Tropomyosin-enriched and Alpha-actinin-enriched Microfilaments Isolated from Chicken Embryo Fibroblasts by Monoclonal Antibodies

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ABSTRACT   Antitropomyosin and anti-alpha-actinin monoclonal antibodies have been used to isolate two classes of microfilaments, i.e., tropomyosin-enriched and alpha-actinin-enriched microfilaments, respectively, from cultured chicken embryo fibroblasts. Electron microscopic studies of the isolated tropomyosin-enriched microfilaments showed periodic localization of tropomyosin along the microfilaments, with a 35-nm repeat. On the contrary, the isolated alpha-actinin-enriched microfilaments showed no obvious periodicity. Many individual alpha-actinin-enriched microfilaments with length >1 μm (ranging from 1 to 10 μm) were aggregated by anti-alpha-actinin monoclonal antibodies. Both of the isolated microfilaments had the ability to activate the Mg^{2+}-ATPase activity of skeletal muscle myosin, although different extents of activation were observed. These two classes of microfilaments also differed in their protein composition. Molar ratios of major identifiable proteins in the isolated microfilaments were alpha-actinin(dimer):actin(monomer):tropomyosin(dimer) = <0.02:8.06:1.00 for tropomyosin-enriched microfilaments and 0.44:13.91:1.00 for alpha-actinin-enriched microfilaments. By two-dimensional gel analysis of the isolated microfilaments, we have found seven spots which possess typical tropomyosin properties including pl 4.5, immunological cross-reaction, lack of proline and tryptophan, and heat stability. Pulse-chase experiments suggested that the assembly of microfilament-associated proteins, at least for alpha-actinin and tropomyosins, was coordinately regulated by the assembly of actin into microfilaments.

Stress fibers observed in cultured living cells are composed ultrastructurally of many individual microfilaments (6). By the use of immunofluorescence microscopy, several contractile proteins such as actin (33), myosin (12, 53), alpha-actinin (13, 32), tropomyosin (29), filamin (23, 52), as well as vinculin (15), have been found to be associated with these microfilament bundles. Microfilaments are thought to be involved in cell spreading and attachment to substratum (6, 33, 54), cytokinesis (47), maintenance of cell shape (8, 17, 18, 24, 42), and cell motility such as locomotion, membrane ruffling, and microspike activity (1, 19, 24, 27, 42, 43, 49).

Microfilaments are reported to exist in several supramolecular forms such as microfilament bundles (or stress fibers) (6, 17, 19), microfilament meshworks (19), polygonal networks (20, 30, 32, 49, 55), and contractile rings (47). The formation of microfilaments and their assembly into these supramolecular forms appear to be transient, dynamic, and interconvertible. For example, microfilament bundles gradu-

ally form after cultured cells are replated (30); a contractile ring forms during cytokinesis and disappears after cell division (47); polygonal networks found in some cell types have been suggested to serve as structural precursors of stress fibers (30, 31). It is unlikely that actin itself can regulate the formation and the interconversion of these supramolecular forms, since it is limited to the formation of filaments. Therefore, the presence of microfilament-associated proteins may be needed for regulating the formation of these supramolecular forms in response to appropriate cellular activities such as spreading, mitosis, and motility, etc.

One approach to explore this control mechanism is to isolate microfilaments from cells having different organization of microfilaments and characterize them both biochemically and morphologically. Schloss and Goldman (46) have reported a method to prepare a population of microfilaments from serum-depleted, cultured cells. Recently, we have developed a rapid method to isolate tropomyosin-containing mi-

crofilaments from a variety of types of cultured cells by using antitropomyosin monoclonal antibodies (38). To extend this microfilament isolation method, we have now used both antitropomyosin and anti-alpha-actinin monoclonal antibodies to fractionate two classes of microfilaments, that is, tropomyosin-enriched and alpha-actinin-enriched microfilaments. We report here on the isolation and characterization of these two classes of microfilaments from chicken embryo fibroblasts. In addition, we examined the rate of assembly of major microfilament-associated proteins, i.e., actin, alpha-actinin, and tropomyosin, into microfilaments by pulse-chase experiments.

MATERIALS AND METHODS

Cell Culture: A population of primary chicken embryo cells (CEF)[1] was prepared from 10-d-old embryos as follows. Skins from legs and backs of embryos were dissected out and treated with 1 ml of 0.05% trypsin in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.3) for 15–20 min. Digestion was terminated by the addition of 9 ml of Dulbecco's modified Eagle's medium (DME) containing 10% fetal calf serum (FCS). Trypsinized cells thus prepared were collected by centrifugation and cultured in DME containing 10% FCS in an atmosphere of 5% CO_2 and 95% air at 37°C. Secondary and tertiary cultures of CEF cells were used for all experiments to isolate microfilaments.

Preparation of Antitropomyosin and Anti-alpha-actinin Monoclonal Antibodies and Polyclonal Antiserum: Antitropomyosin monoclonal antibodies, JLF15, JLH2, and LCK16, have been prepared and characterized as described previously (35). As reported previously (37, 38), ascites fluids of these monoclonal antibodies are suitable for the isolation of tropomyosin-containing microfilaments from a variety of cultured cells. In the present study, LCK16 antibody (IgM, ~7-10 mg/ml of specific antibody in ascites fluid) was used for most experiments.

Anti-alpha-actinin monoclonal antibodies, JLN1, JLN20, JLN23, and JLK13 have been prepared and characterized as described previously (35). In the initial experiments, we found that only JLN20 antibody against alpha-actinin was capable of immunoprecipitating the alpha-actinin-containing microfilaments from CEF cells. This may be due to the suitable affinity of this antibody and/or the proper localization of antigenic determinant on alpha-actinin molecules for this antibody. Thus, the binding of this antibody to alpha-actinin molecules would probably not interfere with the association between alpha-actinin and actin microfilaments. The JLN20 (IgM, ~5-7.5 mg/ml of specific antibody in ascites fluid) antibody was used throughout all experiments for the isolation of alpha-actinin-enriched microfilaments from CEF cells.

Rabbit polyclonal antibody against chicken gizzard tropomyosin was prepared and characterized as described previously (38). Rabbit polyclonal antibodies against beef heart alpha-actinin or chicken gizzard alpha-actinin were generous gifts from Dr. K. Burridge (University of North Carolina at Chapel Hill).

Isolation of Tropomyosin-enriched Microfilaments and Alpha-actinin-enriched Microfilaments from CEF Cells: The microfilament isolation method was essentially similar to that described previously (38). As outlined in Fig. 1, CEF cells (20–30 plates of 100-mm culture dishes) after washing three times with phosphate-buffered saline were treated with Triton X-100/glycerol solution (0.05% Triton X-100, 0.1 M PIPES, 5 mM $MgCl_2$, 0.2 mM EGTA, 4 M glycerol) for 2 min at room temperature. After washing three times with phosphate-buffered saline containing 5 mM $MgCl_2$ and 0.2 mM EGTA, the Triton/glycerol-insoluble residues were scraped off from dishes and homogenized in 20 mM phosphate buffer, pH 7.0, containing 100 mM NaCl, 5 mM $MgCl_2$, 5 mM ATP, 0.2 mM EGTA, and 5 mM phenylmethyl sulfonyl fluoride. The total homogenates were then centrifuged at 12,800 g for 15 min to remove a majority of the intermediate filaments and nuclei. The supernatant (denoted as Sup-1) was incubated with either one-tenth volume of antitropomyosin monoclonal antibody (LKC16) or one-fifth volume of anti-alpha-actinin monoclonal antibody (JLN20) for 1 h at 4°C. The resultant immunoprecipitates were collected and washed three times in phosphate-buffered saline containing 5 mM $MgCl_2$ and 0.2 mM EGTA by low-speed centrifugation (12,800 g for 5 min). The final pellet was designated as either tropomyosin-enriched microfilaments (TM_1-MF) or alpha-actinin-enriched microfilaments (αA_1-MF), respectively. In some experiments, the anti-

body supernatant was further precipitated with either anti-alpha-actinin (JLN20), or antitropomyosin (LCK16) antibody, respectively. The resultant pellets were designated as alpha-actinin-enriched microfilaments after the removal of tropomyosin-enriched microfilaments (αA_2-MF) or tropomyosin-enriched microfilaments after the removal of alpha-actinin-enriched microfilaments (TM_2-MF). In experiments where the microfilaments were precipitated by simultaneous addition of both LCK16 and JLN20 antibodies, the microfilaments were denoted as tropomyosin and alpha-actinin-enriched microfilaments ([TM+αA]-MF).

