Isolation and Characterization of Tropomyosin-containing Microfilaments from Cultured Cells*

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We have developed a new method for the rapid isolation of tropomyosin-containing microfilaments from cultured cells using anti-tropomyosin monoclonal antibodies. Anti-tropomyosin monoclonal antibodies induce the bundle formation of microfilaments, which can be easily collected by low speed centrifugation. Electron microscopic studies of the isolated microfilaments show periodic localization of tropomyosin along the microfilaments of nonmuscle cells with a 33-34 nm repeat. Furthermore, the isolated microfilaments have the ability to activate the Mg2+-ATPase activity of skeletal muscle myosin to almost the same extent as skeletal muscle F-actin (filamentous actin). This microfilament isolation method is applicable to a variety of cell types, including REF-52 cells (an established rat embryo line), L6 myoblasts, 3T3 fibroblasts, Chinese hamster ovary cells, baby hamster kidney (BHK-21) cells, mouse neuroblastoma cells, gerbil fibroma cells, and chicken embryo fibroblasts. Sodium dodecyl sulfate-polyacrylamide gel analysis shows that, in addition to actin, microfilaments isolated from REF-52 cells contain five species of tropomyosin with apparent M, = 40,000, 36,500, 35,000, 32,400, and 32,000, α-actinin, and as yet unknown proteins with apparent M, = 83,000 and 37,000. The molar ratio of total tropomyosin (dimer) to actin in the isolated microfilaments is 1:8. The patterns of these multiple forms of tropomyosin were found to change when REF-52 cells were transformed with SV40 or adenovirus type 5.

It is generally believed that actin-containing microfilaments play central roles in cell motility and cell shape changes of cultured cells (1–7). Over the past decade, studies with immunofluorescent microscopy on cultured cells have led to the conclusion that microfilaments are composed of several proteins in addition to actin, such as myosin (8–10), tropomyosin (11, 12), α-actinin (12–14), and filamin (15, 16). These proteins have also been identified and characterized biochemically in nonmuscle cells (2–5, 17–22). However, it still remains to be shown whether changes in the protein composition and/or structural organization of microfilaments occur concomitantly with changes in biological activities of cultured cells such as spreading, mitosis, movement, differentiation, oncogenic transformation, etc. To begin to answer this question, it is necessary to develop a suitable method for isolation of microfilaments in their intact form from cultured cells under various physiological conditions. Recently, Scioss and Goldman (23) have succeeded in preparing a population of microfilaments from cultured rat embryo cells by utilizing the isolation method for muscle native thin filaments (24, 25). This procedure isolated microfilaments from BHK-21 or 3T3 cells that were first induced to adopt a well spread morphology by subculturing in serum-depleted medium or by selecting only those cells tightly adherent to culture dishes. This method, however, was limited and could not be easily used to isolate microfilaments from a variety of different cell types at various biological stages, such as during mitosis and differentiation.

We have developed a new method for the rapid isolation of microfilaments from a variety of cell types including REF-52, gerbil fibroma (CCL-146), BHK-21, NIH/3T3, Chinese hamster ovary, mouse neuroblastoma, L6 myoblasts and myotubes, and chick embryo fibroblasts. This method is based on the previous observations that anti-tropomyosin monoclonal antibodies cause the aggregation of thin filaments from smooth muscle into ordered bundles which are easily collected by low speed centrifugation (26). Since tropomyosin is one of the major components of microfilaments (11, 12), the anti-tropomyosin monoclonal antibody is suitable to use for the immunoprecipitation of microfilaments. In this report, we describe the method for the isolation of microfilaments from cultured cells and their morphological and biochemical characterization. We report that the isolated microfilaments show a double helical structure similar to the morphology of F-actin, and the tropomyosin is found to be arranged along microfilaments with a periodicity of 33–34 nm. SDS-polyacrylamide gel analysis of the microfilaments from REF-52 cells shows that, in addition to actin, several other proteins including multiple forms of tropomyosin are present.

MATERIALS AND METHODS

Cell Culture—Cultured cells used in the present study were gerbil fibroma cells (American Type Culture Collection, CCL-146), baby hamster kidney (BHK-21/C13) cells (American Type Culture Collection, CCL-101), L6 myoblasts and myotubes, NIH/3T3 fibroblasts, Chinese hamster ovary cells, mouse neuroblastoma cells, REF-52 cells (an established rat embryo line), and an SV40-transformed REF-52 (REF 4A). REF-52 and REF-4A cell lines were kindly provided by guest on March 18, 2020

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by Dr. W. C. Ting, Cold Spring Harbor Laboratory. Cell lines were
maintained in DMEM containing 10% fetal calf serum in an
atmosphere of 5% CO 2 and 95% air at 37 °C unless specified otherwise. For
BHK-21 cells, 10% calf serum supplemented with 10% tryptose
phosphate broth replaced fetal calf serum. All cells were passaged
using 0.05% trypsin in PBS.

