Assembly of Different Isoforms of Actin and Tropomyosin into the Skeletal Tropomyosin-enriched Microfilaments during Differentiation of Muscle Cells In Vitro

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Abstract. We have used a monoclonal antibody (CL2) directed against striated muscle isoforms of tropomyosin to selectively isolate a class of microfilaments (skeletal tropomyosin-enriched microfilaments) from differentiating muscle cells. This class of microfilaments differed from the one (tropomyosin-enriched microfilaments) isolated from the same cells by a monoclonal antibody (LCK16) recognizing all isoforms of muscle and nonmuscle tropomyosin. In myoblasts, the skeletal tropomyosin-enriched microfilaments had a higher content of α-actin and phosphorylated isoforms of tropomyosin as compared with the tropomyosin-enriched microfilaments. Moreover, besides muscle isoforms of actin and tropomyosin, significant amounts of nonmuscle isoforms of actin and tropomyosin were found in the skeletal tropomyosin-enriched microfilaments of myoblasts and myotubes. These results suggest that different isoforms of actin and tropomyosin can assemble into the same set of microfilaments, presumably pre-existing microfilaments, to form the skeletal tropomyosin-enriched microfilaments, which will eventually become the thin filaments of myofibrils. Therefore, the skeletal tropomyosin-enriched microfilaments detected here may represent an intermediate class of microfilaments formed during thin filament maturation. Electron microscopic studies of the isolated microfilaments from myoblasts and myotubes showed periodic localization of tropomyosin molecules along the microfilaments. The tropomyosin periodicity in the microfilaments of myoblasts and myotubes was 35 and 37 nm, respectively, whereas the nonmuscle tropomyosin along chicken embryo fibroblast microfilaments had a 34-nm repeat.

TROPOMYOSIN is an ubiquitous protein associated with the thin filaments of muscle cells and with the microfilaments of nonmuscle cells. In striated muscle, tropomyosin together with the troponin complex plays a central role in regulating the Ca^{++}-dependent interaction between actin and myosin (23, 31–33). Tropomyosin has been shown to exist in many isoforms that are specific to cells and tissues (19, 21). For example, at least 10 isoforms of tropomyosin have been identified and purified from chicken materials (18, 19). These are the cardiac muscle tropomyosin (36,000 mol. wt), the fast-migrating (38,000 mol. wt) and the slow-migrating (45,000 mol. wt) components of gizzard muscle tropomyosin, the α-form (37,500 mol. wt) and β-form (40,000 mol. wt) of skeletal muscle tropomyosin, and the chicken embryo fibroblast (CEF)[1] tropomyosin isoforms 1 (38,000 mol. wt), 2 (36,500 mol. wt), 3 (32,800 mol. wt), a (45,000 mol. wt), and b (43,000 mol. wt). In addition, cardiac and skeletal tropomyosins also exist in their phosphorylated forms (26). It has been shown that different isoforms bind to actin filaments with different affinities (6, 10, 19, 31, 35–37). The functional difference between these isoforms remains to be determined.

The synthesis of tropomyosin isoforms during skeletal muscle differentiation has been studied in the in vitro cultures of primary myogenic cells and clonal cell lines (4, 8, 11, 26, 27). It is clear that during myogenesis the expression of tropomyosin isoforms changes from nonmuscle forms to muscle forms, and that the synthesis of muscle-specific α-actin and other muscle proteins increases coordinately. However, two interesting questions remain to be answered. How are the α-actin and skeletal tropomyosin isoforms assembled into a set of microfilaments during myogenesis, eventually becoming the thin filaments of myofibrils? Do different sets of microfilaments coexist in differentiating muscle cells? Our approach to these questions is to use different monoclonal antibodies (LCK16 and CL2) against tropomyosin isoforms to fractionate different classes of microfilaments from differentiating muscle cells. The fractionation method is similar to the one we have previously reported (20). The CL2 antibody has been shown to recognize only tropomyosin isoforms of striated muscle, whereas the LCK16 antibody appears to react with all isoforms of tropomyosin (17, 18).

1. *Abbreviations used in this paper*: CEF, chicken embryo fibroblast; CL2-MF, skeletal tropomyosin-enriched microfilaments isolated by CL2 monoclonal antibody; LCK16-MF, tropomyosin-enriched microfilaments isolated by LCK16 monoclonal antibody, SFLS, stress fiber-like structures.