The yield of isolated microfilaments was expressed as the actin content. For quantitation, CEF cells were in vivo labeled for 16 h with 250 μCi of [^{35}S]-methionine (1,100 Ci/mmol) in methionine-free DME containing 2.5% FCS and subjected to microfilament isolation as described above. Aliquots of samples from each step of the isolation were analyzed by 12.5% SDS PAGE (2, 28). After staining and destaining, gels were then dried on filter paper. The actin bands of each fraction were cut out and sliced into ~1 mm thickness. Each slice was immersed in 0.2 ml of 2% SDS for 24 h to elute protein. After addition of 3 ml of Aquasol (New England Nuclear, Boston, MA), the radioactivity was measured by a liquid scintillation counter.

Molar ratios of actin to alpha-actinin and major forms of tropomyosin in the isolated microfilaments were calculated from densitometer traces of one-dimensional gels as described (21, 44). Gels were quantitatively stained with fast green (21) and scanned with a Hoefer densitometer (GS 300). The molar amounts of actin, alpha-actinin, and tropomyosin were obtained after correcting values for the differential dye uptake for skeletal muscle actin, gizzard alpha-actinin, and gizzard tropomyosin, respectively (44).

Pulse-chase Experiments: CEF cells were pulse-labeled for 10 min with 1 mCi of [^{35}S]methionine in methionine-free DME containing 2.5% FCS at 37°C. At the end of the pulse period, excess amounts of methionine (2 mg/ml) were added into the culture medium to start the chase. Samples were taken at 0, 10, 30, and 60 min after chase. At each time point, one 100-mm dish of [^{35}S]methionine-labeled cells together with six 100-mm dishes of unlabeled cells was subjected to microfilament isolation as described above. An aliquot of each isolated microfilament fraction was analyzed by SDS PAGE. The radioactivity incorporated into proteins (alpha-actinin, actin, and major tropomyosin) was measured as described above.

One- and Two-dimensional Gel Electrophoresis: One-dimensional SDS PAGE was carried out according to Laemmli (28) with a low concentration of bisacrylamide (12.5% acrylamide and 0.104% bisacrylamide) (2). Two-dimensional gel electrophoresis was performed as described by a modified (14) procedure of O'Farrell (40). The first-dimension tube gel contained 4% acrylamide and pH 5 to 7 ampholytes. The second-dimensional gels were 12.5% SDS polyacrylamide slab gels with a low concentration of bisacrylamide (2). For autoradiography, gels after electrophoresis were stained with 0.15% Coomassie Brilliant Blue in 50% methanol, 10% acetic acid for 2 h and thoroughly destained with 7.5% methanol, 7.5% acetic acid. For fluorography, as a modified method of Bonner and Laskey (5), gels after electrophoresis were soaked twice in DMSO for 30 min each time, once in 8% PPO in DMSO for 90 min and in water overnight. Gels were then dried on filter paper. Radioactive protein spots in the dried gels were detected on Kodak XR-1 film.

Electron Microscopy: Small aliquots of isolated microfilaments were applied onto a carbon-coated formvar grid and negatively stained with 2.5% aqueous uranyl acetate. Samples were observed in a Philips 201E electron microscope at an accelerating voltage of 80 kV. For measurement of periodicity of tropomyosin localization along microfilaments, collagen fibers from a rat tail were mixed with the isolated microfilaments. Micrographs of both microfilament bundles and collagen fibers in the same field were taken, and the periodicity of tropomyosin localization was measured by using the periodicity (64 nm) of collagen fibers as an internal standard.

Assay for Myosin ATPase Activity: Myosin was prepared from rabbit skeletal muscle by the method of Perry (41). F-actin was prepared from acetone powder of rabbit skeletal muscle by the method described previously (38). Myosin ATPase assay was carried out at 24°C in 20 mM imidazole buffer pH 7.0 containing 50 mM KCl, 5 mM $MgCl_2$, 50 μM $CaCl_2$, and 10 mM dithiothreitol with 70 μg/ml of myosin and various amounts of F-actin (0–100 μg) or microfilament fractions. ATP concentration was kept constant (0.1 mM) by using 0.2 mg/ml pyruvate kinase and 2 mM phosphoenolpyruvate as the ATP-regenerating system. The activities were calculated from the initial rate of pyruvate liberation as determined by the method of Reynard et al. (45).

Other Biochemical Procedures: Protein concentration was determined by the method of Lowry et al. (36) with bovine serum albumin as standard. Immunoprecipitation with rabbit antiserum against tropomyosin and against alpha-actinin was carried out as described previously (38) in the buffer containing 50 mM Tris, pH 8.0, 0.05% SDS, 0.5% Triton X-100, 1 mM EDTA, 1 mM PMSF, 100 mM NaCl. Indirect immunofluorescence was carried out as described by Blose (3).

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[1] Abbreviations used in this paper: CEF, chicken embryo cells; DME, Dulbecco's modified Eagle's medium.

RESULTS

Localization of Tropomyosin and Alpha-actinin on Microfilamental Bundles at the Immunofluorescence Microscopic Level

When CEF cells were stained with antitropomyosin monoclonal antibody (JLF15) by indirect immunofluorescence, a typical periodic staining pattern on stress fibers, as described previously (29, 35), was visualized. Whereas some stress fibers (majority of big bundles) do not show a periodicity of fluorescence, the fluorescence in most sheath fibers shows a well resolved periodicity. The average periodicity is 0.94 μm (0.80–1.07 μm) with fluorescent spots 0.65 μm long. Because stress fibers are composed ultrastructurally of many actin-containing microfilaments, stress fibers with no periodical stain by anti-tropomyosin antibody may be due to the alignment of the fluorescent segments in adjacent microfilaments being out of register. No fluorescent staining can be found in the membrane ruffle region.

When CEF cells were stained by indirect immunofluorescence with anti-alpha-actinin monoclonal antibody (JLN20), most of the staining was confined to the stress fibers with a distinct periodicity (32, 35). The overall periodicities are 1.08 μm (0.85–1.31 μm) with fluorescent spots 0.4 μm long. In addition, alpha-actinin is also distributed at regions where many filamental bundles terminate or at adhesion plaque regions (32, 35).

When CEF cells were double-stained with antitropomyosin and anti-alpha-actinin monoclonal antibodies, the periodic staining pattern on stress fibers (microfilament bundles) was no longer observed. Instead, continuous stain on stress fibers was seen. This complementary localization between tropomyosin and alpha-actinin has been previously reported by several investigators (11, 16, 20, 32). Because immunofluorescence microscopy cannot resolve single, individual microfilaments, an interesting question will be raised as to whether tropomyosin and alpha-actinin are complementary on the same individual microfilaments or on different but parallel microfilaments. If they are complementary on different microfilaments that interdigitate, one would expect at least two classes of microfilaments existing in cells. The results from experiments described below support the possibility that two classes of microfilaments, i.e., tropomyosin-enriched and alpha-actinin-enriched microfilaments, are indeed present in CEF cells.

Isolation of Tropomyosin-enriched and Alpha-actinin-enriched Microfilaments

Secondary or tertiary passage of CEF cells at 90% confluence was first treated with Triton/glycerol. The Triton/glycerol residues were collected and homogenized in the presence of Mg^{2+}-ATP to disperse microfilaments. After low-speed centrifugation (Eppendorf centrifuge, 12,800 g for 15 min) removed nuclei and the majority of the intermediate filaments, the supernatant (Sup-1) was used for incubation with either antitropomyosin (JLF15 or LCK16) or anti-alpha-actinin (JLN20) monoclonal antibody at 4°C. As can be seen in Table I, the Sup-1 fraction contained ~60% of total cellular actin. The individual microfilaments were found in the dispersed state in the Sup-1 fraction by electron microscopy. They appeared to be stable in this fraction for at least 2 d,

TABLE I

Distribution of actin in various fractions during Isolation of Tropomyosin- and Alpha-actinin-enriched microfilament from Chicken Embryo Fibroblasts