Isolation of Microfilaments from Cultured Cells—Monolayer cells
were rinsed with PBS at room temperature and then ex-
tracted for 2 min at room temperature with Triton/glycerol solution
(0.1 M PIPES, pH 6.9, 5 mM MgCl 2, 0.2 mM EGTA, 0.05% Triton X-
100, 4 mM glycerol) to stabilize the cytoskeleton. While the extracted
cells were still attached to culture dishes, they were rinsed 3 times
with PBS (pH 7.4, 0.1% MgCl 2, 0.2 mM EGTA). After removal of the
washing solution, the Triton/glycerol residues of cells (10-20
plates of 100-mm culture dish) were collected as follows. To the
first dish 0.2 ml of buffer A was added and then the dish was tilted
to facilitate scrapping the residue into one corner with a rubber
police-
man. The residues plus buffer A from this dish were transferred
to the second dish and the scrapping procedure was repeated. The accu-
mulated residues plus the initial 0.2 ml of buffer A were trans-
ferred to the subsequent dishes until all dishes were scraped of their
residues. This allowed the collection of the residues from 10-20 dishes
to be restricted to a final volume of 1.0-1.5 ml. After addition of ATP
and phenylmethylsulfonyl fluoride to a final concentration of
0.5 mM, respectively, the cell residues were homogenized at 0 °C by
70 strokes in a motor-driven Potter-Elvehjem (glass/Teflon) homogenizer (400
rpm). After centrifugation of the homogenate at 12,800 × g for 15
min at 4 °C in an Eppendorf centrifuge, ½ volume of ascites fluid of
mouse LCK16, LCK15, or LJH2) was added to the supernatant, and the mixture was incubated for 30 min at room
temperature to allow antibody to aggregate microfilaments into
bundles.

The resultant microfilament bundles were collected by centrifug-
ation at 12,800 × g for 5 min at 4 °C in an Eppendorf centrifuge
and washed once with buffer A containing 5 mM ATP, followed by
washing twice with buffer B. The final pellet was resuspended in
50-100 μl of buffer A and used as a microfilament fraction. Protein
concentration was determined by the method of Lowry et al. (27).

There appears to be no way to estimate the yield of microfilaments.
As an alternative way we determined the amount of actin (measured
by radioactivity) in various fractions at each step of the purification
and expressed the yield as a percentage of the original total actin
amount. Cells (one 100-mm culture dish, 10 10 6-10 7 cells in vivo
in vitro) were labeled for 15 h with 250 μCi of [35S]methionine (1110 Ci/mmol) in
methionine-free DMEM containing 2.5% fetal calf serum. Samples
from each step of the isolation were analyzed by SDS-polyacrylamide
gel electrophoresis as shown in Fig. 1. After staining with 0.15%
Coomassie brilliant blue in 50% methanol, 10% acetic acid for 2 h
followed by destaining with 7.5% methanol, 7.5% acetic acid, the gels
were soaked for 30 min with distilled water and then dried on filter
paper. The actin bands were cut out and sliced into about 1-mm
thick slices, which were incubated for 24 h in 0.2% of 2% SDS
dilute protein and the radioactivity was measured by a Beckman liquid
scintillation counter.

Molar ratios of actin to multiple forms of tropomyosin in the
isolated microfilaments were determined from densitometer traces
of one-dimensional SDS gels as described (41, 46). Gels were stained
quantitatively with fast green (56) and scanned with a Hoeffer densi-
tometer (GS 300). The molar ratios were calculated using values
corrected for differential dye uptake between skeletal muscle actin
and smooth muscle tropomyosin (47).

FH)proline-[3H]tryptophan-labeled microfilaments were also
prepared from cells (one 100-mm culture dish, 10 10 4-10 5 cells in vitro
labeled for 15 h with 250 μCi of [3H]proline (108 Ci/mmol) or [3H]
tryptophan (28 Ci/mmol) in DMEM containing 10% fetal calf serum.

Preparation of Muscle Proteins—Myosin was prepared from rabbit
skeletal muscle by the method described by Perry (28). Skeletal
muscle actin was extracted for 10 min at 0 °C from acetone powder
of rabbit skeletal muscle prepared by the method of Ebashi and
Ebashi (29) and polymerized overnight at 4 °C by addition of 3 M
KCl to a final concentration of 30 mM. F-actin was collected by
centrifugation (100,000 × g, 2 h) and treated with 0.6 M KCl according
to the method of Gainer and Watt (40).

