Characterization of the Tropomyosin Present in Various Chick Embryo Muscle Types and in Muscle Cells Differentiated in Vitro*

(Received for publication, December 1, 1980, and in revised form, January 14, 1981)

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Tropomyosin, present in various types of chick embryo muscle, has been characterized by two-dimensional gel electrophoresis. In skeletal muscle, it was found that both the α and β subunits exist as two variants, α',α, and β',β. The most acidic variants (α', and β') could be demonstrated to be phosphorylated and, based upon the facts that 1) after phosphatase treatment α' and β' co-migrate with α' and β' and 2) in vitro translation on the skeletal muscle mRNA produces only α' and β', we suggest that α' and β' merely represent the phosphorylated forms of α and β.

A similar situation is found in differentiated muscle cultures in vitro. In cardiac muscle or in cardiocytes, in culture, the only subunit of tropomyosin which is present (the α subunit) is also phosphorylated. However, in smooth muscle, none of the tropomyosin subunits is phosphorylated. The use of various modifications in the second dimension of two-dimensional gel electrophoresis has allowed us to separate completely the α subunits of slow and fast muscle tropomyosin and to show that: 1) the cardiac α subunit is distinct from either the slow α or the fast α subunit and 2) in vitro differentiated cells synthesize a tropomyosin which, by co-migration under various conditions, is identical with fast muscle tropomyosin.

Tropomyosin is an important component of the contractile apparatus of muscle since in association with a complex of troponin it plays a central role in the regulation of muscle contraction (1). In skeletal muscle, tropomyosin is composed of two polypeptides, α- and β-tropomyosin (2), with apparent molecular weights of ~33,000 and 36,000, which are present in different molar ratios depending on the muscle type (3).

Under non-denaturing conditions, tropomyosin is present as a two-stranded coiled-coil protein and is located in the grooves of the double-stranded structure of actin (4). It forms long filaments by aggregation of individual subunits due to an overlap of eight to nine amino acid residues at the NH2- and COOH-terminal ends (5, 6). Recently, it has been demonstrated that tropomyosin, isolated from frog and rabbit, is phosphorylated at the serine (7). More accurately the tropomyosin molecules which are synthesized by muscle cells undergoing differentiation in vitro. We will show that: 1) in skeletal muscle both α- and β-tropomyosins are phosphorylated; 2) cultured muscle cells differentiated in vitro synthesize a tropomyosin which co-migrates with the tropomyosin isolated from fast muscle; and 3) tropomyosin isolated from various chick embryonic muscle types can be resolved by two-dimensional gel electrophoresis and may therefore represent different gene products.

EXPERIMENTAL PROCEDURES

Purification of Tropomyosin

Tropomyosin was isolated from extracts of ether-dried muscle powder by a combination of (NH4)2SO4 fractionation and isoelectric precipitation at pH 4.6 (2, 8).

Isolation of RNA

Cytoplasmic RNA from differentiated cultures of chick embryo myoblasts was extracted as described elsewhere (9).

To prepare chick muscle RNA, the thigh muscles from chick embryos were immediately frozen in liquid nitrogen and stored at ~70 °C. The frozen muscles were directly homogenized in a mixture of 6 m urea, 3 M LiCl (5–10 ml/g of tissue) in a Waring Blender. This mixture was allowed to stand for a minimum of 4 days at 4 °C. The nucleic acids were pelleted by centrifugation (30 min × 10,000 rpm in the HB4 rotor of the Sorvall) and further extracted three times with a mixture of phenol/chloroform, followed by one extraction with chloroform. The RNA in the aqueous phase was precipitated at ~20 °C with 2 volumes of ethanol. The RNA pellet was washed three times with ethanol, dried, dissolved in sterile water, and stored at ~70 °C.

In Vitro Translation

Reticulocyte lysates were prepared and treated as described by Pelham and Jackson (10). The in vitro translation was performed as described elsewhere (11).

Electrophoresis

Two-dimensional gel electrophoresis was carried out essentially as described by O'Farrell (12). The gels for the first dimension were 12 cm long and contained 2% pH 4–6, ampholines. For the second dimension 12.5% polyacrylamide SDS gels cross-linked with 0.1% bisacrylamide were used and in some cases 3.5 m urea was added. The gels were stained with 0.25% Coomassie blue in methanol/acetic acid/water (45:10:45) or processed for autoradiography (13).