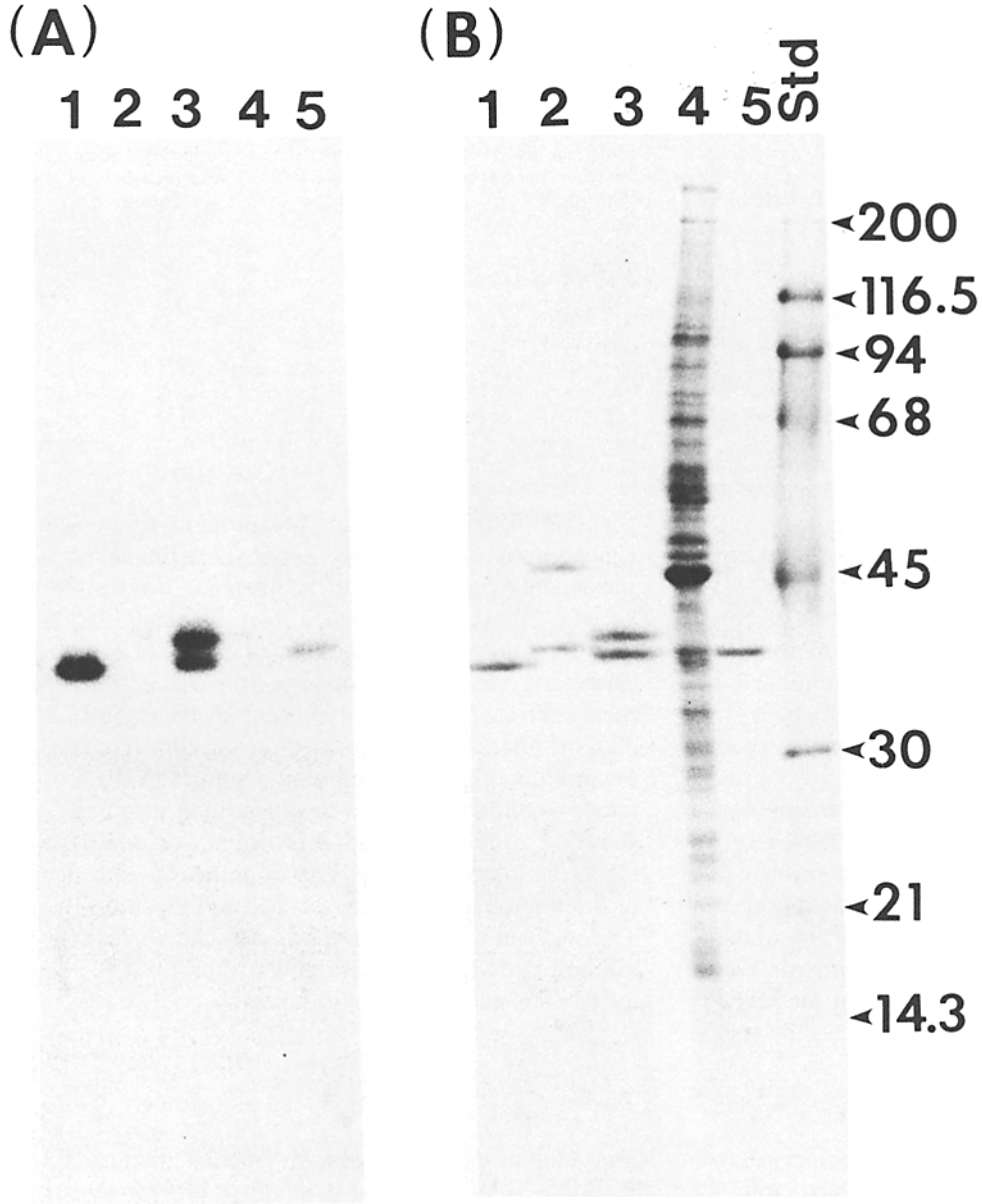

Figure 1. Protein immunoblot analysis of monoclonal antibody CL2 binding to striated muscle tropomyosins. Purified chicken cardiac tropomyosin (lane *1*), chicken gizzard tropomyosin (lane *2*), chicken leg muscle tropomyosin (lane *3*), chicken breast muscle tropomyosin (lane *5*), and total extract of chicken embryo fibroblasts (lane *4*) were separated on SDS polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose papers and either stained with Amido black (*B*) or reacted with CL2 antibody (*A*), followed by ^{125}I-labeled goat anti-mouse IgG (heavy and light chains). Bound antibody was detected by autoradiography. Monoclonal antibody CL2 appeared to react with striated muscle tropomyosin but not with gizzard tropomyosin or CEF tropomyosin. However, in experiments with heavily loaded CEF extract or purified CEF tropomyosin, we have found that the CL2 antibody can weakly bind to CEF tropomyosin isoform 3 (18). Numbers on right indicate molecular mass in kilodaltons.

Based on this specificity, we are now able to use the CL2 antibody to selectively isolate a class of skeletal tropomyosin-enriched microfilaments from differentiating muscle cells. By comparison and characterization of the microfilaments isolated by either LCK16 or CL2 antibody from myoblasts and myotubes, we have reached the conclusion that (*a*) various isoforms of actin and tropomyosin can assemble into the same set of skeletal tropomyosin-enriched microfilaments and (*b*) the skeletal tropomyosin-enriched microfilaments obtained here may represent an intermediate in the maturation of the thin filaments of myofibrils.

Materials and Methods

Cell Culture

Primary cultures of CEF were prepared from 10-d-old embryos as described previously (20). Cells were maintained in Dulbecco's modified Eagle's medium (DME) containing 10% fetal calf serum. Secondary or tertiary cultures of CEF cells were used for the experiments. Primary cultures of chicken embryo myogenic cells were prepared from 11-d-old embryos by a modification (18) of the procedure of Konigsberg (15). Normally, ~85–90% of the cells at the next day of culture were myogenic cells that had a slender spindle shape. Cells were maintained in DME medium containing 15% horse serum and 2% chicken embryo extract. On the second day of culture, myoblasts started to fuse. After 5–7 d, cultures contained >90% myotubes.

Preparation of Monoclonal Antibodies

Anti-tropomyosin monoclonal antibodies LCK16 and CL2 were prepared and characterized as described (17, 18). LCK16 antibody recognized all isoforms of tropomyosin, whereas CL2 antibody preferentially reacted with tropomyosin isoforms from striated muscle (Fig. 1). It should be noted that CL2 antibody could bind weakly to CEF tropomyosin isoform 3 in the case of excess amounts of this antigen presented (18).

Labeling Experiments and Isolation of Microfilaments

In the previous study (18), we have shown that CL2 monoclonal antibody appeared to be able to immunoprecipitate tropomyosin-enriched microfilaments from myotubes, but not from fibroblasts. To further confirm this

specificity, a mixing experiment was designed as follows: CEF or myotubes cells were labeled for 15 h with [^{3}H]methionine (200 μCi; 11.3 Ci/mmol) or [^{35}S]methionine (170 μCi, 1340 Ci/mmol), respectively, in methionine-free DME medium containing 2.5% fetal calf serum. Labeled cells were washed and briefly treated with triton/glycerol solution as described (20, 24). We have previously shown that treatment of cells with Triton/glycerol solution is an essential step in the isolation of microfilaments by monoclonal antibodies. This step appears to stabilize the microfilament structure by extracting some factors that cause disassembly and/or degradation of microfilaments (24). An equal number of counts of Triton/glycerol-insoluble residues from CEF and myotubes were mixed together and subjected to microfilament isolation with CL2 and LCK16 antibody as described (20, 24). The microfilaments isolated by this method do not exchange their components with free exogeneous molecules, such as actin and tropomyosin. Furthermore, the isolation of microfilaments by this method is affected by the biological states of microfilaments in vivo, suggesting that microfilaments isolated by this method may represent those existing in living cells (24). The isolated microfilaments were analyzed on two-dimensional gels. Protein spots corresponding to actin and tropomyosin isoforms were identified and then ^{3}H/^{35}S ratios were determined by cutting spots from the gels and counting them in a liquid scintillation counter.

To analyze the assembly of actin and tropomyosin isoforms into tropomyosin-enriched microfilaments, myoblasts (1–2 d in culture), or myotubes (5–7 d in culture) were first labeled with [^{35}S]methionine as described above and then subjected to the isolation of microfilaments with LCK16 or CL2 antibody. The isolated microfilaments were analyzed on both one- and two-dimensional gels. Bands or spots corresponding to actin and tropomyosin isoforms were identified and their radioactivities were quantified as described previously (19, 21).

One- and Two-dimensional Gel Electrophoresis

One-dimensional SDS PAGE was carried out as described by Laemmli (16) with a low concentration of bisacrylamide (3). Two-dimensional gel electrophoresis was performed by a modification (20) of O'Farrell's procedure (28).

Electron Microscopy

Small aliquots of isolated microfilaments from myoblasts and myotubes were applied to carbon-coated Formvar grids and negatively stained with 2.5% aqueous uranyl acetate. Samples were then observed in a Philips 300 electron microscope at an accelerating voltage of 60 kV. The periodicity of tropomyosin localization along the isolated microfilaments was measured against a stained catalase resolution standard (Polysciences, Inc., Warrington, PA) with a known periodicity of 8.12 nm.

Protein Immunoblotting

Protein immunoblotting was performed as described by Towbin et al. (34). Briefly, purified tropomyosin isoforms from chicken leg, breast, and gizzard muscle, prepared as described previously (19), and total extract from CEF cells were first separated on SDS polyacrylamide gels and then electrophoretically transferred to nitrocellulose papers. One blot was stained with