Smooth muscle tropomyosin was prepared from frozen chicken
gizzards by the method of Ebashi et al. (31) except that tropomyosin
was fractionated by the addition of solid ammonium sulfate to a
concentration of between 30 and 36 g/100 ml rather than between 25
and 28 g. The phosphorus of actin, myosin (95% pure), tropomyosin,
and b-glycerophosphate with a low concentration of bisacrylamide (12.5% acrylam-
10.14% bisacrylamide) (32, 33).

Preparation of Monoclonal and Conventional Antibodies against
Tropomyosin—Hybridoma clones FJL15, JLH2, and LCK16 were
isolated and characterized as described (34, 35). The high titer anti-
bodies obtained from the ascites fluids of hybridoma-bearing mice
were used for all experiments. For all antibodies, only intact
secret IgM antibodies while clone JLH2 secretes IgA antibodies. The antibody JLF15 recognizes only tropomyosin, whereas LCK16 and
JLH2 are found to react with both tropomyosin and vimentin (34,
35).

For preparation of conventional antibody against tropomyosin,
purified smooth muscle tropomyosin (about 0.5 mg in PBS) emulsi-
fied in complete Freund’s adjuvant was injected into rabbits. After
1 month, the rabbits were boosted with similar amounts of antigen in
incomplete Freund’s adjuvant. The booster injection was repeated
once after 2 weeks. Antiserum was collected 1 week after the last
injection and tested by Ouchterlony double diffusion tests against
several purified proteins, including α-actinin, vinculin, filamin, trop-
omyosin, and tropo. Tropomyosin formed a precipitating line with this antibody. The specificity of the antibody was further
tested by Western blots (36) and by immunofluorescence staining.

Preparation of myofibrils—Rabbit skeletal muscle was homogenized
with 0.2 mg/ml of pyruvate kinase and homogenate microfilaments were washed twice with buffer B. The final pellet was resuspended in
50-100 μl of buffer A and used as a microfilament fraction. Protein
concentration was determined by the method of Lowry et al. (27).

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and 28 g. The phosphorus of actin, myosin (95% pure) were determined by SDS-polyacrylamide slab gel electro-

RESULTS

Isolation of Microfilaments—The microfilament isolation method
described here consists of three simple steps, i.e., Triton/glycerol extraction, homogenization in Mg 2+ ·ATP to
The Triton/glycerol-insoluble residues (lane 2 in Fig. 1) were homogenized in 5 mM Mg$^{2+}$-ATP to disperse the microfilaments. This homogenate contained actin, vimentin (the major component of intermediate filaments), and nuclear proteins as well as many other minor proteins. After centrifugation of the homogenate at 12,800 × g for 15 min (Eppendorf), about 50–60% of the total actin remained in the supernatant (lane 4 in Fig. 1). This centrifugation removed the intermediate filaments and nuclei as judged by the disappearance of the vimentin band and many low molecular weight nuclear proteins from the supernatant fraction (lane 4 in Fig. 1). Microfilament bundles (Fig. 3E) produced by incubation of the supernatant with anti-tropomyosin monoclonal antibodies were pelleted by low speed centrifugation at 12,800 × g for 5 min and washed by resuspension. As Table I shows, approximately 30% of total actin was finally recovered in the microfilament fraction. SDS-polyacrylamide gel analysis showed that the microfilament fraction (lane 5 in Fig. 1) contained mainly actin, multiple forms of tropomyosin (which

disperse microfilaments in the supernatant, and immunoprecipitation of microfilaments by anti-tropomyosin monoclonal antibodies in a native condition.

Monolayer cells of REF-52 were subjected to this method to isolate microfilaments. The protein composition of the various fractions arising during the isolation procedure was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1). The Triton/glycerol soluble fraction (lane 1 in Fig. 1) stains very lightly with Coomassie blue due to the volume of the fraction (about 40 times more dilute compared to the other fractions). In an experiment with [35S]methionine-labeled cells, we can detect many soluble proteins including actin in this fraction, although the substantial majority of microfilament-associated proteins remains insoluble. Because there is no way to measure the amount of microfilaments, we alternatively determined the amount of actin in various fractions to estimate the yield (see "Materials and Methods"). As shown in Table I, 12–16% of total actin was extracted by Triton/glycerol treatment. This may represent the nonfilamentous actin fraction in the cells.