Cell Cultures and Labeling

Myoblasts were isolated from 11-day-old embryo thigh muscle and allowed to differentiate into myotubes (14, 15). Labeling with [35S]Methionine—Myotubes were pulse-labeled for 2 h with [35S]methionine (>1000 Ci/mmol; 50 μCi/ml) in methionine-free medium supplemented with 10% fetal calf serum. At the end of the labeling period, either the culture was washed and cells were

* This work has received financial support from the Délégation Générale à la Recherche Scientifique et Technique, Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, Commissariat à l'Energie Atomique, Ligue Nationale Française contre le Cancer, Fondation pour la Recherche Médicale et la Muscular Dystrophy Associations of America. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Chick Embryonic Muscle Tropomyosin

...treated to prepare a total cell extract (15) or myofibrils (16), or the tropomyosin was purified.

Labeling with $^{32}$P—Myotubes were labeled with $[^{32}]P$phosphate (1 mCi/ml) for 24 h. At the end of the labeling period either a total cell extract was prepared (15) or tropomyosin was purified.

Phosphatase Treatment

Digestion with alkaline phosphatase from calf intestine (Boehringer 500 units/mg, 1 mg/0.2 ml) was carried out in 50 mM Tris-HCl, pH 8, 0.2 mM ZnCl$_2$, 0.15 M NaCl for 3 h at 35 °C. The enzyme to substrate ratio was approximately 1:100. At the end of the incubation the samples were prepared for two-dimensional gel electrophoresis by adding NP40, pH 4-6 ampholines, β-mercaptoethanol, and urea to a final concentration of, respectively, 2%, 2%, 5%, and 9.5 M.

RESULTS

Two-dimensional Gel Electrophoresis of Tropomyosin from Embryonic Skeletal Muscle—Tropomyosin isolated from embryonic thigh muscle migrates as two components on SDS-polyacrylamide gels. These correspond to the known α and β subunits (Fig. 1, lane 3) which, when compared with proteins of known molecular weight, have molecular weights of 34,000 and 36,000, respectively. When analyzed on two-dimensional gel system, using ampholines giving a pH range of 4-6 for the first dimension, both α- and β-tropomyosins are now resolved into two variants which we call α$'$ and β$'$. No radioactivity is associated with α$'$ or β$'$.

In these myofibrils, one other protein is strongly labeled with $^{32}$P and it has been identified as α-desmin. To determine further if the charge differences between α$'$ and α or β$'$ and β$'$ are related exclusively to a phosphorylation, the effect of phosphatase treatment on the electrophoretic migration of

![Fig. 1. SDS-polyacrylamide gel electrophoresis of tropomyosin isolated from chick embryonic heart, gizzard, and skeletal muscle. 1, 2.5 μg of tropomyosin isolated from the heart of 17-day-old chick embryo; 2, 12 μg of tropomyosin isolated from the gizzard of the same embryo; 3, 30 μg of tropomyosin isolated from the thigh muscle.](image)

![Fig. 2. Two-dimensional electrophoresis of skeletal muscle tropomyosin. A, tropomyosin was purified from the thigh muscle of 17-day-old chick embryo as described under "Experimental Procedures"; B, total cell extract was prepared from the thigh muscle from 17-day-old chick embryo and analyzed by two-dimensional gel electrophoresis; C, myofibrils were prepared from a well fused culture of chick embryo myoblasts.](image)