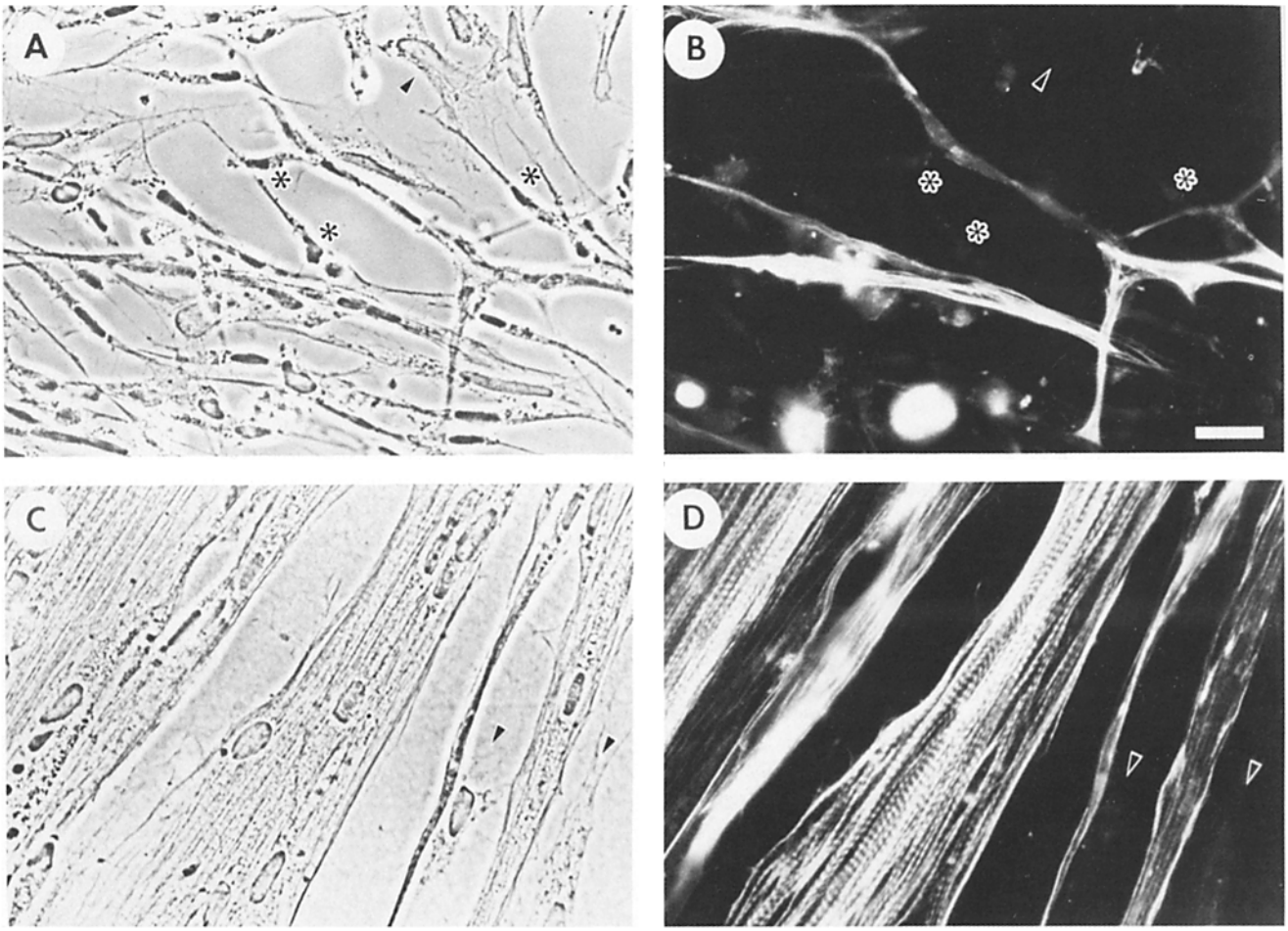

Figure 2. Immunofluorescence analysis of striated muscle isoform of tropomyosin in chicken embryo myoblasts and myotubes. Indirect immunofluorescence with monoclonal antibody CL2 was performed on myoblast (*A* and *B*) and myotube (*C* and *D*) cultures. (*A* and *C*) Phase-contrast micrographs; (*B* and *D*) fluorescent micrographs. There are few negative-stained cells with fibroblastic appearance (*arrowheads*) in both cultures. In myoblast cultures, most mononuclear cells with spindle shape are weakly stained (*asterisks*) and some fused cells show strong staining with CL2 antibody. At myotube stage, the striated staining patterns are prominent. Bar, 10 μm.

Table I. $^3H/^{35}S$ Ratios of Actin and Tropomyosin Isoforms in the Microfilaments Isolated by CL2 or LCK16 Antibody from the Mixture of [^{3}H]Methionine-labeled CEF Cells and [^{35}S]Methionine-labeled Myotubes

	(a) Ratio in CL2-MF	(b) Ratio in LCK16-MF	(a)/(b)
Total actin	0.56	1.96	0.30
α-actin	0.45	1.71	
β-actin	0.65	2.17	
γ-actin	0.54	1.87	
Total TM	0.25	0.93	0.27
β-TM	0.06	0.15	
Pβ	0.05	0.04	
α-TM + TM-1	0.30	1.15	
Pα	0.14	0.28	
TM-2	1.67	4.89	
TM-3a	0.28	0.85	
TM-3b	0.38	2.00	

Ratios of actin and tropomyosin isoforms were obtained by quantifying the two-dimensional gels of the isolated microfilaments. Tropomyosin (TM) isoforms can be separated into muscle forms (α-TM and β-TM), phosphorylated muscle forms (Pα and Pβ), and nonmuscle forms (TM-1, TM-2, TM-3a, and TM-3b) on two-dimensional gels. However, the spots of α-TM and TM-1 are too close together on the two-dimensional gel to be quantified separately (19).

Amido black to detect proteins, and a replicate blot was immunoblotted with CL2 antibody as detailed previously (18).

Results

Specificity of CL2 Monoclonal Antibody: Selective Isolation of the Skeletal Tropomyosin-enriched Microfilaments

Hybridoma clone CL2 was previously obtained by the in vitro immunization method (18). The antigens recognized by this antibody were identified as the striated muscle isoforms of tropomyosin by both solid-phase radioimmunoassay and protein immunoblotting. As shown in Fig. 1 *A*, the CL2 antibody reacted with cardiac muscle tropomyosin (lane *1*), α- and β-isoforms of leg muscle tropomyosin (lane *3*) and breast muscle tropomyosin (lane *5*), but not with gizzard tropomyosin (lane *2*) or CEF tropomyosin (lane *4*). Indirect immunofluorescence of myoblasts and myotubes with CL2 antibody also supported this specificity. The CL2 antibody did not stain fibroblasts in both myoblast and myotube cultures (arrowheads in Fig. 2, *B* and *D*). Although the antibody reacted weakly with mononuclear myoblasts, strong staining was observed on all myotubes.

As reported previously (18), the CL2 antibody was capable of immunoprecipitating microfilaments from cultured myotubes with a yield (estimated by the actin content) similar to that of the LCK16 antibody, which was known to recognize all isoforms of tropomyosin. On the other hand, the yields of microfilaments isolated from fibroblastic cells by CL2 antibody were very low (about one-tenth) as compared with that obtained by LCK16 antibody (18). These results suggested that the CL2 antibody could be used to selectively isolate the skeletal tropomyosin-enriched microfilaments but not the fibroblastic tropomyosin-enriched microfilaments from differentiating muscle cells.

In order to determine whether the CL2 microfilaments represent a class of skeletal muscle microfilament, we have used CL2 and LCK16 antibodies to isolate microfilaments from a mixed population of [^{3}H]methionine-labeled CEF cells and [^{35}S]methionine-labeled myotubes. If the CL2 microfilaments (CL2-MF) represent skeletal tropomyosin-enriched microfilaments, the $^3H/^{35}S$ ratio in the CL2-MF should be lower than that in the LCK16 microfilaments (LCK16-MF). This type of analysis will also establish the extent of background contamination by fibroblastic tropomyosin-enriched microfilaments in the isolated skeletal tropomyosin-enriched microfilaments. The initial mixture, with a $^3H/^{35}S$ ratio of 0.91, was subjected to microfilament isolation with either CL2 or LCK16 antibody. The protein components of the isolated microfilaments (CL2-MF and LCK16-MF) were resolved on two-dimensional gels. The radioactivity of each protein spot was determined by cutting spots from the gels and counting them as described (18). As can be seen in Table I, the $^3H/^{35}S$ ratios (*a*) of protein components in CL2-MF were significantly smaller than that (*b*) in LCK16-MF. Because both CL2 and LCK16 antibodies immunoprecipitate roughly equal amounts of ^{35}S-labeled microfilaments from myotubes, the *(a)/(b)* ratios (Table I) of total actin and total tropomyosin would represent the extent of fibroblastic tropomyosin-enriched microfilament contamination in skeletal tropomyosin-enriched microfilaments. Thus, ~27–30% of the fibroblastic microfilaments were present as contaminants in the CL2-MF after the microfilament isolation by CL2 antibody. We have previously shown that during microfilament isolation the filaments do not exchange their components with exogeneous molecules, such as F-actin (24). This would argue against the possibility that the contamination may be due to the recombination occurring between two types of microfilaments during isolation.

