

A Molecular Defect in Virally Transformed Muscle Cells that Cannot Cluster Acetylcholine Receptors

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Abstract. Muscle cells infected at the permissive temperature with temperature-sensitive mutants of Rous sarcoma virus and shifted to the non-permissive temperature form myotubes that are unable to cluster acetylcholine receptors (Anthony, D. T., S. M. Schuetze, and L. L. Rubin. 1984. *Proc. Natl. Acad. Sci. USA*. 81:2265-2269.). Work described in this paper demonstrates that the virally-infected cells are missing a 37-kD peptide which reacts with an anti-tropomyosin

antiserum. Using a monoclonal antibody specific for the missing peptide, we show that this tropomyosin is absent from fibroblasts and is distinct from smooth muscle tropomyosins. It is also different from the two previously identified striated muscle myofibrillar tropomyosins (alpha and beta). We suggest that, in normal muscle, this novel, non-myofibrillar, tropomyosin-like molecule is an important component of a cytoskeletal network necessary for cluster formation.

A characteristic feature of the vertebrate neuromuscular junction is its enormously high concentration of acetylcholine receptors (AChRs)¹. AChRs are uniformly distributed along embryonic muscle fibers, but accumulate at high concentrations at junctional sites in response to innervation. This accumulation is probably initiated by a factor of neural origin (12, 28, 43). In mature muscle, clusters are maintained by a component of the muscle's basal lamina, which has been studied in some detail (11, 39). How the muscle cell responds to molecules that induce clustering is not well understood, although it might be anticipated that cytoskeletal elements are involved. A variety of cytoskeletal molecules are present in either the postsynaptic region of adult muscle or beneath AChR clusters on cultured muscle cells. These include alpha-actinin, filamin, vinculin, talin, an intermediate filament-like molecule, nonmuscle actin, a 300-kD protein, and a 58-kD protein (5-7, 19, 22, 23, 33, 47, 49, 56; reviewed in reference 20). In addition, a 43-kD AChR-associated protein, thought to be capable of binding to both the AChR (10) and actin (53), is concentrated near AChR clusters (8, 21, 42, 46, 50).

The mere presence of a cytoskeletal molecule, however, does not guarantee its participation in clustering. Other structural changes occur during muscle cell development that could also require cytoskeletal reorganization. For instance, the synaptic region of muscle is characterized by extensive membrane folds. In addition, we have shown that AChR clustering initiates the sub-membrane localization and immobilization of a set of myonuclei and Golgi apparatus. This process probably results from a cytoskeletal reorganiza-

tion beneath the cluster (17). To establish that particular cytoskeletal molecules have a direct role in clustering, evidence of a more functional nature must be obtained.

Previously, we reported that chick myotubes that are infected at the permissive temperature with a temperature-sensitive mutant (tsNY68) of Rous sarcoma virus (RSV) and allowed to fuse at the nonpermissive temperature do not cluster their AChRs in response to a variety of factors that increase greatly the number of AChR clusters in normal muscle cells (3). This suggests that the transformed cells have a functional defect in clustering. We have tried to identify differences between normal and transformed cells that might account for this defect. In this paper, we demonstrate that transformed cells have greatly decreased levels of a non-myofibrillar protein that is labeled by an anti-tropomyosin antibody. This molecule could function in AChR clustering by stabilizing actin filaments.

Materials and Methods

Cell Culture

Preparation of chick myotube cultures and viral infection were carried out as described previously (3), except that cultures were grown in 35-mm tissue culture dishes in DME (Gibco, Grand Island, NY) with 10% horse serum (Gibco) and 5% chick embryo extract in an atmosphere containing 10% CO₂. Rat muscle cell cultures were prepared as described previously, and cells were maintained in DME with 10% horse serum, 2% chick embryo extract, and 33 mM additional glucose (45). For immunofluorescence experiments, cells were grown on acid-washed, collagen-coated glass coverslips.

One-dimensional SDS-PAGE

Cultures were rinsed three times with ice-cold PBS and homogenized in SDS-PAGE sample buffer (5% SDS, 10% glycerol, 62.5 mM Tris, pH 6.8,

1. *Abbreviations used in this paper:* AChR, acetylcholine receptor; ALD, anterior latissimus dorsi; HRP, horseradish peroxidase; PLD, posterior latissimus dorsi; RSV, Rous sarcoma virus.

and 5% 2-mercaptoethanol). Lysates were microfuged and stored at -20°C until use. Protein was determined by the method of Schaffner and Weissman (48). One dimensional SDS-PAGE was performed according to the method of Laemmli (29).

Western Blot Analysis

Proteins resolved by SDS-PAGE were transferred to nitrocellulose by electroelution for 18–20 h at 150 mA constant current at room temperature in 20 mM Tris-HCl, 150 mM glycine, 20% methanol (52). Gels and nitrocellulose sheets were pre-soaked in the transfer buffer for at least 20 min before electroelution. Proteins transferred to nitrocellulose were reversibly stained with 0.2% Ponceau S (Serva Fine Biochemicals Inc., Garden City Park, NY) in 3% trichloroacetic acid.