![Fig. 3. Two-dimensional electrophoresis of $^{32}$P-labeled myofibrils from differentiated muscle cells. Chick embryo myoblasts were isolated from the thigh muscle of 11-day-old chick embryo and allowed to differentiate in vitro. Two days after the formation of the myotubes, the cells were labelled for 24 h with $^{32}$P (1 mCi/ml). Myofibrils were purified and analyzed by two-dimensional gel electrophoresis.](image)
purified tropomyosin was examined. Tropomyosin was purified from 15-day-old embryo thigh muscle and was phosphorylated protein was treated with phosphatase as described under "Experimental Procedures." In the absence of phosphatase (Fig. 4A), α1 and β2 are more intense than α2 and β1. After treatment by phosphatase for 3 h at 35 ºC only two spots are seen (Fig. 4B). These spots can be identified as α1 and β1 since this tropomyosin preparation is not absolutely pure and a contaminant (labeled with an arrowhead) located below α1 and β1 is also located below the two remaining spots. The same experiment performed with 32P-labeled tropomyosin isolated from fused muscle cell cultures showed that after phosphatase treatment the radioactivity had completely disappeared (result not presented).

All these experiments, therefore, clearly demonstrate that the α2 and β2 species are phosphorylated forms of tropomyosin.

**Phosphorylation of Other Muscle Tropomyosin.**—Since we had found that tropomyosin from embryonic thigh muscle were phosphorylated, we decided to check whether tropomyosin isolated from other muscle types presented the same characteristic. We have therefore analyzed tropomyosin isolated from embryonic gizzard and embryonic heart. As can be seen in Fig. 1 (lane 2), gizzard tropomyosin consists of two subunits, α and β, with apparent molecular weight of 35,000 and 43,000, respectively. When analyzed by two-dimensional gel electrophoresis, each subunit is resolved as a single spot (Fig. 5A). After phosphatase treatment (Fig. 5B) the migration of the two subunits remains unchanged. These subunits still co-migrate with untreated tropomyosin (not shown) which suggests that neither the α nor the β subunit of gizzard tropomyosin is phosphorylated.

As shown in Fig. 1 (lane 1), embryonic cardiac tropomyosin consists exclusively of the α subunit which has an apparent molecular weight of 33,500. When analyzed by two-dimensional gel electrophoresis, this α subunit is resolved into two variants (Fig. 5C). The more acidic species is phosphorylated as can be demonstrated either by phosphatase treatment (not shown here) or by [32P]phosphate labeling of cultured cardiac cells (Fig. 5D).

To extend this analysis further we decided to check whether tropomyosins isolated from slow skeletal muscle or fast skeletal muscle were also phosphorylated. As slow muscle we used the anterior latissimus dorsi and as fast muscle the posterior latissimus dorsi. As shown in Fig. 6, both ALD tropomyosin (lane 1) and PLD tropomyosin (lane 2) consist of two subunits, α and β. However, by comparing the two lanes it can be seen that the difference in molecular weight between αPLD and βPLD on the one hand and αALD and βALD on the other is larger in the case of ALD, suggesting that the fast muscle (PLD) tropomyosin could be separated from the slow muscle (ALD) tropomyosin. This is demonstrated in Fig. 6B and C, where both samples have been mixed and analyzed together by two-dimensional gel electrophoresis. Under normal conditions (SDS present alone in the second dimension) no difference can be detected between the β subunit while a very minor difference is visible between the α subunit (Fig. 6B). This difference can be enhanced by using SDS-urea in the second dimension. Under these conditions it is very easy to differentiate the two α subunits (Fig. 6C), whereas there is still no separation between the two β subunits. This analysis also demonstrates that both subunits of either ALD tropomyosin or PLD tropomyosin are resolved into two variants, suggesting that both the α and the β subunits of these two muscles are phosphorylated.

Finally we have compared the cardiac α tropomyosin to the tropomyosins isolated from both PLD and ALD. The α-tropomyosin subunits from PLD and cardiac muscle can be readily distinguished (Fig. 7A) under normal conditions. The separation is much smaller, however, when the cardiac species is compared to ALD tropomyosin (Fig. 7B). This difference
can be considerably increased when urea is present in the second dimension (Fig. 7C). In this case the two α subunits are completely separated with the α cardiac migrating between the α and β subunits of ALD tropomyosin. In this experiment the ALD which was used for purification of tropomyosin was taken from a 15-day-old chick embryo. At that stage the ALD is not exclusively slow and also contains muscle-specific proteins which are characteristic of fast muscle. Therefore, the tropomyosin isolated from that muscle consists of both the slow and fast types. This explains the presence of the α fast variants which migrate slightly more rapidly than the cardiac α-tropomyosin.