Table II. Yield and Tropomyosin/Actin Ratio of Various Microfilaments Isolated from Myoblasts, Myotubes, or CEF

	Yield	
	Percent of total actin	TM/A*
Myoblasts		
CL2-MF	8.5	0.19
LCK16-MF	9.2	0.17
Myotubes		
CL2-MF	27.2	0.26–0.29
LCK16-MF	25.9	0.26–0.30
CEF		
LCK16-MF	24.3–32.5‡	0.17–0.19‡

The yield of isolated microfilaments was expressed as a percentage of total actin in the cells. Amounts of [^{35}S]methionine incorporation into the actin band of various fractions during microfilament isolation were used for calculation of percent.
* TM/A, radioactive ratio of tropomyosin to actin. Values were calculated from [^{35}S]methionine incorporation into tropomyosin and actin bands of the isolated microfilaments separated on SDS polyacrylamide gels.
‡ Values obtained from references 19 and 20.

Assembly of Actin and Tropomyosin Isoforms into the Microfilaments of Myoblasts and Myotubes

It has been shown that during muscle differentiation, the expression of actin and tropomyosin isoforms changes from nonmuscle forms to muscle forms (4, 8, 11). What is the underlying mechanism for this transition? Do different sets of microfilaments exist in the differentiating muscle cells?

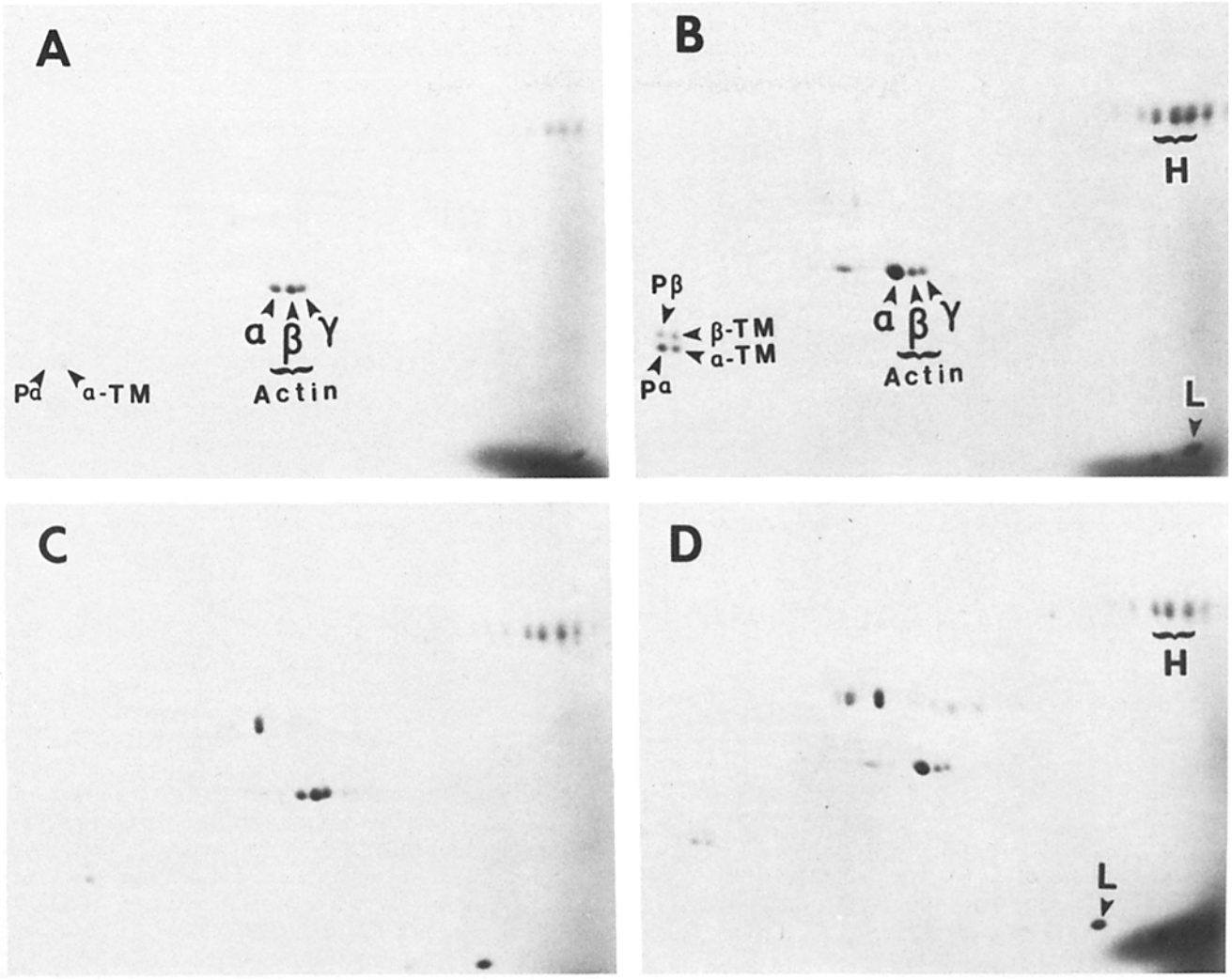

Figure 3. Two-dimensional gel analysis of the isolated microfilaments from chicken embryo myoblasts and myotubes. Coomassie Blue–stained protein patterns of the gels are shown here, with the acidic end to the left. (*A* and *B*) Skeletal tropomyosin-enriched microfilaments isolated by CL2 antibody from myoblasts and myotubes, respectively. (*C* and *D*) Tropomyosin-enriched microfilaments isolated by LCK16 antibody from myoblasts and myotubes, respectively. *H* and *L* indicate the positions of heavy and light chains of monoclonal antibodies. *Pα* and *Pβ* refer to the phosphorylated forms of skeletal muscle α-tropomyosin (*α-TM*) and β-tropomyosin (*β-TM*), respectively.

These questions are still not completely answered, but our ability to isolate skeletal tropomyosin-enriched microfilaments by CL2 antibody should provide a new approach to these problems. Therefore, we carried out experiments to fractionate and characterize myoblast and myotube microfilaments with CL2 and LCK16 antibodies. Two classes of microfilaments, i.e., the skeletal tropomyosin-enriched (CL2-MF) and the tropomyosin-enriched (LCK16-MF) microfilaments, were obtained.