![Fig. 1. SDS-polyacrylamide gel analysis of the microfilament isolation fractions from REF-52 cells. Lane 1, Triton X-100/glycerol-soluble fraction; lane 2, homogenate of Triton X-100/glycerol-insoluble residues; lane 3, precipitate of the homogenate by low speed centrifugation (12,800 × g for 15 min); lane 4, supernatant of the homogenate after low speed centrifugation (12,800 × g for 15 min); lane 5, isolated microfilaments, i.e. the immunoprecipitate of proteins in lane 4 with anti-tropomyosin monoclonal antibody LCK16; lane 6, supernatant after antibody precipitation. Asterisks indicate the heavy and light chains of IgM monoclonal antibody. a, 250,000-Da protein; b, α-actinin; c, 83,000-Da protein; d, actin; e, tropomyosin 1 (TM-1, 40,000 Da); f, 37,000-Da protein; g, tropomyosin 2 (TM-2, 36,500 Da); h, tropomyosin 3 (TM-3, 35,000 Da); i, tropomyosins 4 and 5 (TM-4, 32,400 Da; TM-5, 32,900 Da). Four arrowheads on the right of lane 5 indicate the positions of migration of rabbit skeletal and chicken gizzard tropomyosin bands (from top to bottom: chicken gizzard slower moving component, rabbit skeletal slower moving component, chicken gizzard faster moving component, rabbit skeletal faster moving component). An equivalent amount of each sample except for proteins in lane 1 (40 times more dilute) and in lane 4 (14 times more concentrate) was loaded on the gel. After electrophoresis, the gel was stained with Coomassie blue and de-stained with 7.5% acetic acid, 7.5% methanol.

![Fig. 2. Comparison of the microfilament isolation methods. Supernatant (Sup-1) fraction was prepared from REF-4A cells as described under "Materials and Methods" and subjected to the isolation of microfilaments by anti-tropomyosin monoclonal antibody LCK16 (lane 1), polyclonal anti-tropomyosin antibody (lane 2), or Mg$^{2+}$ precipitation (lane 3). Lane 4 is the supernatant of Sup-1 after Mg$^{2+}$ precipitation. Asterisks indicate the heavy and light chains of monoclonal antibody (lane 1) and polyclonal antibody (lane 2). Arrowheads indicate the tropomyosin species.](http://www.jbc.org/)

| Table I | Distribution of actin in various fractions during microfilament isolation from REF-52 |
|---------|-------------------------------------------------|
| Experiment 1 | Experiment 2 |
| Total counts | Yield | Total counts | Yield |
| cpm | % | cpm | % |
| Total cells | 7.6 × 10⁶ | 100 | 2.5 × 10⁵ | 100 |
| Triton/glycerol residues | 6.4 × 10⁵ | 84 | 2.2 × 10⁵ | 88 |
| Supernatant | 3.8 × 10⁵ | 50 | 1.5 × 10⁵ | 60 |
| Microfilament fraction | 2.0 × 10⁴ | 27 | 0.8 × 10⁴ | 32 |

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FIG. 3. Electron micrographs of the isolated microfilament bundles from smooth muscle and cultured cells. A, taenia coli of guinea pig; B, BHK-21 cells; C, L6 myoblasts; D, REF-52 cells; E, gerbil fibroma cells. The microfilament bundles induced by anti-tropomyosin monoclonal antibody were negatively stained with uranyl acetate and observed with an electron microscope. Arrowheads indicate the localization of tropomyosin molecules visualized by the binding of anti-tropomyosin monoclonal antibody JLF15 (A–C) or LCK16 (D and E). All of the micrographs are at the same magnification.

will be identified later), and the heavy and light chains of the anti-tropomyosin IgM antibody. Several other minor proteins such as α-actinin (105,000 Da) and an 83,000-Da protein can be also detected in this fraction. The characterization of these microfilament-associated proteins will be discussed later.

The supernatant (lane 6 in Fig. 1) after the precipitation of microfilaments with anti-tropomyosin monoclonal antibodies contained significant amounts of actin, approximately 20% of the total actin. No significant amount of tropomyosin could be found in this antibody-supernatant fraction, as determined by two-dimensional gel electrophoresis and by further immunoprecipitation using conventional rabbit anti-tropomyosin antiserum. However, half of the actin in the antibody-supernatant could be pelleted by high speed centrifugation at 100,000 × g for 2 h. This suggests that this supernatant may contain microfilaments devoid of tropomyosin and/or membrane-associated actin.

The formation of microfilament bundles appears to be specifically caused by the anti-tropomyosin monoclonal antibodies. In order to show the specificity, the microfilaments in supernatant were incubated either with ascites fluid of other monoclonal antibodies (57), such as FM28 (against skeletal muscle myosin) and JLT12 (against troponin-T), or with heat-denatured anti-tropomyosin monoclonal antibodies. In all cases, no microfilament bundles were detected by electron microscopy or by SDS-polyacrylamide gel analysis.