In Vitro Translation of Cardiac and Skeletal Muscle RNA—Total RNA was extracted from either hearts or thigh muscles of 15-day-old embryos and translated in the reticulocyte lysate as described under “Experimental Procedures.” The polypeptides synthesized in vitro were analyzed by two-dimensional gel electrophoresis together with authentic tropomyosin purified from hearts or thigh muscles. Fig. 8A shows that among the many polypeptides, the synthesis of which is directed by heart RNA, there is one which co-migrates exactly with the unphosphorylated variant of cardiac tropomyosin (α'). This polypeptide is the only one to be detected when the purification scheme for tropomyosin is applied to the in vitro translation mixture of polypeptides (not shown). As shown in Fig. 8B the same holds true when thigh muscle RNA is translated in vitro. In that case the polypeptides which are detected co-migrate with the unphosphorylated variant of the various subunits of skeletal muscle tropomyosin: β', α fast and α slow.

What Type of Tropomyosin Is Being Synthesized by Muscle Cells in Culture?—Since we have shown that it is possible to distinguish between fast and slow tropomyosin, we have studied whether the tropomyosin synthesized by cultured muscle cells is of the fast or the slow type. Chick embryo muscle cells in culture were labeled with [35S]methionine and tropomyosin was purified. The radioactive preparation was mixed with PLD and ALD tropomyosin and then analyzed by two-dimensional gel electrophoresis with urea included in the second dimension. As shown in Fig. 9 the radioactive tropomyosins correspond precisely to those found in the PLD sample. In contrast, the α subunits of ALD tropomyosin are not found in the cultured cell preparation, whereas the ALD β subunits do co-migrate with the radioactive β proteins. This experiment therefore suggests that chick embryo muscle cells in culture synthesize tropomyosins which comigrate with the fast muscle tropomyosin.

DISCUSSION

The use of two-dimensional gel electrophoresis of proteins as first described by O’Farrell (12) has proved to be a very powerful tool to analyze very complex mixtures of proteins. Furthermore, it has allowed the demonstration of subtle differences between isozymic forms of the same proteins (17, 18).

We have used this technique to analyze the tropomyosin molecules present in various chick embryo muscle tissues and the tropomyosin which is synthesized by chick embryo muscle cells differentiated in vitro.

We have shown that thigh muscle of chick embryo contains α- and β-tropomyosins, both of which are phosphorylated. In mammals, only the α subunit of skeletal muscle has been previously shown to be phosphorylated (7). In this case it was demonstrated that phosphorylation occurred on a serine located at position 283. Preliminary experiments suggest that
and analyzed by two-dimensional electrophoresis with heart tropomyosin and analyzed by two-dimensional electrophoresis. Aliquots of the reaction mixture were mixed with purified tropomyosin which was mixed with tropomyosin purified from ALD muscle cells were allowed to differentiate in culture. Well fused both the α and β subunits of chicken tropomyosin are also phosphorylated on a serine since partial HCl hydrolysis of the mixture yields only one phosphorylated amino acid, O-phosphoserine. Experiments are in progress to locate precisely the position of the phosphorylated serine(s).

Phosphorylation of α- and β-tropomyosin has also been observed by O’Connor et al. (19). However, this group found that the phosphorylated tropomyosin represented a minor component when compared to the nonphosphorylated form. When we compared the situation found in 15-day-old embryo and newborn or adult chick muscle, we observed a significant variation in the ratio between phosphorylated and nonphosphorylated tropomyosins. This variation was similar for both the α and β subunits. It was found that the ratios of either α1/α2 or β1/β2 increase from 40:60 in the 15-day-old embryo to 70:30 in the newborn and finally to 95:5 in the adult (data not shown). Therefore, the situation which has been described by O’Connor et al. (19) (10-day-old chick) seems to be very similar to that which is found in the adult bird. The significance of the decrease in the amount of phosphorylated tropomyosin which we observe during the development of the chick is not known and experiments are in progress in our laboratory to further analyze this process.