Because there did not appear to be a suitable way to estimate the yield of microfilaments, 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 total cellular actin. As seen in Table II, the yields for both CL2-MF and LCK16-MF isolated from either myoblasts or myotubes are very close (8.5–9.2% for myoblasts and 25.0–27.2% for myotubes). The yield of tropomyosin-enriched microfilaments isolated from myoblasts seems to be relatively low. This may reflect the true amounts for myoblast tropomyosin-enriched microfilaments, because indirect immunofluorescence microscopy on these cells with anti-tropomyosin antibody generally reveals a weak and diffuse staining pattern (data not shown). As cells differentiated into myotubes, we readily obtained a comparable amount of tropomyosin-enriched microfilaments, as we did from CEF cells (Table II). When the tropomyosin/actin ratios (TM/A in Table II) were calculated from the radioactivities of both proteins, a reasonable result was found. The TM/A ratios of tropomyosin-enriched microfilaments from myoblasts were similar to that from CEF cells. On the other hand, myotube microfilaments contained a higher tropomyosin/actin ratio.

Protein components of these two microfilament classes were further analyzed on two-dimensional gels. Fig. 3 shows the Coomassie Blue–stained protein profiles of the isolated microfilaments. In addition to heavy and light chains of immunoglobulins, actin isoforms (α, β, and γ) and skeletal tropomyosin isoforms (α-TM, β-TM, Pα, and Pβ) were readily separated. As expected, the amounts of α-actin in the microfilaments isolated from myotubes (Fig. 3, *B* and *D*)

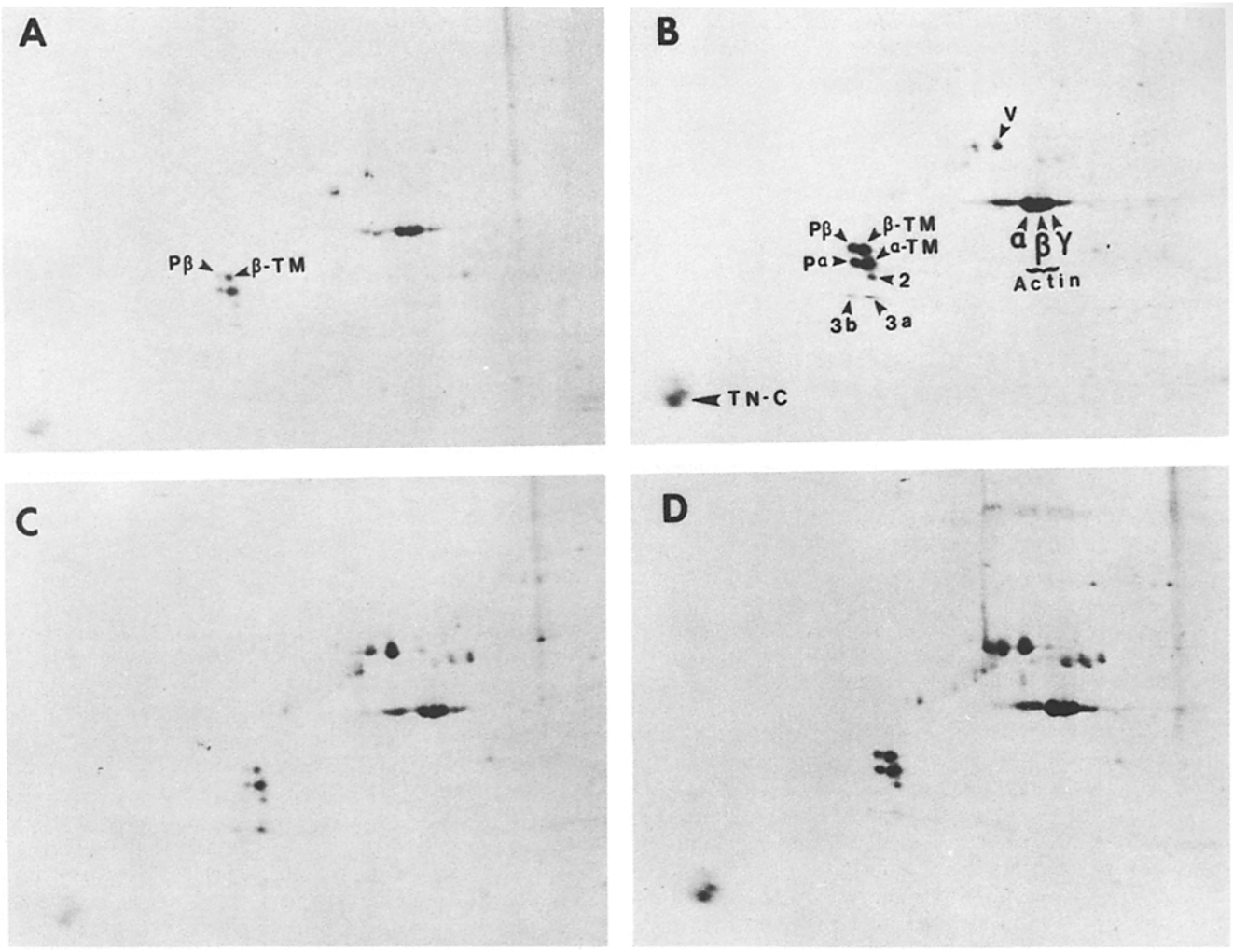

Figure 4. Two-dimensional gel analysis of the isolated microfilaments from [^{35}S]methionine-labeled chicken embryo myoblasts and myotubes. Fluorographs of the gels are shown here, with the acidic end to the left. (*A* and *B*) Skeletal tropomyosin-enriched microfilaments isolated by CL2 antibody from myoblasts and myotubes, respectively. (*C* and *D*) Tropomyosin-enriched microfilaments isolated by LCK16 antibody from myoblasts and myotubes, respectively. *Pα* and *Pβ*, phosphorylated forms of skeletal muscle α-tropomyosin (*α-TM*) and β-tropomyosin *β-TM*), respectively. *v*, Vimentin; *TN-C*, troponin C; *2*, *3a*, and *3b*, CEF tropomyosin isoforms 2, 3a, and 3b, respectively.

were greatly increased, as compared with those in the myoblast microfilaments (Fig. 3 *A* and *C*). There was no obvious qualitative difference between CL2-MF and LCK16-MF, except that vimentin and its variants were coprecipitated with LCK16-MF. The association of vimentin with LCK16-MF appeared to be due to the cross-reaction of LCK16 antibody to both tropomyosin and vimentin (17). These same gels were further processed for autoradiography. Fig. 4 shows [^{35}S]methionine-labeled protein profiles of the isolated microfilaments. In addition to actin isoforms and skeletal tropomyosin isoforms, nonmuscle tropomyosin isoforms 1, 2, 3a, and 3b, as identified previously (20), were also synthesized and assembled into the skeletal tropomyosin-enriched microfilaments (CL2-MF, Fig. 4, *A* and *B*) and the tropomyosin-enriched microfilaments (LCK16-MF, Fig. 4, *C* and *D*). However, the content of these nonmuscle tropomyosin isoforms in CL2-MF was significantly lower than that in LCK16-MF. The radioactive incorporation into each isoform of actin and tropomyosin was measured by cutting spots from the gels and counting them. We felt very comfortable in cutting the spots of actin isoforms, because the well-separated spots could be readily identified from the Coomassie-Blue–stained gels. Therefore, the counts of actin did not include the long trailing edge on the side of α-actin (Fig. 4). However, the α-TM spot was contaminated by nonmuscle tropomyosin isoform 1. After normalization with the radioactivity of γ-actin or total actin, relative rates of [^{35}S]methionine incorporation into actin and tropomyosin isoforms from the isolated microfilaments were obtained and summarized in Table III.