Fractions	% of total actin*			
	Exp 1	Exp 2	Exp 3	Exp 4
Total cells	100	100	100	100
Triton/glycerol extract	20.0	14.3	10.6	11.2
Triton/glycerol residues	80.0	85.7	89.4	88.8
Pellet-1	17.6	19.3	16.7	ND
Sup-1	57.3	58.6	64.7	65.4
TM₁-MF	30.0	24.3	31.9	32.5
αA₁-MF	2.5	2.1	2.4	2.2
αA₂-MF	0.2	0.4	0.1	0.2
TM₂-MF	20.0	15.6	19.2	23.0
Antibody supernatant	15.8	15.9	ND	14.7
40,000-rpm supernatant	9.8	ND	ND	ND
40,000-rpm pellet	6.0	ND	ND	ND

Pellet-1, pellet after the first low-speed centrifugation (12,800g, 15 min). Sup-1, supernatant after the first low-speed centrifugation (12,800g, 15 min). TM₁-MF, tropomyosin-enriched microfilaments. αA₁-MF, alpha-actinin-enriched microfilaments. αA₂-MF, alpha-actinin-enriched microfilaments after the removal of tropomyosin-enriched microfilaments (TM₁-MF) from Sup-1 fraction. TM₂-MF, tropomyosin-enriched microfilaments after the removal of alpha-actinin-enriched microfilaments (αA₁-MF) from Sup-1 fraction. Antibody supernatant, supernatant after the immunoprecipitations with both anti-tropomyosin and anti-alpha-actinin. 40,000-rpm supernatant and pellet, supernatant and pellet, respectively, after the high speed centrifugation (100,000g, 2 h) of the antibody supernatant. ND, Not determined.
* Amounts of [^{35}S]methionine incorporation into the actin band of each fraction were used for calculation of percent (see Materials and Methods).

suggesting that the Triton/glycerol treatment of cells was very effective for stabilizing microfilaments. The Triton/glycerol extraction released ~15% of total cellular actin to the supernatant. This fraction of actin may represent the monomeric form of actin or G-actin in cells. After low-speed centrifugation of homogenates of the Triton/glycerol residue, ~20% of total actin was found to be lost from the pellet 1. It is likely that the actin in the pellet fraction may be in a highly cross-linked form of microfilaments and/or in membrane-associated microfilaments.

As described in previous report (37, 38), antitropomyosin monoclonal antibodies (LCK16 or JLF15) caused the lateral aggregation of tropomyosin-containing microfilaments in the Sup-1 fraction to form ordered bundles which could then be easily collected by low-speed centrifugation (12,800 g, 5 min). About 30% of total cellular actin was associated with this tropomyosin-enriched microfilament (TM₁-MF, see Fig. 1) in CEF cells. This yield is comparable to that described in the previous paper for rat embryo REF-52 cells (38).

For the isolation of alpha-actinin-enriched microfilaments (αA₁-MF), we first screened several monoclonal antibodies against alpha-actinin (JLN1, JLN20, JLN23, and JLK13; see reference 35) in order to obtain the antibody that was able to efficiently precipitate microfilaments from the Sup-1 fraction of CEF cells. The criteria used for determining whether microfilaments were effectively precipitated were: (a) SDS polyacrylamide gel analysis to show the coexistence of alpha-actinin, actin and immunoglobulins; and (b) electron microscopic observation of actin-containing microfilaments in the precipitates. JLN20 anti-alpha-actinin monoclonal antibody was the only one that fit these criteria. As can be seen in Table I, alpha-actinin-enriched microfilaments obtained by JLN20 antibody contained ~2.3% of total cellular actin. The
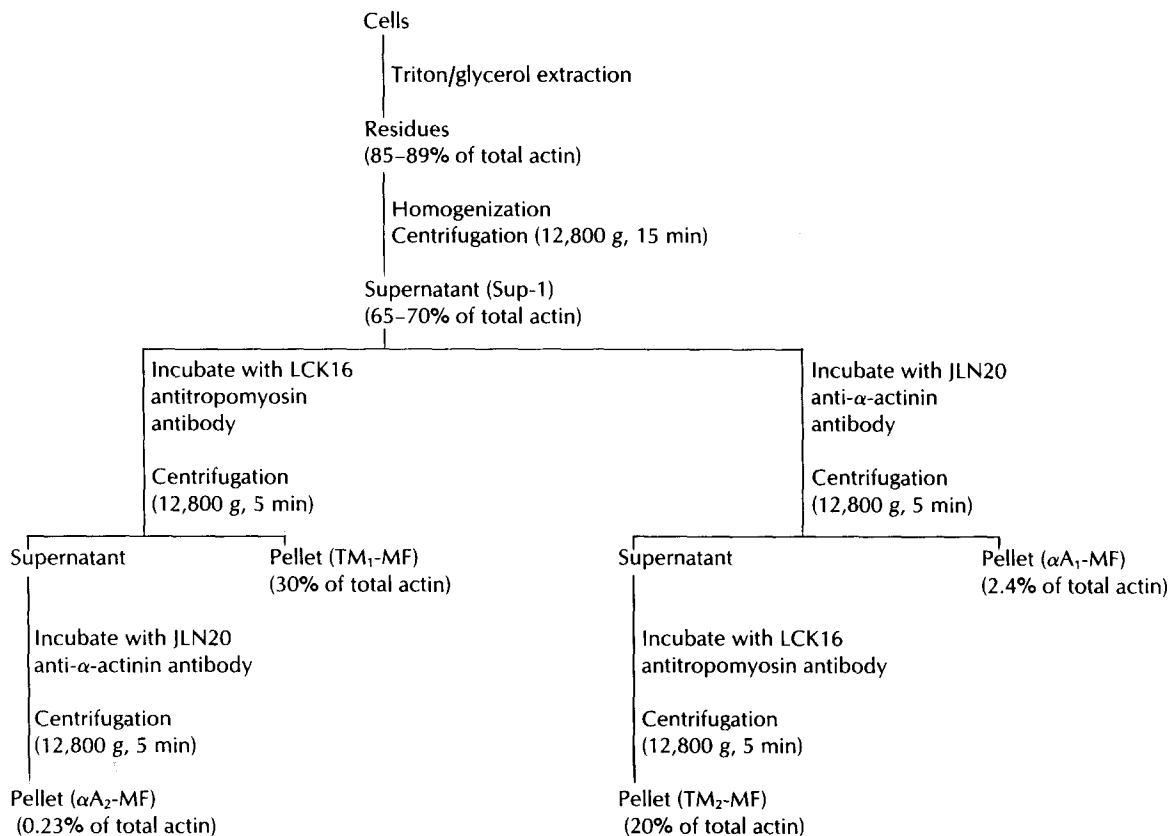
Cells

Triton/glycerol extraction

Residues
(85–89% of total actin)

Homogenization
Centrifugation (12,800 g, 15 min)

Supernatant (Sup-1)
(65–70% of total actin)

Incubate with LCK16
antitropomyosin
antibody

Centrifugation
(12,800 g, 5 min)

Supernatant — Pellet (TM₁-MF)
(30% of total actin)

Incubate with JLN20
anti-α-actinin antibody

Centrifugation
(12,800 g, 5 min)

Pellet (αA₂-MF)
(0.23% of total actin)

Incubate with JLN20
anti-α-actinin
antibody

Centrifugation
(12,800 g, 5 min)

Supernatant — Pellet (αA₁-MF)
(2.4% of total actin)

Incubate with LCK16
antitropomyosin antibody

Centrifugation
(12,800 g, 5 min)

Pellet (TM₂-MF)
(20% of total actin)

FIGURE 1 Schematic outline for the isolation of tropomyosin-enriched and alpha-actinin-enriched microfilaments from chicken embryo fibroblasts.

yield of (αA_1-MF) is less than one-tenth of (TM_1-MF), based on their actin content.

The supernatant after the removal of tropomyosin-enriched or alpha-actinin-enriched microfilaments was further incubated with anti-alpha-actinin (JLN20) or anti-tropomyosin (LCK16) antibody, respectively, to precipitate the other class of microfilaments (see Fig. 1). About 0.2% of total cellular actin was recovered in the fraction called alpha-actinin-enriched microfilaments after the removal of tropomyosin-enriched microfilaments (αA_2-MF), whereas 19.5% of total cellular actin was found in the fraction called tropomyosin-enriched microfilaments after the removal of alpha-actinin-enriched microfilaments (TM_2-MF). The fact that we have been able to further precipitate the other class of microfilament from the supernatant after the removal of one class of microfilament may suggest that two classes of microfilaments exist in cells.