As described in a previous paper (26), the anti-actin monoclonal antibody JLA20 (34) morphologically distorted thin filaments or microfilaments, which lost their double helical structure and showed a fuzzy coats of antibody binding. This distortion may also cause a dissociation of minor proteins from the microfilaments. Therefore, even though actin is the major component of microfilaments of all eukaryotic cells, the monoclonal antibody against actin is not suitable for microfilament isolation.

We have shown that both IgM (LCK16 or JLF15) and IgA (JLH2) monoclonal antibodies against tropomyosin are equally efficient to use for the isolation of thin filaments (26) or microfilaments. Furthermore, we are also able to isolate troponin-T-containing microfilaments from chicken embryo myotubes by using similar method with an IgG monoclonal antibody (JLT12) against troponin-T (data not shown). Thus, the microfilament isolation method described here appears to be independent of classes of antibody.
Comparison with Other Methods for Microfilament Isolation—In the method described here, we used the monoclonal antibodies for immunoprecipitation of microfilaments because of their high specificity and high titer. For comparison, we also used rabbit polyclonal antibody instead of the monoclonal antibodies. When the antisera were used for the isolation of microfilaments from REF-4A cells, only tropomyosin but no detectable amount of actin was found in the immunoprecipitates (lane 2 in Fig. 2), indicating that no microfilaments were precipitated. This may be due to the presence of actin-depolymerizing factors or actin filament length regulators (gelsolin, brevin, etc.) in the crude serum (see, for review, Ref. 55). When the IgG fraction of polyclonal antibody purified by ammonium sulfate precipitation (45% saturation) was used for the immunoprecipitation, the immunoprecipitate contained actin and multiple forms of tropomyosin. However, the immunoprecipitate with polyclonal antibodies showed no F-actin-like filaments but amorphous clumps when examined by electron microscopy. On the other hand, the immunoprecipitate with monoclonal antibodies showed ordered bundles of microfilaments (Fig. 3) with typical F-actin-like structure. The binding of polyclonal antibodies to tropomyosin molecules at too many sites probably caused this distortion of microfilaments and might cause a dissociation of minor proteins from microfilaments.

We also compared the present method with a second microfilament isolation protocol similar to that described by Schloss and Goldman (23). This method is based on the fact that a high concentration of Mg2+ (10 mM) forms paracrystals of smooth muscle actin filaments (44), which can be easily collected by low speed centrifugation. As Fig. 2 (lane 3) shows, actin, tropomyosin, and several other proteins were enriched by this procedure relative to the supernatant. However, the protein pattern is much more complex than that of microfilaments isolated by the monoclonal antibodies (lane 1). Moreover, the yield of microfilaments is less than half of that of microfilaments isolated by the monoclonal antibodies.

**Fig. 4. Two-dimensional gel analysis of multiple forms of tropomyosin from REF-52 and REF-4A cells.** The microfilaments isolated from [35S]methionine-labeled REF-52 (A) and REF-4A (B) cells and the immunoprecipitate (C) from [35S]methionine-labeled REF-4A total cell extract by rabbit antiserum against smooth muscle tropomyosin were analyzed by two-dimensional gel electrophoresis. After electrophoresis, gels were processed for fluorography. Only parts of two-dimensional gels containing tropomyosin and actin are shown here. The first dimensional gels contained pH 5-7 ampholites and the second dimensional gel contained 12.5% polyacrylamide. Numbers indicate multiple forms (TM-1, TM-2, TM-3, TM-4, and TM-5, respectively) of tropomyosin.

### Table II

Periodicity of tropomyosin binding to thin filaments or microfilaments from different kinds of tissue and cells

| Tissue or cell           | Periodicity value (nm) |
|--------------------------|------------------------|
| Skeletal muscle (rabbit psoas) | 38.3 ± 1.8 (n = 46)*   |
| Smooth muscle (guinea pig tibiae) | 37.2 ± 3.2 (n = 32)    |
| BHK-21                   | 33.3 ± 2.3 (n = 59)    |
| CCL gerbil fibroma       | 33.1 ± 1.6 (n = 42)    |
| REF-52                   | 34.2 ± 2.0 (n = 38)    |

* n, number of bundles measured which contained an average of 10 cross-striations.