As seen in Table III A, the skeletal tropomyosin-enriched microfilaments (CL2-MF) isolated from both myoblasts and myotubes contained higher relative amounts of α-actin than the respective tropomyosin-enriched microfilaments (LCK16-MF). Furthermore, the relative proportions of actin isoforms remained very similar in the CL2-MF of myoblasts and myotubes (Table III A, columns a and c), except a significant increase in the assembly of α-actin onto the myotube microfilaments. Judging from the Coomassie-Blue–stained gels (Fig. 3, *A* and *B*), the chemical amounts of α-actin in the myotube CL2-MF were much higher than that in the myoblast microfilaments. These results suggested that the coassembly of new-

Table III. Relative Rates of [^{35}S]Methionine Incorporation into Actin and Tropomyosin Isoforms in the Microfilaments Isolated by CL2 Antibody or LCK16 Antibody from Cultured Chicken Embryo Myoblasts and Myotubes

		Myoblasts			Myotubes		
		CL2-MF (a)	LCK16-MF (b)	a/b	CL2-MF (c)	LCK16-MF (d)	c/d
A	α-Actin	1.26	0.81	1.55	1.39	1.06	1.31
	β-Actin	2.19	1.99	1.10	2.28	1.86	1.22
	γ-Actin	1.00	1.00	1.00	1.00	1.00	1.00
B	Total actin	100 (7,876)	100 (8,564)		100 (19,030)	100 (18,195)	
	β-TM	3.46	2.52	1.37	8.67	8.16	1.06
	*Pβ	1.50	0.33	4.55	1.94	1.87	1.04
	α-TM + TM-1	9.53	8.73	1.09	10.12	10.61	0.95
	Pα	2.62	0.93	2.82	3.07	2.69	1.14
	TM-2	0.84	1.45	0.58	1.26	1.79	0.70
	TM-3a	1.21	2.10	0.58	0.47	0.74	0.64
	TM-3b	0.28	0.56	0.50	0.21	0.33	0.64

Note that the spots of α-TM and TM-1 are too close together on the two-dimensional gel to be quantified separately (19). Numbers in the parenthesis are the counts (cpm) of total actin (α, β, and γ) in different microfilament preparations.
*Pβ, Phosphorylated isoform of β-tropomyosin (β-TM); Pα, phosphorylated isoform of α-tropomyosin (α-TM); TM-1, TM-2, TM-3a, and TM-3b, CEF tropomyosin isoforms 1, 2, 3a, and 3b, respectively.

ly synthesized actin isoforms into pre-existing microfilaments and that the pre-existing microfilaments could be enriched in β-actin amount, as in the case of myoblast CL2-MF (Fig. 3 *A*) or in α-actin amount, as in the case of myotube CL2-MF (Fig. 3 *B*).

In Table III B, the ratio of each tropomyosin isoform was expressed per 100 parts of total actin counts in the microfilaments. It was clear that skeletal tropomyosin-enriched (CL2-MF) and tropomyosin-enriched (LCK16-MF) microfilaments from myoblasts had different compositions of tropomyosin isoforms. The spots for muscle isoform α-TM and nonmuscle isoform 1 of tropomyosin were too close together on the two-dimensional gel to be quantified separately. Therefore, we always treated them as a unit. However, one should keep in mind that in the differentiating muscle cells, the α-TM is predominant to the nonmuscle tropomyosin isoform 1. The content (Table III B, column a) of nonmuscle tropomyosin isoforms (TM-2, TM-3a, and TM-3b) in the myoblast CL2-MF was less than that (Table III B, column b) in the LCK16-MF of the same cells. However, the CL2-MF still contained ~50–58% (column a/b) of the nonmuscle tropomyosin isoforms found in LCK16-MF. These ratios (50–58%) were greater than the calculated contamination (27–30%) during isolation, as described above. This comparison appears to be reasonably valid, since the tropomyosin/actin ratios for myoblast and fibroblast microfilaments and the total amounts of actin precipitated by CL2 and LCK16 antibodies are very similar (Table II). Therefore, the nonmuscle tropomyosin isoforms were a significant component of the skeletal tropomyosin-enriched microfilaments of myoblasts. As muscle cells differentiated into myotubes, tropomyosin isoform contents of the CL2-MF and LCK16-MF became very similar except for nonmuscle isoforms. Again, a significant amount of nonmuscle tropomyosin isoforms was associated with the skeletal tropomyosin-enriched microfilaments of myotubes.

When one compared the changes in tropomyosin isoforms within the CL2-MF during development (Table III B, columns a and c), the increases in muscle α- and β-TM and the decreases in nonmuscle TM-3a and 3b were evident. However, an unexpected increase in nonmuscle TM-2 isoform from myoblasts to myotubes was repeatedly obtained. At present there is no reasonable explanation for this increase.

In myoblasts, a striking difference between the tropomyosin content of CL2-MF and LCK16-MF was the relative amount of phosphorylated forms (Pα and Pβ in Table III B) of muscle tropomyosin. The skeletal tropomyosin-enriched microfilaments (CL2-MF) contained 4.5- and 2.8-fold higher concentrations of the phosphorylated forms of β- and α-tropomyosins, respectively, than the tropomyosin-enriched microfilaments of myoblasts (LCK16-MF). This difference appeared to diminish as the muscle cells differentiated into myotubes. In another experiment with myoblasts pulse-labeled for 4 h, we also obtained a higher content of the phosphorylated forms of muscle tropomyosin in the skeletal tropomyosin-enriched microfilaments (data not shown). The physiological significance of this finding remains to be determined.

It should be noted that the chemical amount (estimated by the Coomassie Blue staining intensity) of the phosphorylated form of α-tropomyosin was greater than the unphosphorylated form in myotube microfilaments (Fig. 3, *B* and *D*), whereas the reverse relation was found in the myoblast microfilaments (Fig. 3, *A* and *C*). However, the rate of assembly ([^{35}S]methionine incorporation) of the phosphorylated form of α-tropomyosin into myotube microfilaments was much lower than that of the unphosphorylated form (Fig. 4, *B* and *D*, Table III B).

Electron Microscopic Characterization of Isolated Microfilaments from Myoblasts and Myotubes

When the isolated microfilaments from myoblasts and myotubes were negatively stained and observed with the electron microscope, ordered bundles of microfilaments formed by anti-tropomyosin monoclonal antibodies can be readily seen (Fig. 5). In addition, cross-striations along whole bundles are apparent. These cross-striations represent the location of antibodies, which in turn indicate the position of tropomyosin molecules. The periodicity may further reflect the size of the tropomyosin rod, assuming every tropomyosin dimer lying in the grooves of actin filaments does not overlap. As can