The actin remaining in the final antibody supernatant was ~15% of total cellular actin (Table I). Of this, about 40% was precipitated by centrifugation at 100,000 g for 2 h.

Protein Compositions of Tropomyosin-enriched and Alpha-actinin-enriched Microfilaments

Protein components of the isolated microfilaments were analyzed by 12.5% SDS PAGE (2, 28). Fig. 2 shows protein profiles of Sup-1 and various isolated microfilaments. In addition to heavy and light chains of immunoglobulins (indicated by asterisks in Fig. 2), several protein bands such as those of 105,000 mol wt, 58,000 mol wt, 38,000 mol wt, 36,500 mol wt, and 32,800 mol wt are clearly enriched in the

isolated microfilament fractions as compared to protein composition of the Sup-1 fraction (lane sup-1 in Fig. 2). The 58,000-mol-wt protein was most likely vimentin (the major subunit of the fibroblastic intermediate filaments) because of its mobility in the two-dimensional gel (see Fig. 5). The association of vimentin with tropomyosin-enriched microfilaments (lanes LCK16 and JLN20 → LCK16 in Fig. 2) appeared to be due to the cross-reaction of LCK16 antibody to both tropomyosin and vimentin (35). When we used another IgM anti-tropomyosin monoclonal antibody (JLF15) to isolate tropomyosin-enriched microfilaments, no detectable amount of vimentin was observed. On the other hand, vimentin in alpha-actinin-enriched microfilaments (lane JLN20 in Fig. 2) might be real, since we could also find the intermediate filaments in this microfilaments fraction by electron microscopy. This possibility may be supported by recent reports that intermediate filaments are associated with dense bodies of smooth muscle cells (4) and with adhesion plaques of cultured fibroblasts (51).

The protein band with apparent molecular weight of 105,000 was identified as alpha-actinin by co-migration with purified chicken gizzard alpha-actinin on two-dimensional gels (data not shown) and by immunoprecipitation with rabbit antiserum against chicken gizzard alpha-actinin (lane 4 in Fig. 3) and against beef heart alpha-actinin (lane 5 in Fig. 3). The proteins with apparent molecular weights of 38,000, 36,500, and 32,800 were identified as multiple forms of tropomyosin from chicken embryo fibroblasts by the criteria of two-dimensional gel analysis (Figs. 4 and 5), immunoprecipitation with rabbit antiserum against chicken gizzard tropomyosin in the presence of SDS (lane 2 in Fig. 3), lack of
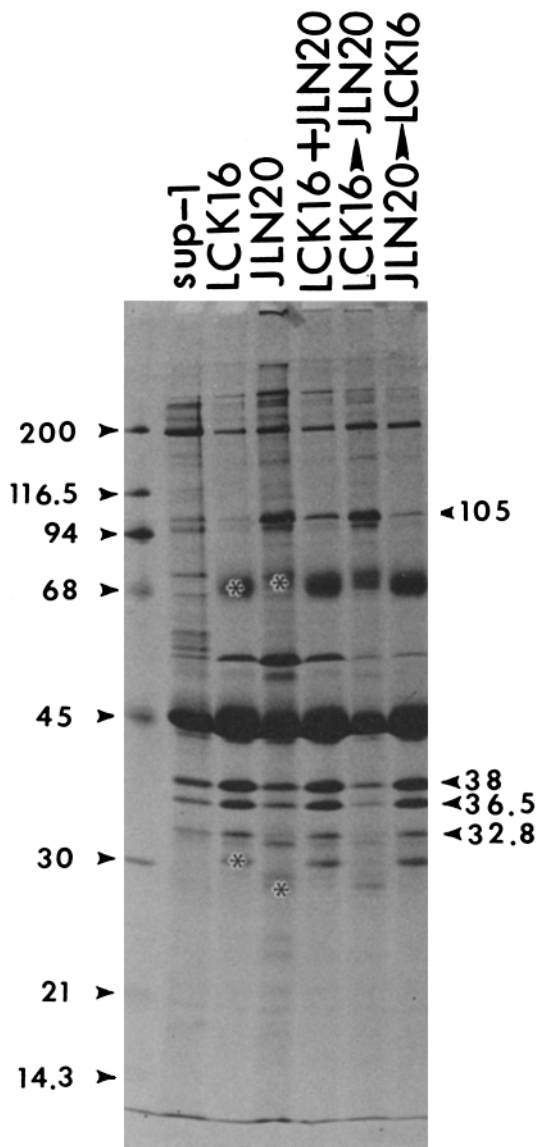
FIGURE 2 SDS polyacrylamide gel analysis of the isolated microfilaments from CEF cells. *Sup-1*, the supernatant fraction after the removal of nuclei and intermediate filaments from the homogenates of Triton/glycerol residues; *LCK16*, tropomyosin-enriched microfilaments (TM₁-MF); *JLN20*, α-actinin-enriched microfilaments (αA₁-MF); it should be noted that a protein with a molecular weight of 32,000 was preferentially enriched in this microfilament fraction. *LCK16 + JLN20*, tropomyosin and α-actinin-enriched microfilaments ([TM+αA]-MF). *LCK16 → JLN20*, alpha-actinin-enriched microfilaments after the removal of tropomyosin-enriched microfilaments from Sup-1 (αA₂-MF); *JLN20 → LCK16*, tropomyosin-enriched microfilaments after the removal of α-actinin-enriched microfilaments (TM₂-MF). Asterisks indicate the heavy and light chains of IgM monoclonal antibodies. Molecular weights, × 10⁻³.

proline and tryptophan (Fig. 4), and heat stability (data not shown). Two-dimensional gel analysis of the isolated microfilaments reveals more tropomyosin spots which have a similar pI value of 4.5 (Figs. 4 and 5). Spots *1* and *2* in Fig. 4 correspond to 38,000- and 36,500-mol-wt proteins detected in one-dimensional gels (Figs. 2 and 3), whereas the 32,800-mol-wt tropomyosin is now resolved into two distinguished spots (indicated by *3a* and *3b* in Figs. 4 and 5). In addition, minor spots indicated by *a* (45,000 mol wt), *b* (43,000 mol wt), and *c* (38,000 mol wt) in Figs. 4 and 5 are also identified

as tropomyosin by the same criteria described above. For unknown reasons, tropomyosins a, b, 1, and 2 are usually found to be split into two molecular weight variants in two-dimensional gels with pH 5–7 ampholytes but not with pH 3.5–10 ampholytes. Therefore, at least seven distinct tropomyosin spots are present in the chicken embryo fibroblasts. These tropomyosin spots could also be found in two-dimensional gel patterns of the total cell extract (Fig. 5A), which was prepared by quickly solubilizing cells in SDS gel sample buffer (containing 2% SDS) and boiling for 3 min to prevent modification by proteolytic digestion. Moreover, the relative ratio of these tropomyosin spots (except *3a* and *3b*) was similar to that obtained from the isolated microfilament (Fig. 5C), suggesting that these seven tropomyosin forms were not derived from proteolytic fragments of other proteins during isolation of the microfilament. As will be described below, pulse-chase experiments also ruled out the possibility that there were precursor-product relationships between these multiple forms of tropomyosin in CEF cells.

Among these seven forms of tropomyosin (see Figs. 4 and 5), spots *a* (45,000 mol wt) and *c* (38,000 mol wt) appeared to have identical mobility on two-dimensional gels with slow-migrating and fast-migrating components, respectively, of chicken gizzard tropomyosin (data not shown). Because these muscle forms of tropomyosin were found to be still present in the long-term culture of CEF cells (more than 10 passages), and because we prepared CEF cells from trypsinized skins of legs and backs of embryos, the contamination by either smooth or skeletal muscle cells would be minimized. This may further suggest that CEF cells prepared here express a trace but significant amount of smooth muscle tropomyosin.