### Table III

Activation of skeletal muscle myosin ATPase activity by microfilament fraction isolated from REF-52

| Components                  | ATPase activity (μmol/mg min) | Activation factor |
|-----------------------------|------------------------------|-------------------|
|                            | −DTT | +DTT | −DTT | +DTT |
| Myosin (110 μg/ml)          | 0.0165 | 0.0183 | 0.0165 | 0.0183 |
| Myosin + actin (2.54 μg/ml) | 0.0262 | 0.0275 | 1.6 | 1.5 |
| actin (12.7 μg/ml)          | 0.0889 | 0.0950 | 5.4 | 3.2 |
| actin (25.4 μg/ml)          | 0.135  | 0.0902 | 8.2 | 4.9 |
| actin (127 μg/ml)           | 0.186  | 0.139  | 11.2 | 7.6 |
| Myosin + microfilament fraction (30 μg/ml) | 0.0288 | 0.0500 | 1.8 | 2.7 |
| Microfilament fraction (30 μg/ml) | 0.0 | 0 | 0 | 0 |
| Ascites fluid (2000 μg/ml)  | 0 | 0 | 0 | 0 |
myosin (26, 45–47). This observation agrees well with reports that tropomyosin paracrystals from nonmuscle cells such as platelet (17, 21), brain (18), and BHK-21 cells (23) showed a shorter repeat (33–34 nm) than that (40 nm) of muscle tropomyosin (49). These observations might mean that the non-muscle tropomyosin is shorter by one actin monomer than muscle tropomyosin.

Activation of Skeletal Muscle Myosin ATPase Activity by the Isolated Microfilaments—It is well known that F-actin can activate the skeletal muscle myosin Mg2+-ATPase activity (48). Therefore, we asked whether the microfilaments isolated from REF-52 cells also activate the myosin ATPase activity (Table III). When activities were measured in the absence of DTT, the activation of myosin ATPase by REF-52 microfilaments (30 μg/ml) was 1.8-fold. The same extent of activation required 2.3 μg/ml of skeletal muscle F-actin; that is, the isolated microfilaments only contained 2.3 μg/ml of actin which were able to activate the myosin ATPase activity. This is equivalent to 15% of the total actin in the isolated microfilaments assuming that 50% of total protein in the isolated microfilaments is actin (based on the electrophoretic pattern of microfilaments shown in lane 5 of Fig. 1). This inefficient activation of the myosin ATPase activity may be due to the fact that most of the actin in the microfilament bundles is sterically prevented from reacting with myosin by the monoclonal antibodies. Therefore, we have introduced 10 mM DTT into the microfilament bundles to dissociate the bound antibody and then measured the activation of ATPase activity. In this case, up to 80% of the actin in the microfilament fraction was as active as F-actin (Table III). In another experiment with microfilaments isolated from L6 myoblasts, 70% of the actin was found to be active. Therefore, we have concluded that microfilaments isolated by this method can activate the myosin Mg2+-ATPase activity to nearly the same extent as skeletal muscle F-actin.

Identification of Multiple Forms of Tropomyosin and Other Microfilament-associated Proteins in the Microfilaments Isolated from Normal (REF-52) and SV40-transformed (REF-4A) Cells—Actin and immunoglobulin (IgM) heavy and light chains are the major bands of the microfilament fraction from REF-52 cells (lane 5 in Fig. 1) and REF-4A cells (lane 1 in Fig. 2). In addition, the microfilament fraction contained several proteins with apparent M. = 250,000, 105,000, 83,000, 40,000, 37,000, 36,500, 35,000, 32,400, and 32,000 (see Fig. 1).

Of these polypeptides, proteins of 40,000, 36,500, 35,000, 32,400, and 32,000 Da were identified as tropomyosin by the criteria of two-dimensional gel analysis, immunoprecipitation with conventional rabbit anti serum in the presence of SDS, lack of proline and tryptophan, heat stability, and actin binding as shown below. These five proteins were found to have an isoelectric point around pH 4.6 (Fig. 4, A and B) by two-dimensional gel analysis, which is the known pl for tropomyosin (17, 18, 21). Immunoprecipitation of total cell lysates of REF-4A cells with rabbit anti-tropomyosin antiserum in the presence of 0.05% SDS showed five spots by two-dimensional gel analysis (Fig. 4C), whose electrophoretical mobilities were identical to those of the five proteins of microfilaments isolated from both normal (REF-52) and SV40-transformed (REF-4A) cells (Fig. 4). It should be noted that both 32,400- and 32,000-Da proteins were not completely recovered in the immunoprecipitate with rabbit antisera (compare Fig. 4, B and C). This may be due to the lower affinity of the antisera to both proteins.