Before antibody incubation, nitrocellulose blots were blocked with 10% horse or FCS (Gibco) in PBS for 1 h. The blots were incubated with the appropriate primary antibody overnight at 4°C or for 2 h at room temperature in PBS containing 10% serum, 0.1% Triton X-100, 0.02% SDS (buffer A). They were then washed at room temperature three times for 10 min each in Buffer A minus serum. The appropriate second antibody-horseradish peroxidase (HRP) conjugate (Hyclone Laboratories, Logan, UT) was diluted in Buffer A, incubated with the blots at room temperature for 2 h, and washed as above. Blots were rinsed in water or 10 mM sodium citrate, pH 4.5, and then reacted with 0.01% hydrogen peroxide and 0.5 mg/ml 4-chloro-1-naphthol in the citrate buffer (24, 30). The reaction was stopped by rinsing the blots in water.

Monoclonal Antibodies

To prepare antigen for immunization of BALB/C mice, proteins from noninfected myotubes were resolved by SDS-PAGE, and the gels were stained with Coomassie Blue. The 37-kD band which was found by Western blotting analysis to react with an anti-tropomyosin antibody (see Results) was excised and washed in alternate 10-min cycles of 1 M ammonium bicarbonate, pH 8.8, and water. The gel slice was minced, and protein was extracted by overnight incubation in 1% SDS, 10 mM ammonium bicarbonate, pH 8.8, and 1 mM EDTA. About 50 μg protein was extracted into 1 ml of this buffer. Next, 0.5 ml of this solution was mixed with 0.5 ml Alu-Gel-S adjuvant (Serva) and injected into the peritoneum of mice at 5 w, 1.5 w, and 4 d before the fusion procedure. Mice were chosen for fusions by bleeding from the orbital sinus and checking titers against low molecular mass tropomyosins on mini-Western blots.

One day before the fusion, macrophage feeder layers were prepared (40). Fusion of spleen cells with NS-1 mouse myeloma cells was done using slight modifications (30) of standard procedures (40) with polyethylene glycol in 75 mM Hepes buffer (Boehringer Mannheim, Indianapolis, IN). HAT selection was carried out according to standard protocols. Hybridoma cells were grown in RPMI medium (Gibco) containing 10% FCS (Hyclone) and 10 mM sodium pyruvate, and supernatants were assayed by mini-Western blots to detect wells that were producing antibodies predominantly against the desired molecular weight band. These cells were cloned in 0.5% Sea-plaque soft agar (FMC BioProducts, Rockland, ME) over feeder layers of rat embryo fibroblasts in the above medium. After ~ 2 w, clones were picked and diluted into medium in 96-well microtiter plates. The supernatants were again analyzed by mini-Western blot analysis.

Purification of Smooth Muscle Tropomyosin

Chicken gizzard tropomyosin was prepared by the method of Ebashi (16) modified by Dr. Fumio Matsumura of Cold Spring Harbor Laboratory (Cold Spring Harbor, NY) (35). Briefly, 100 gm frozen chicken gizzard (Pel-Freez Biologicals, Rogers, AK) was homogenized in 400 ml of a buffer containing 0.1 M NaCl, 20 mM Tris, pH 7.4, 1 mM EDTA, and 5 mM 2-mercaptoethanol, and centrifuged at 13,800 g for 20 min. The supernatant was saved, and the pellet was re-extracted and homogenized in 100 ml of the same buffer, but with 1 M NaCl, and again centrifuged at 13,800 g for 20 min. Both supernatants were heated separately at 100°C for 10 min, cooled on ice for 30 min and centrifuged as before. An additional 5 mM 2-mercaptoethanol was added to each supernatant, which was subjected to 28–36% ammonium sulfate fractionation. The pellets were redissolved to a final protein concentration of 5 mg/ml in 20 mM Tris, pH 7.4, containing 5 mM 2-mercaptoethanol.

Myofibril Preparation

Chicken pectoral myofibrils were generously provided by Dr. Jim Dennis

(Department of Anatomy and Cell Biology, Cornell University Medical College, New York, NY). Myofibrils were prepared from fascicles of anterior latissimus dorsi (ALD) and posterior latissimus dorsi (PLD), provided by Dr. Dennis, that had been stored at -20°C in buffer with 0.5% Triton X-100. The fascicles were minced into 1 mm pieces in K-Buffer, which consisted of 0.1 M KCl, 10 mM sodium phosphate pH 7.0, 5 mM MgCl_2 , 1 mM EGTA, 1 mM dithiothreitol (DTT), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co., St. Louis, MO). The mince was then homogenized before centrifugation at 3,000 rpm for 20 min in a Sorvall SS-34 rotor. The pellets were washed three times with 40 ml of the same buffer and centrifuged as above. The final myofibril pellets were stored at -20°C in glycerol: buffer, 1:1. ALD and PLD muscle do not yield clean myofibrils because of the large amount of connective tissue in these muscles compared with that in pectoral muscle.