We have also investigated whether phosphorylation of tropomyosin was restricted to skeletal muscle or could be also demonstrated with other muscle tissue. It was found, as for mammalian muscle, that the α-tropomyosin isolated from heart muscle was also phosphorylated since phosphatase treatment eliminates the more acidic variant. On the other hand, as already shown by O’Connor et al. (19), we found no phosphorylation of the tropomyosin isolated from gizzard muscle. We also analyzed the tropomyosin isolated from either fast twitch muscle, the posterior latissimus dorsi or a slow tonic muscle, the anterior latissimus dorsi. In both cases, α and β-tropomyosins were phosphorylated. Furthermore, it was also possible to distinguish between the PLD α subunit and the ALD α subunit since these two polypeptides have different mobility in the SDS-urea gels (Fig. 6C). We have taken advantage of the possibility to differentiate between the α subunits of fast and slow muscle to characterize the type of tropomyosin which is synthesized by chick muscle cells in culture. A number of reports have stressed the point that the program which is expressed by these cells is similar to that of fast muscle (20). However, recently Reeburgh-Keller and Emerson have provided evidence that both fast and slow myosin are synthesized by muscle cells in culture (21). From our studies we found that the tropomyosin isolated from differentiated cultures of chick myoblasts co-migrate exactly with tropomyosin isolated from fast muscle (Fig. 8) and we found no evidence of a polypeptide co-migrating with the α subunit of slow muscle. We conclude therefore that newly differentiated muscle cells and skeletal fast muscle synthesize the same tropomyosin.

Lastly we compared the subunits of tropomyosins isolated from heart muscle, fast skeletal muscle, or slow skeletal muscle. We found that it was possible to distinguish between the three types of α subunits by using either SDS-polyacrylamide gels for cardiac and fast muscle or SDS-urea polyacrylamide gels for cardiac and slow muscle. This result raises the question of whether these various α subunits represent different gene products or simply post-translational modification of the same protein. Evidence that the former hypothesis may be correct is provided by experiments in which RNAs were isolated from these various types of muscle and translated in vitro using the reticulocyte lysate. As shown in Fig. 9 the various α subunits of tropomyosin can be identified among the products made in vitro and in all cases they co-migrate exactly with the corresponding α subunits isolated from the tissue from which the RNA has been purified. However, two major differences between the in vitro product and the in vivo protein exist since, first, no phosphorylated variant can be detected in vitro and, second, the amounts of tropomyosin made in vitro are not in the same ratios when compared with the in vivo situation. The first point seems to be due to a lack of phosphorylation in the reticulocyte lysate since another phosphorylated protein, the α-desmin (19), is also found exclusively in its unphosphorylated form when RNA is translated in the reticulocyte lysate. As to the second point, the simplest explanation is that the messenger RNAs coding for the various α and β subunits must have different efficiency of translation and, therefore, the amounts of the various subunits made in vitro cannot be used to quantitate the amount of message present in the muscle. These results suggest, however, that the various subunits of tropomyosin are different gene products. Our observations are in contradistinction with other published results since, according to Dhoot and Perry (22), skeletal muscles contain only one type of α subunit of tropomyosin which is exclusively associated with fast muscle fibers while the β subunit is found in both fast and slow muscle fibers. Also, in mammals, the primary sequences of α tropomyosin from heart and skeletal muscle seem to be very similar, therefore suggesting that they are the product of the same gene (22). However, assuming that our observation is correct for avian muscles, one can calculate the number of genes which are required for the synthesis of various muscle tropomyosins. Such a calculation gives a final number of six different genes.

| Type of muscle | Tropomyosin subunit |
|---------------|-------------------|
| Skeletal muscle | Fast α-fast β-fketal |
| Cardiac muscle | Slow α-slow β-skeletal |
| Smooth muscle | α-cardiac β-skeletal |
| Smooth muscle | α-smooth β-smooth |

In addition to these six different genes one should also add certain extra genes, the exact number of which is unknown for the moment and which would code for the nonmuscle tropomyosin (23, 24).

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2. L. Cohen-Solal and M. Y. Fiszman, unpublished observation.
Acknowledgments—We wish to thank Dr. J. P. Bourgeois for his help in dissecting slow and fast muscles and C. Minty for technical assistance.

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