The absence of two amino acids, proline and tryptophan, is known to be characteristic of tropomyosin (17, 50, 51). Microfilaments were separately isolated from REF-4A cells labeled in vivo with either [35S]methionine, [3H]tryptophan, or [3H]proline, and were analyzed by two-dimensional gel electrophoresis. While fluorograms of [35S]methionine-labeled microfilaments had five spots of 40,000, 36,500, 35,000, 32,400, and 32,000 Da (Fig. 5A), [3H]proline-labeled microfilaments did not show any of these five spots (Fig. 5B). [3H]Trypto-
Tropomyosin-containing Microfilaments

One well known characteristic for tropomyosin is its heat stability (52). The putative tropomyosins were tested by boiling the microfilament of REF-4A cells at 100 °C for 10 min. The supernatant after heat treatment contained all five polypeptides (Fig. 6), suggesting that these five proteins were heat-stable. Preliminary binding experiments have shown that these five polypeptides are able to bind to skeletal muscle F-actin (data not shown). Based on these pieces of evidence, we have concluded that tropomyosin in REF-52 cells exists in filaments. Therefore, the increase in the amount of minor tropomyosins TM-1 and TM-2 was indicated by light microscopy traces of fast green-stained gels. From the periodicity data obtained by us (Table II) and by others (17, 18, 21, 23), it is likely that 1 nonmuscle tropomyosin dimer binds 6 actin monomers. These results together suggest that some of the actin in the isolated microfilaments are not fully covered with tropomyosin molecules.

The protein band with an apparent $M_r = 105,000$ that also coisolated in our preparation was identified as $\alpha$-actinin by two-dimensional gel analysis (co-migration with authentic $\alpha$-actinin isolated from chicken gizzard) and by immunooautoradiography on SDS-polyacrylamide gels with a rabbit antiserum against beef heart $\alpha$-actinin (a generous gift from Dr. K. Burridge, University of North Carolina).

Proteins with apparent $M_r = 250,000, 83,000$, and 37,000 were also present in the microfilament fraction of REF-52 and REF-4A cells. We have found that the 83,000-Da protein, like tropomyosin, was heat-stable (i.e. remained in the supernatant after heat treatment of the microfilament fraction) (Fig. 6b). Two-dimensional gel analysis, however, showed that the 83,000-Da protein, unlike tropomyosin, is a basic protein which focused as four variants around pH 7-8. Preliminary actin-binding experiments have shown that the 83,000-Da protein can bind to skeletal muscle F-actin (data not shown). Thus, the 83,000-Da protein may be a new actin-binding protein. The purification and further characterization of this protein are in progress. At the present time, the properties of the 250,000- and 37,000-Da proteins remain unclear. The amount of 37,000-Da protein appeared to be increased in the microfilaments isolated from SV40-transformed cells (compare lane 5 in Fig. 1 with lane 1 in Fig. 2). In addition, the 37,000-Da protein appeared to be rather basic on two-dimensional gels.

Application of the Method—The method we describe here is applicable to isolation of microfilaments from a variety of different cultured cells including BHK-21 cells, chicken embryo fibroblasts, gerbil fibroma cells, REF-52 cells, NIH/3T3 cells, L6 myoblasts, normal rat kidney cells, mouse neuroblastoma cells, and Chinese hamster ovary cells. Among cell lines we have examined, HeLa cells are so far the only cell line from which we failed to isolate microfilaments. The reason for this is unknown. It might suggest that HeLa cells have few tropomyosin-containing microfilaments.

We have applied the method to examine changes in the protein composition upon DNA virus-induced cell transformation. One such example is shown in Fig. 4, A and B. On transformation by SV40, the levels of both TM-1 and TM-2 were decreased while the minor tropomyosins TM-3 and TM-5 were increased. Similar but more drastic changes in tropomyosin were found in adenovirus type 5-transformed cells. In the microfilaments of adenovirus type 5-transformed cells (Ad5D.lA), TM-1 was missing entirely and TM-3 was found to increase 10 times more than that of the normal cell microfilaments. Therefore, the increase in the amount of minor tropomyosin and the decrease of major tropomyosin appeared to commonly occur on cells transformed by SV40 or adenovirus type 5. Detailed analysis of these changes in tropomyosin patterns will be described elsewhere.