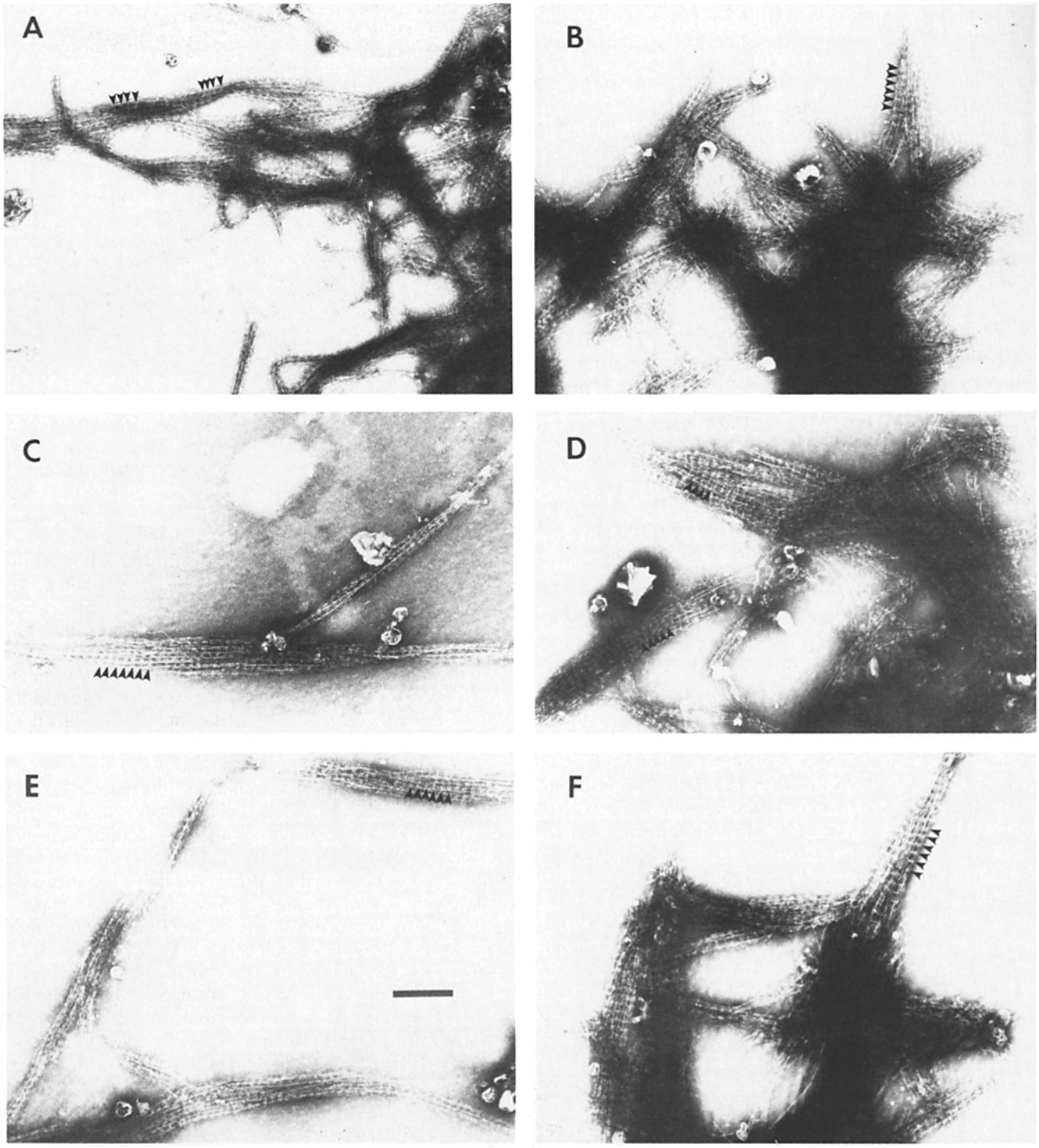

Figure 5. Electron micrographs of isolated microfilaments from chicken embryo myoblasts (*A* and *B*) and myotubes (*C–F*). (*A*, *C*, and *E*) Tropomyosin-enriched microfilaments isolated by LCK16 antibody, which recognized all isoforms of tropomyosin. (*B*, *D*, and *F*) Skeletal tropomyosin-enriched microfilaments isolated by CL2 antibody. The isolated microfilaments 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. Bar, 0.1 μm.

be seen in Fig. 5, both CL2-MF and LCK16-MF isolated from myotubes usually have more ordered bundles and more obvious periodicity than the microfilaments isolated from myoblasts. An increase in the tropomyosin/actin ratio (Table II) may explain this observation.

To measure the tropomyosin periodicities in both CL2-MF and LCK16-MF of myoblasts and myotubes, we have used a catalase standard with a periodicity of 8.12 nm for calculation. Measurements were always made on bundles that contain between 5 and 10 continuous cross-striations. Histo-

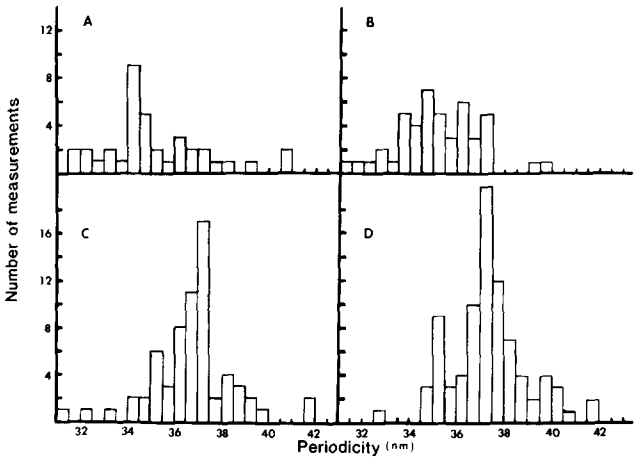

Figure 6. Histogram of the measurements of tropomyosin periodicities along the isolated microfilaments from myoblasts (*A* and *B*) and myotubes (*C* and *D*). (*A* and *C*) Skeletal tropomyosin-enriched microfilaments isolated by CL2 antibody. (*B* and *D*) Tropomyosin-enriched microfilaments isolated by LCK16 antibody. The isolated microfilaments were negatively stained and observed with an electron microscope. For measurement, a stained catalase resolution standard with a periodicity of 8.12 nm was used to calibrate the tropomyosin periodicity. Each measurement of tropomyosin periodicity was made from a bundle that contained 5–10 cross-striations visualized by the binding of monoclonal antibody.

grams from such measurements are shown in Fig. 6, and the mean values of tropomyosin periodicities along CL2-MF and LCK16-MF of myoblasts and myotubes are summarized in Table IV. No significant difference between the tropomyosin periodicities of skeletal tropomyosin-enriched microfilaments (CL2-MF) and tropomyosin-enriched microfilaments (LCK16-MF) could be detected. However, the tropomyosin periodicity (37 nm) along myotube microfilaments was considerably longer ($P < 0.001$) than those of myoblast microfilaments (35 nm) or CEF microfilaments (34 nm). Early studies on tropomyosin paracrystals have shown that the repeat distance (38–40 nm) in the paracrystals obtained from muscle tropomyosin is consistently longer than that obtained from nonmuscle tropomyosin (5, 7, 10, 30). These observations may imply that the nonmuscle tropomyosin is shorter than muscle tropomyosin.

Discussion

In the present study, a striated muscle tropomyosin isoform-specific monoclonal antibody (CL2) was used to selectively isolate a class of microfilaments, i.e., skeletal tropomyosin-enriched microfilaments (CL2-MF), from differentiating muscle cells. CL2-MF differ from another class of microfilaments, i.e., tropomyosin-enriched microfilaments (LCK16-MF), isolated from the same cells by a monoclonal antibody (LCK16) against all isoforms of tropomyosin.