Different Protein Contents Found in Tropomyosin-enriched and Alpha-actinin-enriched Microfilaments

For quantitation we calculated molar ratios of alpha-actinin and actin to tropomyosin by scanning the fast green-stained gels as described under Materials and Methods. The results are shown in Table II. In tropomyosin-enriched microfilaments (TM₁-MF and TM₂-MF) every 7–8 mol of actin monomer were covered with 1 mol of tropomyosin dimers (38,000, 36,500, and 32,800 mol wt), whereas in alpha-actinin-enriched microfilaments (αA₁-MF and αA₂-MF) every 14 to 15 mol of actin monomers contained only 1 mol of tropomyosin dimer. A small amount of alpha-actinin was associated with the tropomyosin-enriched microfilaments, whereas the alpha-actinin-enriched microfilaments contained 20–40-fold higher amounts of alpha-actinin (Table II). These results suggest that there are at least two classes of microfilaments in CEF cells. One class of microfilaments is enriched in tropomyosin content (called tropomyosin-enriched microfilaments, TM₁-MF or TM₂-MF). The other has high content of alpha-actinin and less amount of tropomyosin (called alpha-actinin-enriched microfilaments, αA₁-MF or αA₂-MF). In addition to a difference in alpha-actinin and tropomyosin contents, a protein band with molecular weight of 32,000 was found preferentially enriched in alpha-actinin-enriched microfilaments (lane JLN20 in Fig. 2). Although this protein has not been identified, the difference also supports the idea that two classes of microfilaments exist in the cells.

Molar ratios of alpha-actinin dimer and actin monomer to tropomyosin dimer in the microfilaments isolated by simul-

(A)
Coomassie blue
stain

(B)
Autoradiogram

1 2 3 4 5   1 2 3 4 5

200 ➤

116.5 ➤

94 ➤

68 ➤

45 ➤

30 ➤

21 ➤

14.3 ➤

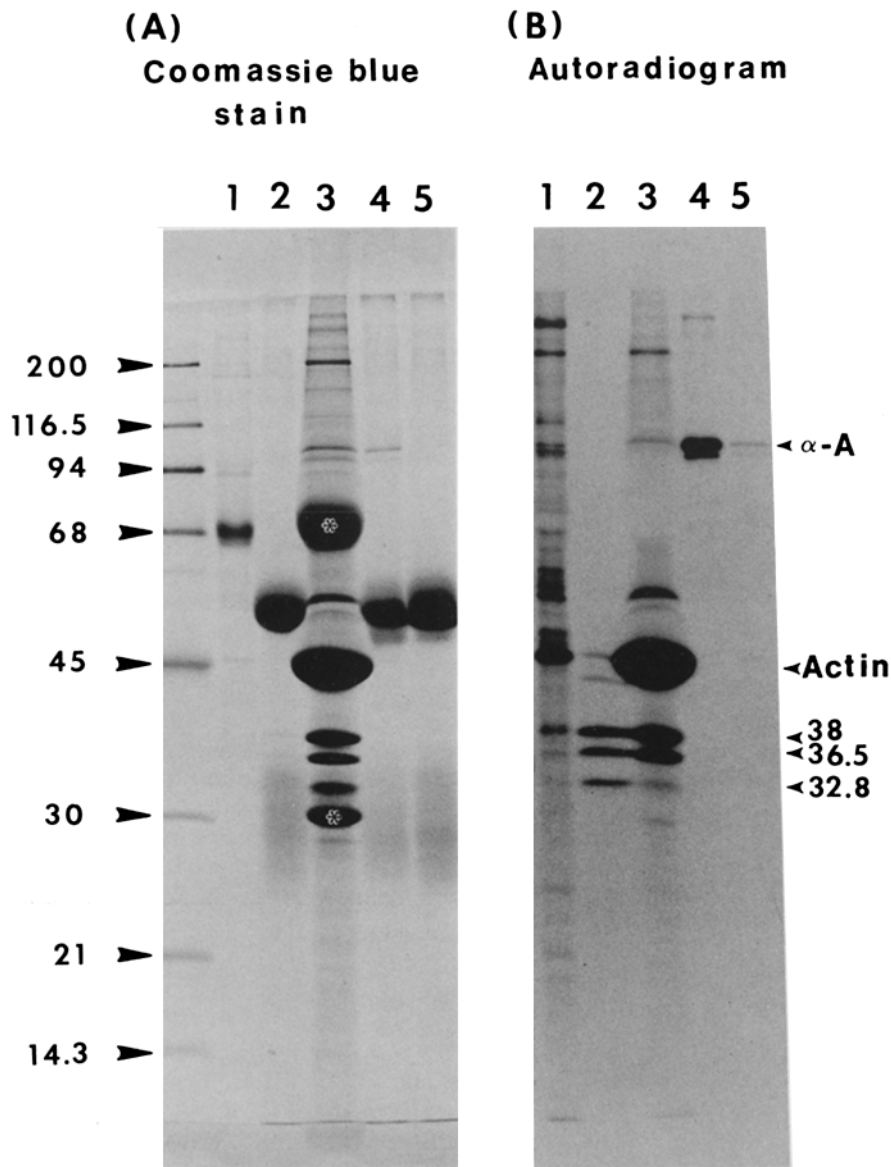◄ α-A

◄Actin

◄38
◄36.5

◄32.8

FIGURE 3 SDS polyacrylamide gel analysis of the isolated tropomyosin-enriched microfilaments and the immunoprecipitates of total cell extracts with rabbit antiserum against tropomyosin and against α-actinin. (A) Coomassie-Blue-stained gel. (B) Autoradiogram of the same gel. Lane *1*, total extract of [^{35}S]methionine-labeled CEF cells; lane *2*, immunoprecipitate of total extract by rabbit antiserum against chicken gizzard tropomyosin. In addition to 38,000, 36,500, and 32,800-mol-wt forms of tropomyosin, two other forms with molecular weights of 45,000 and 43,000 were also immunoprecipitated by this antibody. These two proteins have mobilities on two-dimensional gels identical to that of spots *a* and *b* (see Fig. 5*A*) of the isolated microfilaments. Lane *3*, the isolated tropomyosin-enriched microfilaments. Asterisks in Coomassie-Blue-stained gel indicate the heavy and light chains of monoclonal antibody. Lanes *4* and *5*, immunoprecipitates of total extracts by rabbit antiserum against chicken gizzard alpha-actinin and against beef heart α-actinin, respectively. α-A, α-actinin (105,000 mol wt).

taneous addition of anti-alpha-actinin and antitropomyosin antibodies (called tropomyosin and alpha-actinin-enriched microfilaments [TM+αA]-MF) were 0.04:7:1, which was similar to those for tropomyosin-enriched microfilaments. This is because total actin content in the alpha-actinin-enriched microfilaments is only ¹⁄₁₂ of that in the tropomyosin-enriched microfilaments (see Table I). It should also be noted that molar ratios between 38,000-, 36,500-, and 32,800-mol-wt tropomyosin forms were very similar for all the isolated microfilaments.

Activation of Myosin ATPase Activity by Isolated Microfilaments

We have previously shown that tropomyosin-containing microfilaments isolated from rat embryo cells (REF-52) and L6 myoblasts have the ability to activate skeletal muscle myosin ATPase activity (38). It is interesting to test whether the two classes of microfilaments isolated here show any difference in the extent of activation of myosin ATPase activity. As Table III shows, both tropomyosin-enriched mi-

crofilaments (TM$_1$-MF and TM$_2$-MF) and alpha-actinin-enriched microfilaments (αA$_1$-MF and αA$_2$-MF) are able to activate myosin ATPase activity. This suggests that filamentous actin in the microfilaments isolated by monoclonal antibodies remains native in terms of its ability to activate myosin ATPase activity. However, the microfilaments precipitated by the tropomyosin antibodies have more myosin-activating activity per actin molecule than the microfilaments precipitated by the alpha-actinin antibodies (Table III).

