	11	11	8	14
Phe	7	4	7	13
His	9	8	4	12
Lys	87	98	79	71
Arg	59	55	61	56

Compositions for platelet and brain are expressed as moles per 10^5 grams. Values for BHK-21 and RE samples are expressed so that the total number of residues equals that in platelets.

* See reference 24.

‡ See reference 26.

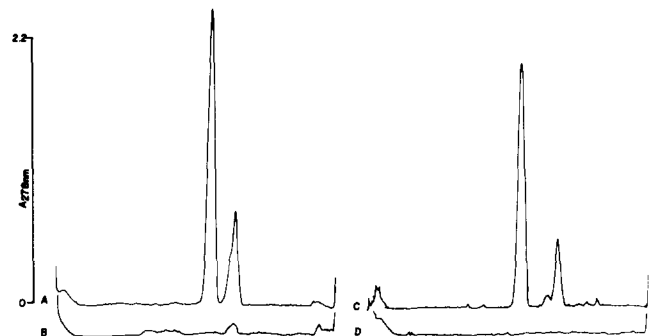

FIGURE 8 Tropomyosin binding to actin. Skeletal muscle actin and tropomyosin and BHK-21 cell tropomyosin were dialyzed against 50 mM NaCl, 5 mM $MgCl_2$, 0.2 mM DTT, 0.1 mM EGTA, 10 mM imidazole-Cl, pH 7.0. The tropomyosins were centrifuged for 3 h at 160,000 *g* to remove aggregated material. The following samples were then prepared: (*A*) 500 μg actin plus 220 μg muscle tropomyosin; (*B*) 220 μg muscle tropomyosin; (*C*) 500 μg actin plus 225 μg BHK-21 tropomyosin; (*D*) 225 μg BHK-21 tropomyosin. Each tube contained a total volume of 1 ml. The samples were centrifuged for 3 h at 160,000 *g*, the supernates were removed, and the pellets were rinsed. The pellets were then suspended in sample buffer, and an equal volume of each was run on SDS-polyacrylamide gels (37). Gels were stained quantitatively with fast green FCF (43) and scanned at 635 nm. Relative amounts of stained material were determined by cutting out and weighing relevant peaks. Scan *B* was subtracted from scan *A*, the values were corrected for differential dye uptake (46, 47), and the molar ratios of actin:tropomyosin were calculated. The assay was based on the method of Fine et al. (26).

Table II

BHK-21 Tropomyosin in a Calcium-sensitive Hybrid Assay System

	Specific activity		Calcium sensitivity*
	$+Ca^{++}$	$-Ca^{++}$	
	µmol P_i/ mg myosin/ min		
A·M	0.081	0.077	4.9
A·M·TN	0.126	0.120	4.8
A·M·TM·TN	0.114	0.010	91.2
A·M·BHK TM	0.104	0.106	0
A·M·BHK TM·TN	0.113	0.020	82.3
	0.121	0.014	88.4

ATPase was assayed for 15 min at 24°C in 2 ml of 30 mM KCl, 5 mM $MgCl_2$, 25 mM Tris-HCl, pH 7.5, and 2.5 mM ATP (added to initiate the reaction). For assays in the absence of calcium, 1 mM EGTA was added. Protein concentrations were: 52 µg rabbit actin/ml, 252 µg rabbit myosin/ml, 15 µg rabbit tropomyosin/ml, 20 µg BHK-21 tropomyosin/ml, and 22 µg rabbit troponin/ml. *A*, actin; *M*, myosin; *TN*, troponin; *TM*, muscle tropomyosin; *BHK TM*, nonmuscle tropomyosin.

* $(1-[SA_{-Ca^{++}}]/[SA_{+Ca^{++}}]) \times 100$.

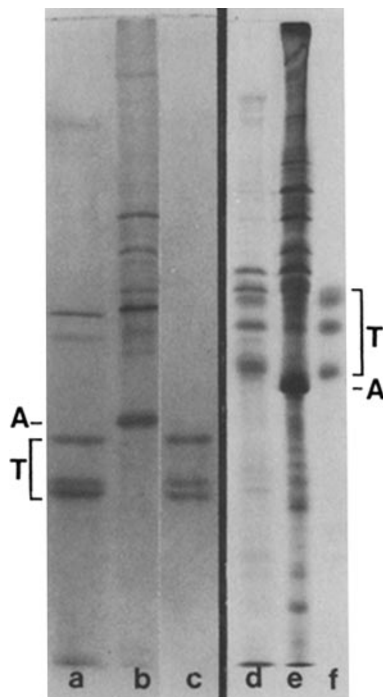

Figure 9 SDS- and urea/SDS-polyacrylamide gel analysis of RE cell tropomyosin and the heat-stable fraction of isolated RE cell microfilaments. The microfilament sample was fractionated as described in the text. Samples were applied to a SDS-12.5% polyacrylamide (30:0.8 acrylamide:bis) slab gel (*a–c*; see reference 38) and to a similar gel (*d–f*) with 6 M urea in the separating gel, 3 M urea in the stacking gel, and 5 M urea in the sample (28). (*a* and *d*) Supernatant and (*b* and *e*) pellet fractions of heated microfilament sample. (*c* and *f*) RE cell tropomyosin fraction. *A*, actin; *T*, tropomyosin.