Since the experimental approach used in this study (see also references 20 and 24) allows us to directly isolate the skeletal tropomyosin-enriched microfilaments, the relative ratios of [^{35}S]methionine incorporation should partly reflect the relative ratios of assembly into microfilament fractions. The assembly of α-actin appears to be lower than that of

Table IV. Periodicity of Tropomyosin Binding to Microfilaments Isolated from Chicken Embryo Myoblasts and Myotubes by CL2 or LCK16 Monoclonal Antibody

	Periodicity value (mean ± SD)	
	CL2-MF	LCK16-MF
Myoblasts	35.2[a] ± 2.0 (n = 46)*	35.3[c] ± 2.2 (n = 37)
Myotubes	37.4[b] ± 1.6 (n = 85)	36.8[d] ± 1.8 (n = 66)
CEF	–	33.9 ± 2.1 (n = 11)

Using the two-sample t test for means between a and b ($P < 0.001$, equal standard deviations) or between c and d ($P < 0.001$, unequal standard deviations), it is clear that the difference in tropomyosin periodicity between myoblasts and myotubes is significant.
* n, Number of bundles measured that contained 5–10 cross-striations.

β-actin in both myoblast and myotube CL2-MF (Table III A). However, the increase in the assembly of α-actin from myoblasts to myotubes is significantly greater than that in β-actin or γ-actin (Table III A, columns a and c). Therefore, the total amounts (estimated from Coomassie Blue–stained gels) of α-actin accumulated in myotube microfilaments can be greater than β-actin or γ-actin (Fig. 3 *B*). Another possible reason for the preferential accumulation of α-actin in myotube microfilament may be due to a slower turnover rate for α-actin as compared with β-actin or γ-actin. More pulse-chase experiments are needed to test this possibility.

The skeletal tropomyosin-enriched microfilaments reported here contained not only muscle isoforms of actin and tropomyosin but also their nonmuscle counterparts. The amounts of nonmuscle isoforms associated with this class of microfilament could not be totally accounted for by contamination with the fibroblastic microfilaments during isolation (Tables I–III) or by contamination of fibroblasts in the culture we used. This finding suggests that different isoforms of actin and tropomyosin can be assembled into the same set of skeletal tropomyosin-enriched microfilaments.

An early study on the development of myofibrils by electron microscopy has demonstrated that new sarcomeres appeared to form on a pre-existing bundle of thin filaments, suggesting that the microfilament bundles may be precursors to myofibrils (29). From studies of temporal and topographical relationships between stress fiber–like structures (SFLS) and nascent myofibrils in both ethyl methanesulfonate–recovering skeletal myosheets and normal cultured heart cells, Holtzer and his colleagues have further shown that at the earliest stage of myofibrillogenesis, individual nascent myofibrils appear to be part of or juxtaposed to pre-existing individual SFLS and that at the later stages all SFLS have disappeared and are replaced with mature myofibrils (1, 9, 14). Although a transient one-on-one relationship between individual SFLS and newly emerging individual nascent myofibrils has been suggested and proposed, these investigators could not determine whether the earliest muscle-specific myofibrillar proteins form transitory heteropolymers with the nonmuscle isoforms or homopolymers of thick and thin filaments in the vicinity of the pre-existing SFLS. In the present biochemical study, we have demonstrated that the skeletal tropomyosin-enriched microfilaments contain not only muscle isoforms but also nonmuscle isoforms of actin and tropomyosin, suggesting that they can assemble into heteropolymers. This result supports the idea that there is a gradual transition of one set of microfilaments into another during

maturation of thin filaments. Coassembly of muscle isoform of actin into stress fiber of fibroblasts has also been demonstrated by microinjection of purified muscle α-actin (12) or by transfection of α-actin gene sequence (13) into live cells.

The myoblast CL2-MF are enriched not only in α-actin content but also in the phosphorylated isoforms of skeletal α- and β-tropomyosins when compared with the myoblast LCK16-MF. The difference in phosphorylated form content cannot be due to the possibility that the LCK16 antibody used in the isolation of microfilaments contains higher phosphatase activity than the CL2 antibody, because both CL2-MF and LCK16-MF isolated from myotubes contain a similar amount of these phosphorylated isoforms. At the present time, the physiological significance of tropomyosin phosphorylation is not known. Mak et al. (22) have speculated that the phosphate group of serine-283 on one molecule of tropomyosin could form a salt linkage with lysine-6 on the other molecule. This linkage could participate in stabilizing the head-to-tail overlap of the tropomyosin dimers that are believed to mediate a degree of cooperation in the control of muscle contraction (25, 32). However, Barany et al. (2) have demonstrated that phosphorylation of tropomyosin is independent of muscle contraction in frogs.

In the present work, we have consistently found a higher content of phosphorylated isoforms of tropomyosin in the skeletal tropomyosin-enriched microfilaments of myoblasts. This suggests that during muscle differentiation in vitro, the conversion of microfilaments (which initially contain either nonmuscle forms of tropomyosin or no tropomyosin) into skeletal tropomyosin-enriched microfilaments is accompanied by phosphorylation of muscle tropomyosin. Whether this phosphorylation occurs before or after the assembly of muscle isoforms in microfilaments remains unclear. As can be seen in Figs. 3 and 4, myotube CL2-MF contains a higher chemical amount of the phosphorylated form of α-tropomyosin and less [^{35}S]methionine incorporation into this phosphorylated form as compared with the unphosphorylated form. This result may be explained by a slower turnover rate of the phosphorylated form of α-tropomyosin. However, it may also suggest that phosphorylation, at least of α-tropomyosin, occurs after assembly into microfilaments. Further experiments with pulse-chase labeling may establish this precursor–product relationship. Although the function of the phosphorylated form of tropomyosin remains unclear, it is possible that the phosphorylated isoforms of tropomyosin in the skeletal tropomyosin-enriched microfilaments may serve as a label or marker on microfilaments to allow further assembly of muscle-specific isoforms of actin and tropomyosin. The phosphorylated isoforms of tropomyosin have been shown to be the major forms of tropomyosin in the leg muscle of 10-d-old embryos (27). These phosphorylated isoforms found in embryonic muscle may also play a marking function in the maturation of thin filaments. During embryonic development, the amount of phosphorylated isoforms of both α- and β-tropomyosins decreases so that after hatching <20% of each isoform is phosphorylated.

What molecular mechanism can account for the transition from nonmuscle to muscle forms of actin and tropomyosin during myogenesis? A pre-existing microfilament, either with nonmuscle tropomyosin or devoid of tropomyosin, may serve as a precursor for the assembly of muscle actin and tropomyosin. We and other investigators have previously shown that nonmuscle tropomyosins have lower binding affinity for actin filaments than do muscle tropomyosins (6, 10, 19). Thus, competition between muscle and nonmuscle tropomyosins for binding to microfilaments may well be a part of the transition mechanism. Moreover, the phosphorylation of muscle tropomyosins may amplify this competition, presumably by enhancing the head-to-tail polymerization of tropomyosin dimers. Once enough molecules of muscle tropomyosins are assembled, the microfilaments can become the skeletal tropomyosin-enriched microfilament, as defined by the CL2 antibody. In addition to being rich in α-actin content, the skeletal tropomyosin-enriched microfilament contains a higher content of phosphorylated forms of muscle tropomyosin. These phosphorylated isoforms may serve as a marker on the skeletal tropomyosin-enriched microfilament for further assembly of muscle actin, tropomyosin, and other thin filament proteins to eventually form mature thin filaments of myofibrils. However, more experiments are needed to test this hypothesis.

It is now known that nonmuscle tropomyosin is shorter than muscle tropomyosin (Table IV, references 5, 7, 10, 30). Thus, we should expect to have a larger periodicity for tropomyosin in the myoblast CL2-MF as opposed to that in the myoblast LCK16-MF. The fact that we do not detect any difference is totally surprising. A possible explanation is that tropomyosin dimer in myoblast microfilaments may organize differently. For example, they may have more region for head-to-tail overlap as compared with that in myotube microfilaments.

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