Electron Microscopic Characterization of Isolated Microfilaments

When tropomyosin-enriched microfilaments were negatively stained and examined by electron microscopy, ordered bundles of microfilaments formed by antitropomyosin monoclonal antibodies can be readily observed (Fig. 6*A*). In addition, cross-striations along whole bundles are evident. These cross-striations represent the location of monoclonal antibodies, which in turn indicates the position of antigenic determinant on tropomyosin molecules. By using the perio-
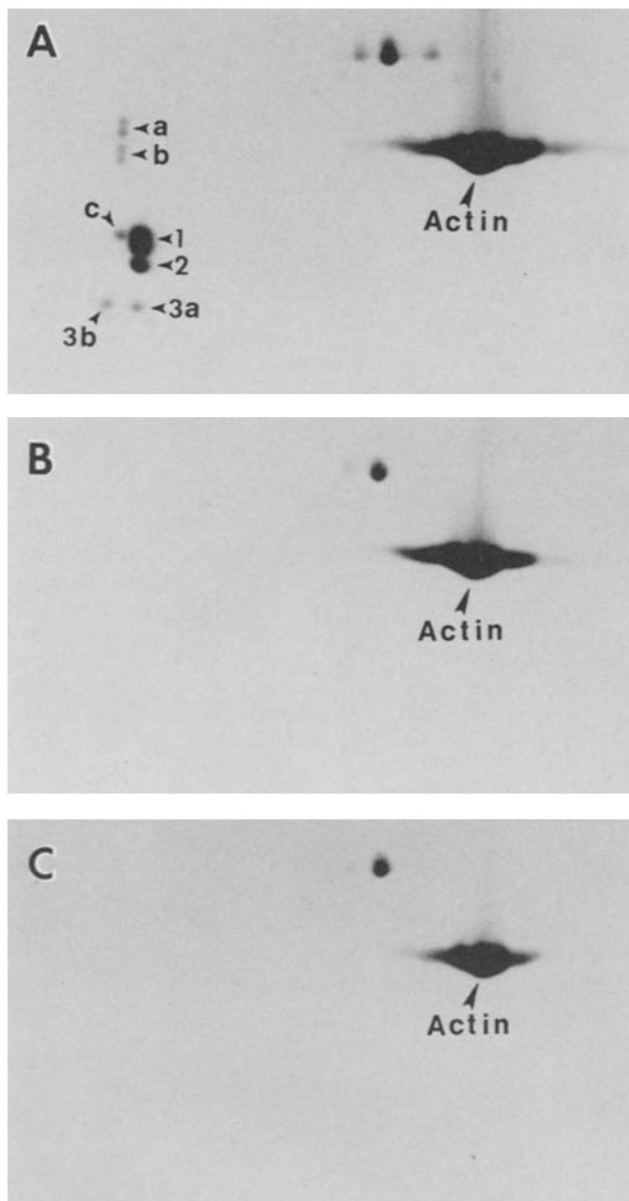
Figure 4 Two-dimensional gel analysis of the tropomyosin-enriched microfilaments of CEF cells. (A) The tropomyosin-enriched microfilaments isolated from [^{35}S]methionine-labeled cells. (B) The tropomyosin-enriched microfilaments isolated from [^{3}H]proline-labeled cells. (C) The tropomyosin-enriched microfilaments isolated from [^{3}H]tryptophan-labeled cells. After electrophoresis, gels were processed for fluorography. Only those parts of the gels containing tropomyosin and actin are shown here, with the acidic end to the left. Multiple forms of tropomyosin were indicated by a, b, c, 1, 2, 3a, and 3b. Note that all these polypeptides were not labeled with [^{3}H]proline or [^{3}H]tryptophan, which is one of the characteristics of tropomyosin.

dicity of 64 nm for collagen fibers as an internal standard, we have obtained the tropomyosin periodicity of 35 ± 1 nm (n = 80) for CEF cell microfilaments.

Alpha-actinin-enriched microfilaments isolated by anti-alpha-actinin monoclonal antibodies show no obvious periodicity. In contrast, one can observe many aggregates converged from 10 to 20 individual microfilaments with length >1 μm (ranging from 1 to 10 μm) (Fig. 6B). At the higher

magnification (Fig. 6C), IgM monoclonal antibodies can be recognized by their star-like structure (9, 10) along the single microfilaments as well as the aggregated region (as indicated by arrowheads in Fig. 6C). The aggregated region may represent an alpha-actinin cluster.

Assembly of Microfilament-associated Proteins into Microfilaments

Having developed a rapid and reproducible method for the isolation of tropomyosin and alpha-actinin-enriched microfilaments ([TM+αA]-MF), we used this technique to examine the rates of assembly for actin, alpha-actinin, and different forms of tropomyosin (38,000, 36,500, 32,800 mol wt) into microfilaments from CEF cells. Cells were pulse-labeled for 10 min with [^{35}S]methionine. At various time points (0, 10, 30, and 60 min) of chase, aliquots of labeled cells were mixed with unlabeled cells and subjected to isolation of microfilaments ([TM+αA]-MF). The isolated microfilaments were then analyzed on SDS polyacrylamide gels. The [^{35}S]methionine incorporation into each protein was measured by cutting the Coomassie-Blue-stained protein bands out and counting them in a liquid scintillation counter. The assembly of alpha-actinin, actin and tropomyosins (38,000, 36,500, and 32,800 mol wt) into microfilaments appeared to reach a maximum within 30 min. Moreover, the relative ratios of each protein (i.e., alpha-actinin and tropomyosin) to actin appear to be constant during the chase period, suggesting that the assembly of microfilament-associated proteins, at least for alpha-actinin and tropomyosins (38,000, 36,500, and 32,800 mol wt), was coordinately regulated by the assembly of actin into microfilaments.

DISCUSSION

In the present study, we used antitropomyosin and anti-alpha-actinin monoclonal antibodies to isolate two classes of microfilaments from chicken embryo fibroblasts. Two classes of microfilaments, i.e., tropomyosin-enriched and alpha-actinin-enriched microfilaments, differ in their protein composition and their extent of activation for myosin ATPase activity. The molar ratios of alpha-actinin(dimer), actin(monomer), and tropomyosin(dimer) in the isolated tropomyosin-enriched (TM$_1$-MF) and alpha-actinin-enriched (αA$_1$-MF) microfilaments were <0.02:8.06:1.00 and 0.44:13.91:1.00, respectively. The low content of tropomyosin in the alpha-actinin-enriched microfilament may be responsible for the relative low activity in activation of skeletal muscle myosin ATPase, compared to the extent of activation by the tropomyosin-enriched microfilament. This is supported by the recent observation that both smooth muscle and nonmuscle tropomyosins can stimulate more myosin ATPase activity in reconstituted skeletal actomyosin (7, 48). Therefore, tropomyosin-enriched microfilaments would stimulate more ATPase activity than alpha-actinin-enriched microfilaments.

Although it is impossible to assess the purity of microfilaments, the isolation method described here appears to be very specific. Neither class of microfilaments could be obtained if a specific monoclonal antibody was replaced by either a heat-denatured monoclonal antibody or another monoclonal antibody such as JLT12 against troponin T, which was known to be absent in microfilaments of nonmuscle cells. Furthermore, as described in previous reports (38), the isolation of
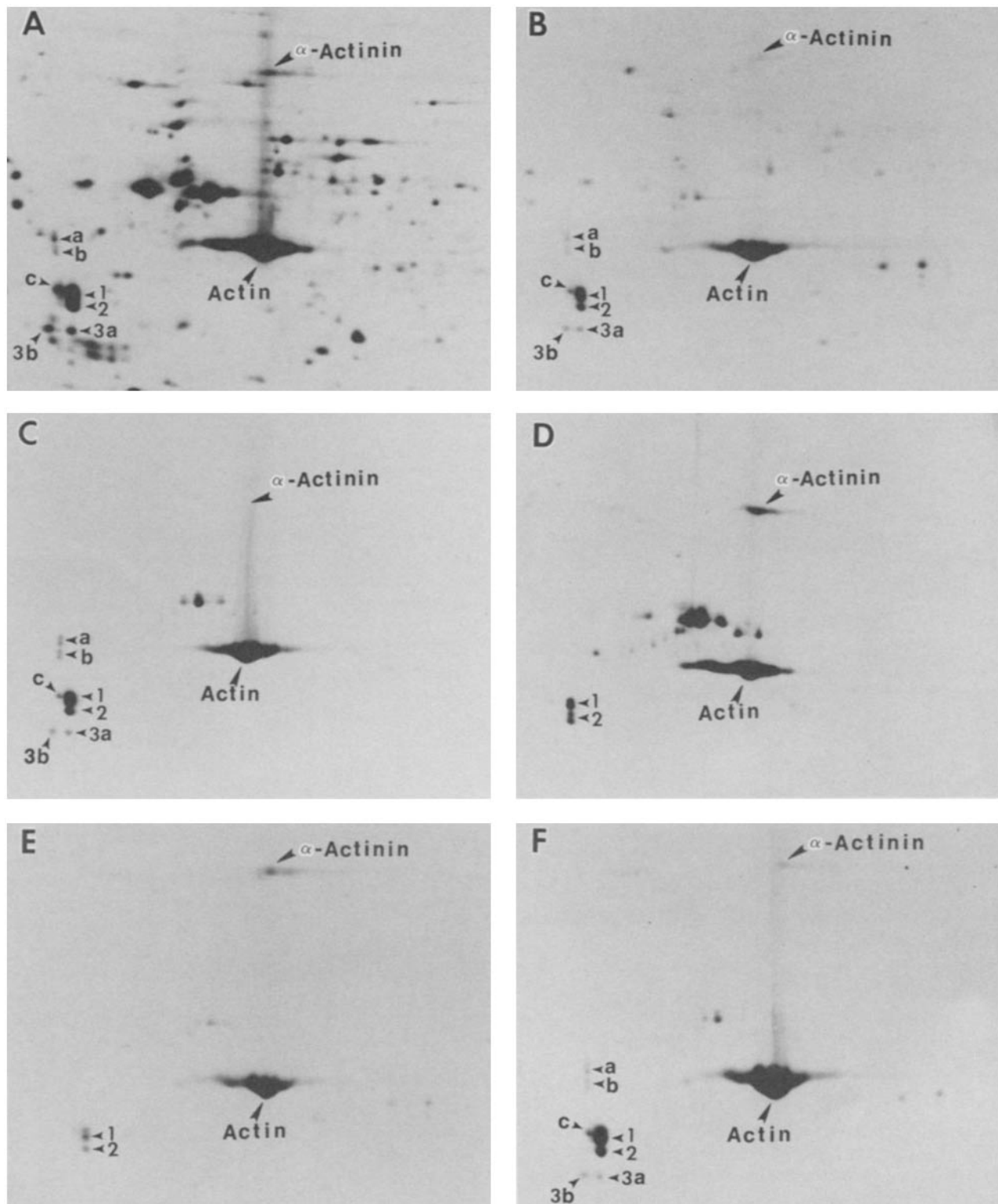
FIGURE 5   Two-dimensional gel analysis of the isolated microfilaments from [^{35}S]methionine-labeled CEF cells. (A) Total extract. (B) The supernatant fraction (Sup-1) after the removal of nuclei and intermediate filaments from the homogenates of Triton/glycerol residues. (C) The tropomyosin-enriched microfilaments (TM$_1$-MF); (D) the alpha-actinin-enriched microfilaments (αA$_1$-MF). (E) The α-actinin-enriched microfilaments after the removal of the tropomyosin-enriched microfilaments from Sup-1 fraction (αA$_2$-MF). (F) The tropomyosin-enriched microfilaments after the removal of the alpha-actinin-enriched microfilaments from Sup-1 fraction (TM$_2$-MF). Multiple forms of tropomyosin were indicated by a, b, c, 1, 2, 3a, and 3b. Fluorographs of the gels were shown here, with the acidic end to the left.