DISCUSSION

This method of microfilament isolation provides us with a sensitive way to detect new microfilament-associated pro-
Teins. As Fig. 1 and 6 show, microfilaments of REF-52 and REF-4A contained an 83,000-Da protein which has not yet been reported. The presence of an 83,000-Da protein in total cell lysates by two-dimensional gel analysis suggests that this protein is not a proteolytic fragment during isolation of microfilaments. Preliminary studies have shown that this protein is also present in the microfilaments isolated from L6 myoblasts but is missing in the microfilaments of L6 myotubes. This may suggest that the 83,000 Da protein may play an important role in myogenensis. Further characterization of the protein is in progress. Furthermore, we also identified five polypeptides (M_r = 40,000 (TM-1), 36,500 (TM-2), 35,000 (TM-3), 32,400 (TM-4), and 32,000 (TM-5)) as tropomyosin in the microfilaments from REF-52 cells. These multiple forms of tropomyosin appeared to be commonly present in rat cell lines since microfilaments from L6 myoblasts and normal rat kidney cells were found to contain the same five polypeptides judged by the criteria of the co-migration on two-dimensional gels, heat stability, and immunoprecipitation with conventional anti-tropomyosin antiseraum. L6 myoblasts were reported to have three species of tropomyosin (53), which appeared to correspond to the tropomyosins TM-1, TM-2, and TM-4 of REF-52 cells. The microfilament isolation method described here allowed us to identify minor proteins with apparent M_r = 35,000 (TM-3) and 32,000 (TM-5) as tropomyosins, because these proteins were greatly enriched in the microfilament fraction. It remains to be investigated whether these multiple forms of tropomyosin are functionally different and whether they bind to microfilaments randomly or selectively.

Any of the methods for the isolation of microfilaments have the problem of whether or not the isolated microfilaments are precisely the same structures that exist in living cells. To assess the nativeness of isolated microfilaments, we addressed two major questions associated with the method described here: 1) Does Triton/glycerol extraction immediately freeze the microfilaments at a state identical to that occurring in vivo? 2) Does an exchange reaction occur between microfilament components and free molecules during isolation?

To address the first question, we examined the effect of Triton/glycerol extraction on microfilament isolation. We observed microfilaments in the homogenates of cells treated with or without Triton/glycerol using electron microscopy. These two sets of filaments were morphologically identical. However, while microfilaments in the homogenate of cells without Triton/glycerol extraction disappeared quickly, within hours, microfilaments in the homogenates of extracted cells were stable for at least 1 day. In addition, if Triton/glycerol extraction was omitted, the yield of microfilaments was reduced to only one-fourth of that obtained by the original method. These results mean that Triton/glycerol treatment stabilizes the microfilament structure by extracting some factors which cause disassembly and/or degradation of microfilaments. We also examined the effect of the concentration of Triton X-100 on the extractability of tropomyosin from cells. While 20–40% of tropomyosin was extracted with 0.5% Triton X-100 from chick embryo fibroblasts, no significant amounts of tropomyosin were extracted with 10-fold diluted Triton X-100 (0.05%) as judged by two-dimensional gel analysis.

To address the second problem, we examined whether an exchange reaction occurs between the actin or tropomyosin of microfilaments and free actin or tropomyosin externally added during isolation. Cells were first labeled in vivo with [35S]methionine. Purified smooth muscle tropomyosin or skeletal muscle actin was added to the Triton/glycerol residues of the cells to a final concentrations of 30 or 100 µg/ml, respectively, and microfilaments were isolated in the same way. Autoradiography of microfilament components analyzed by SDS-polyacrylamide gels showed no significant changes in protein patterns as compared to that of the control (data not shown). This suggests that no exchange reactions occur between free molecules and microfilaments.

Finally, the isolation of microfilaments by this method was affected by the biological states of microfilaments in vivo. For example, the treatment of cells with cytochalasin B, which is known to disorganize microfilament bundles in cells (7, 54), reduced the yield of microfilaments isolated to one-third of that isolated from control cells, although there were no apparent differences in the protein composition of the microfilaments between the two experiments. The detailed results will be described elsewhere. Another such example is that the tropomyosin patterns of isolated microfilaments were changed in the cells transformed by DNA virus as described under "Results." These results suggest that microfilaments isolated by this method may represent, at least in part, those existing in living cells.

It should be noted that our method only detects the tropomyosin-containing microfilaments from cells. The supernatant after immunoprecipitation of microfilaments with anti-tropomyosin monoclonal antibodies still contains a significant amount of actin but no obvious tropomyosin. The observation that half of the actin was precipitable by high speed centrifugation may suggest the presence of other classes of actin-containing microfilaments devoid of tropomyosin. This notion is further supported by a preliminary result that a monoclonal antibody JLN20 against α-actinin (54) is able to precipitate a second class of microfilaments from the supernatant remaining after the removal of the tropomyosin-containing microfilaments from chicken embryo fibroblasts. Consequently, we are now using monoclonal antibodies against other contractile proteins such as α-actinin, filamin, and vinculin to isolate other classes of microfilaments by a similar approach. Such an approach will provide us with the detailed information regarding the protein composition and perhaps organization of microfilaments and will help us understand the function of microfilaments in cell motility and cell shape changes.

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