Although myosin was present in these fractions, presumably bound to actin-containing filaments, myosin filaments were not seen in negative stains. Only after prolonged dialysis were myosin-like thick filaments observed. The appearance of these filaments probably resulted from the gradual decrease in ATP concentration during dialysis under conditions of low ionic strength. The presence of a myosin-like component in the microfilament fraction, despite the inclusion of ATP in the buffer B step, was consistent with observations of other workers, who have reported a prerequisite for nonmuscle actomyosin dissociation, namely actin depolymerization by chaotropic agents (54, 55).

The presence of tropomyosin as a component of microfilaments was suggested by two morphological (as well as the biochemical) observations. First, negative stains of isolated microfilaments revealed straighter fibers than are seen in preparations of purified F-actin filaments. Second, our finding that transverse banding was generally not seen in magnesium-induced microfilament aggregates, was similar to the observation that magnesium-induced paracrystals of actin-tropomyosin complexes do not, whereas pure actin paracrystals do, display transverse banding in negative stain (41).

At the present time, it is impossible to demonstrate rigorously that the isolated microfilaments existed in precisely the same form in the living cell. Several factors argue qualitatively in that direction. The isolation method was based on procedures for purifying muscle thin filaments. When gel profiles of thin filaments from vertebrate, mollusc, annelid, and brachiopod muscle were compared with myofibrils from the same organisms, similar relative amounts of actin and associated proteins were observed (56). Because of the complexity of SDS-polyacrylamide gels containing whole nonmuscle cell homogenates (large number of closely migrating proteins, large variation in abundance of proteins, etc.), similar analysis for nonmuscle systems is difficult. We cannot rule out the possibility that some of the proteins present in the supernate after magnesium precipitation, were bound to microfilaments in the initial fraction and were released during dialysis. Perhaps under other conditions the 98,000, 75,000, 62,000, and 58,000 mol wt proteins that remained in the supernate could be shown to have an affinity for microfilaments. However, the stoichiometries determined for the major proteins in the magnesium-precipitated samples support the possibility that isolated microfilaments were similar to structures that existed in vivo. Assuming that our assignments of protein identity were correct, then, for the microfilament population isolated, actin was saturated with tropomyosin, and one myosin or HMW protein molecule was present for every two to three turns of the actin helix.

The cells from which microfilaments were isolated contained prominent phase-dense stress fibers, the equivalent of microfilament bundles (e.g., references 32, 34, 57). Corresponding phase-dense fibers were seen after scraping the cells from the culture dishes in buffer A (not shown). F-actin was not seen in negatively stained preparations of the buffer A washes. These fractions contained 50–70% of total cell protein, and a considerable portion of the cells' 42,000 mol wt protein (Fig. 2*b*), but attempts to induce actin to polymerize in these fractions were unsuccessful.[1] This may be explained by the presence of a polymerization inhibitor, such as profilin (58). Unless all of the microfilaments that remained in the cells were depolymerized during homogenization and centrifugation and then replaced by in vitro polymerized F-actin, to which other proteins then bound (a possibility that we cannot formally exclude), it is reasonable to assume that the isolated microfilaments originated from microfilament bundles originally present in the cells.

SDS-polyacrylamide gel profiles obtained from isolated microfilaments revealed an unexpected pattern of putative tropomyosin subunits. We observed that RE and 3T3 cell micro-

[1] J. A. Schloss. Unpublished observations.

filaments contained polypeptides that migrated at 29,000, 35,000, and 37,000 mol wt. Earlier work based on nonmuscle tropomyosin isolation, immunological identification, or partial purification resulted in reported molecular weights of either 29,000–30,000 (20, 24–28, 59, 60) or 34,000–35,000 (9, 48, 61). In a few instances, a minor 34,000 mol wt subunit was observed in addition to the major 30,000 mol wt species. However, the former was considered to be a contaminating protein (24) or a smooth muscle tropomyosin subunit, present because of blood vessels contaminating the tissue under investigation (25, 26, 59). Our studies show that proteins similar to "muscle" (35,000) and "nonmuscle" (30,000) tropomyosins are present within a single culture of either morphologically homogeneous (rat embryo) or cloned (3T3) nonmuscle cells. These findings agree with the recent report that three tropomyosin species are present in cultures of L6 myoblasts (62). Whether tropomyosin-like proteins observed in RE, 3T3, BHK-21, and L6 cells are similar and whether they are, in fact, expressed simultaneously within a single cell remains to be demonstrated.

We feel that the ability to isolate microfilaments represents a new approach to analysis of their properties and those of their associated proteins in nonmuscle cells. The techniques will be used to study regulation of microfilament organization in cells. For example, a protein such as the HMW protein might play a role in the conversion of meshwork microfilaments into bundles (23). Studies are underway to determine whether consistent differences exist between the microfilaments isolated from cells in various physiological states.

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