TABLE II

Molar Ratios of Alpha-actinin and Actin to Tropomyosin in
Microfilaments Isolated from Chicken Embryo Fibroblasts*

	TM_1-MF	αA_1-MF	αA_2-MF	TM_2-MF	$(TM + \alpha A)$-MF
$\alpha A^{\ddagger}$ (dimer)	<0.02	0.44	0.80	<0.02	0.04
Actin (monomer)	8.06	13.91	15.34	7.51	7.41
$TM^{\ddagger}$ (dimer)					
38,000	0.52	0.58	0.61	0.56	0.54
36,500	0.31	0.31	0.28	0.32	0.33
32,800	0.17	0.11	0.11	0.12	0.13

See Table I legend for symbols.
* Average values used here were obtained from four experiments by scanning fast-green-stained gels (see Materials and Methods). Molar ratios provided are given relative to the total amount of the tropomyosin (38,000, 36,500, and 32,800).
‡ αA, alpha-actinin; TM, tropomyosins (38,000, 36,500, and 32,800-mol-wt proteins).

TABLE III

Activation of Skeletal Muscle Myosin ATPase Activity by
Tropomyosin- and Alpha-actinin-enriched Microfilaments
Isolated from Chicken Embryo Fibroblasts

Components	ATPase activity*	Active actin content§
	(umol/mg/min)	(µg/ml)
Myosin (70 µg/ml)‡	0.008	0
Myosin + actin	0.017	2.5
actin	0.036	10
actin	0.056	25
actin	0.099	100
Myosin + TM_1-MF (58 µg/ml)‡	0.055	23.5
αA_1-MF (20 µg/ml)‡	0.022	4.5
αA_2-MF (80 µg/ml)‡	0.040	13
TM_2-MF (71 µg/ml)‡	0.066	35

See Table I legend for symbols.
* Myosin ATPase activity was measured at 24°C and in the presence of 10 mM dithiothreitol. No ATPase activity can be detected in either one of the microfilament fractions alone or in ascites fluid alone.
‡ Parentheses indicate amounts of total proteins in each microfilament fraction. The total actin content in these fractions was estimated as about half of the total protein content, based on SDS polyacrylamide gel analysis of these fractions.
§ Active actin content was expressed as the equivalent amount of muscle actin to show the same extent of activation on myosin ATPase activity.

microfilaments by this method appeared to be affected by the biological state of microfilaments in vivo, such as a reduced yield of microfilaments obtained from cytochalasin B-treated cells.

The microfilament isolation method described here consistently recovered 30% of total cellular actin in the tropomyosin-enriched microfilament (TM_1-MF) and 2.3% of total cellular actin in the alpha-actinin-enriched microfilament (αA_1-MF). The tropomyosin content in both of these microfilaments was considerably higher than that reported in the isolated microfilaments of rat embryo cells by Schloss and Goldman (46). This discrepancy is not due to the difference in cell species, because we have also obtained a similar tropomyosin content in tropomyosin-enriched microfilaments from rat embryo REF-52 cells (38). It is quite likely that the microfilament isolated by Schloss and Goldman (46) may represent a different class of microfilament in vivo, such as tropomyosin-depleted microfilaments or those enriched in tropomyosin-free microfilaments. It should be noted that in

our microfilament isolation method, a significant amount (15.8%) of actin remained in the antibody supernatant after the removal of both TM_1-MF and αA_2-MF. Further analysis of this antibody supernatant indicated that ~40% of this actin could be pelleted by high-speed centrifugation (100,000 g for 2 h). This may suggest that this actin exists in filamentous and/or membranous forms. Moreover, very little, if any, or no detectable amount of tropomyosin was found in the antibody supernatant fraction. Thus, in addition to tropomyosin-enriched and alpha-actinin-enriched classes of microfilaments described here, a third class of microfilaments such as a tropomyosin-depleted or tropomyosin-free microfilament may exist in cells. On the basis of immunofluorescence studies, Lazarides (31) has previously suggested that there may indeed be classes of actin filaments in cultured fibroblasts that are either tropomyosin-free or tropomyosin-depleted. Additionally, Singer et al. (23) have similarly suggested that there are classes of myosin-free actin filaments in tissue culture cells. Therefore, many classes of microfilaments may indeed exist in the cultured cell. These classes of microfilaments may play different roles in cell motility and maintenance of cell shape.

Electron microscopic studies of the isolated tropomyosin-enriched microfilaments led to the suggestion that tropomyosin molecules are arranged along actin filaments with a periodicity of 35 nm. This value is significantly less than that reported for rabbit skeletal muscle tropomyosin (38), guinea pig smooth muscle (38), and chicken gizzard tropomyosin (37). This further suggests that one tropomyosin dimer may bind to six actins, instead of seven actins in the case of skeletal muscle tropomyosin.

Alpha-actinin-enriched microfilaments isolated here showed many aggregates converged from 10 to 20 individual microfilaments. It is likely that these aggregates are caused by anti-alpha-actinin monoclonal antibody (IgM). Therefore, the aggregated region may represent an alpha-actinin cluster. In addition, few alpha-actinin molecules are distributed along single microfilaments, as revealed by the star-like structure of IgM (Fig. 6C). It is unlikely that alpha-actinin-enriched microfilaments are derived from a breakdown of tropomyosin-enriched microfilaments during isolation. The length of individual microfilaments found in alpha-actinin-enriched microfilaments ranges from 1 to 10 µm, which is longer than the length of fluorescent spots revealed by antitropomyosin antibody in immunofluorescence micrographs. If they were derived from pieces of tropomyosin-enriched microfilaments, they should have the same ratio of actin to tropomyosin. This is certainly not the case because the actin to tropomyosin ratio in alpha-actinin-enriched microfilaments (αA_1-TM and αA_2-TM) is much larger than that in tropomyosin-enriched microfilaments (TM_1-MF and TM_2-MF).

A schematic representation of two classes of microfilaments of CEF cells is shown in Fig. 7. Both classes of microfilaments are made up of a double-helical array of actin polymers (indicated by a long, straight line in Fig. 7), which have lengths ranging from 1 to 10 µm. Clusters of tropomyosin (indicated by shaded rectangles) and alpha-actinin (indicated by shaded circles) are distributed periodically at about a 1-µm repeat along the tropomyosin-enriched microfilaments (Fig. 7A, equivalent to TM_2-MF in our preparation) and the alpha-actinin-enriched microfilaments (Fig. 7B, equivalent to αA_1-MF in our preparation), respectively. Based on our biochemical data, the amounts of actin in the tropomyosin-enriched
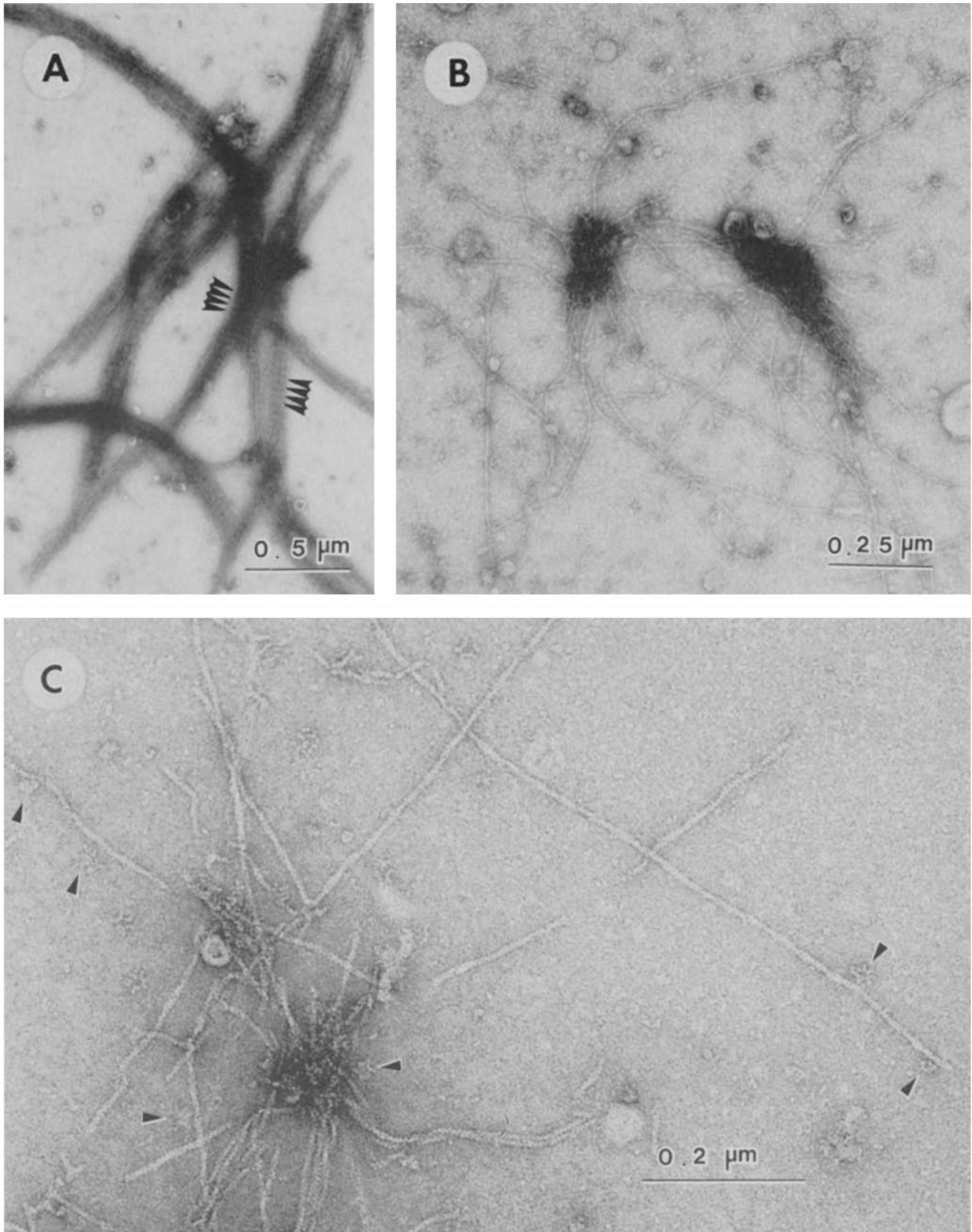
Figure 6   Electron micrographs of the isolated microfilaments from CEF cells. (A) The tropomyosin-enriched microfilaments (TM₁-MF). (B and C) The α-actinin-enriched microfilaments (αA₁-MF). The isolated microfilaments were negatively stained with uranyl acetate and observed with an electron microscope. Arrowheads in A indicate the localization of tropomyosin molecules visualized by the binding of antitropomyosin monoclonal antibody. Arrowheads in C show the star-like structure (9, 10) of IgM antibody, which may represent the localization of α-actinin molecules.
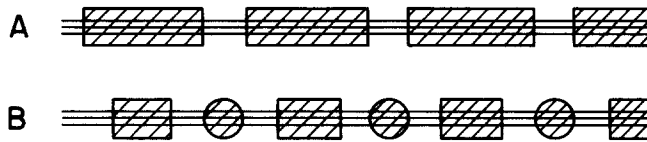
FIGURE 7 Schematic representation of (A) tropomyosin-enriched microfilaments (TM_2-MF) and (B) α-actinin-enriched microfilaments (αA_1-MF). The long, straight lines represent double-helical arrays of actin-containing microfilaments. Shaded rectangles and circles indicate tropomyosin-rich and α-actinin-rich regions, respectively, along microfilaments.

(TM_2-MF) and alpha-actinin-enriched (αA_1-MF) microfilaments possess 19.5% and 2.3%, respectively, of total cellular actin. Our preparation of TM_1-MF should be the summation of TM_2-M and αA_1-MF, which contain ~30% of total cellular actin. Although smaller amounts of tropomyosin are found in the alpha-actinin-enriched microfilament (αA_1-MF), this amount of tropomyosin appears to be enough for antitropomyosin monoclonal antibody to immunoprecipitate it (αA_1-MF) together with the tropomyosin-enriched microfilament (TM_2-MF). On the other hand, alpha-actinin in the tropomyosin-enriched microfilament (TM_2-MF) is not detectable or there is very little, if any, that cannot be immunoprecipitated by anti-alpha-actinin monoclonal antibody together with the alpha-actinin-enriched microfilament (αA_1-MF). The protein composition of αA_2-MF is similar to that of αA_1-MF, except for the higher alpha-actinin content. This αA_2-MF may represent the microfilaments localized at the focal contact or adhesive plaque regions. Because αA_2-MF could not be immunoprecipitated by anti-tropomyosin monoclonal antibody, tropomyosin on this microfilament would appear to be arranged randomly. However, more experiments are needed to prove this possibility.

It has been known that skeletal muscle tropomyosin is a rod-like dimer, which interacts in register to form a coiled-coil molecule (26, 34, 50). These molecules further aggregate in a head-to-tail fashion to form a polymer (39), which lies along each of the two grooves of F-actin filaments (22). Each skeletal muscle tropomyosin dimer has possible interactions with seven actin monomers (25). It is reasonable to assume that nonmuscle tropomyosin has a similar organization along the microfilament, except that each dimer may bind to six actin monomers. From data obtained by immunofluorescence and electron microscopy analysis, one can calculate the ratio of actin to tropomyosin in the tropomyosin-enriched microfilaments based on the model shown in Fig. 7A. The unit length of the tropomyosin molecule is 35 nm. In one periodical length of microfilament, the portion of microfilament containing tropomyosin is 0.65 μm and the rest (0.29 μm) of the microfilament does not contain tropomyosin. From the assumptions described above for the nonmuscle tropomyosin organization along the microfilament, one can calculate the number of actin molecules per one periodical length of the microfilament as the following. One periodical length of microfilament is equal to the length of (650/35 + 290/35) molecules of tropomyosin dimer. This will contain (650/35 + 290/35) × 6 molecules of actin. Thus, the ratio of actin (monomer) to tropomyosin (dimer) is (650/35 + 290/35) × 6/650/35 = 8.68. This ratio is very close to that obtained from a quantitation of the isolated tropomyosin-enriched microfilaments by scanning the fast green-stained gels (Table II).

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