Immunofluorescence

Cultured Cells. Muscle cell cultures grown on glass coverslips were fixed at room temperature for 20 min in 3% paraformaldehyde in PBS containing 1 mM CaCl_2 and 1 mM MgCl_2 , washed three times with PBS, permeabilized for 3 min in 0.1% Triton in PBS, and again washed with PBS three times. Nonspecific binding sites were blocked in 10% horse serum in DME for 15 min at room temperature. Hybridoma supernatants or rabbit antisera diluted in 10% horse serum were added to the coverslips for 30–60 min. The cells were washed three times with PBS, blocked for an additional 15 min in 10% horse serum in DME and then labeled with the appropriate FITC-conjugated second antibody (Cooper Biomedical, Malvern, PA; diluted 1:150 in 10% horse serum in PBS) for 30 min before washing three times in PBS, once in water and mounting with UV inert mountant (Atomergic Chemetals Corp., Farmingdale, NY). Photographs were taken using appropriate filter combinations for fluorescein and rhodamine on a Zeiss ICM 405 microscope equipped with epifluorescence illumination (Carl Zeiss, Inc., Thornwood, NY).

Myofibrils

Aliquots of the myofibril suspension were absorbed to gelatin-coated slides and incubated in 1% BSA in K-Buffer for 30 min to block nonspecific binding sites. Antibodies diluted in 1% BSA in K-buffer were incubated with the tissue for 1 h. The slides were washed with K-buffer 3 times for 5 min each. The myofibrils were again incubated in 1% BSA in K-Buffer for 10 min before incubating them with the appropriate FITC-conjugated anti-mouse or anti-rabbit immunoglobulin (diluted 1:150 in 1% BSA in K buffer) for 30 min. The slides were washed and rinsed as before and mounted in 90% glycerol in PBS.

Results

Identification of Myotube Tropomyosin Isoforms

Our interest in muscle cells transformed with temperature-sensitive mutants of RSV arose from our observation that these cells are unable to cluster AChRs even when maintained at the nonpermissive temperature, 42°C (3). We, therefore, compared these cells with normal muscle cells grown at 42°C . SDS-PAGE revealed, as anticipated, that proteins of the two cell types were quite similar. There were some differences, though. The most striking change was that one protein with an apparent molecular weight of 37 kD was virtually absent in infected myotubes that had been shifted to 42°C for 3–4 d (Fig. 1). This protein was also absent from cells shifted back to 37°C (data not shown). It seemed unlikely that this difference between the two types of culture was due to vastly differing numbers of myotubes. For all experiments, tsNY68-infected and uninfected cultures were inspected to determine that comparable numbers of myotubes were present. The numbers of cell surface AChRs in both types of cultures were also determined in some cases to ensure that they were comparable (3).

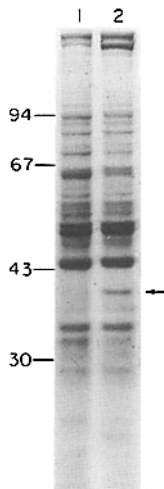


Figure 1. Coomassie Blue stained SDS-polyacrylamide gel of total protein from noninfected (lane 2) and tsNY68-infected (lane 1) muscle cell cultures grown at 42°C. The two types of cells are quite similar except that the infected cells are missing a peptide with an apparent molecular mass of 37 kD (arrow). Positions of molecular mass standards are indicated on the left.

The identity of the 37-kD peptide was suggested by experiments in which Western blots of equal amounts of total protein from cultures of normal and transformed myotubes were probed with a rabbit polyclonal antibody, TM#6 (kindly provided by Dr. Fumio Matsumura, Cold Spring Harbor Laboratories), prepared against chicken gizzard tropomyosin which cross-reacts with many tropomyosin isoforms (35). The 37-kD peptide in normal cells is labeled with TM#6 (Fig. 2). This immunoreactive peptide, however, is missing from tsNY68-infected myotubes kept at 42°C (Fig. 2) as well as from those shifted back to 37°C (data not shown). These experiments suggest that a molecule of 37 kD, which bears some structural similarity to a tropomyosin, is present in normal muscle cells, but absent from transformed ones. We will refer to this molecule as tropomyosin 2 since it is the peptide with the second highest molecular weight labeled by the anti-tropomyosin antibody.

Generation of a Monoclonal Antibody Against Tropomyosin 2

To characterize tropomyosin 2 more completely, we isolated it from SDS-gels prepared from cultures of normal chick myotubes. We immunized mice with the gel-eluted material for the production of monoclonal antibodies. From one of our fusions, we obtained an antibody, D3-16, which preferentially labeled this peptide on Western blots of total muscle culture protein. In fact, D3-16 labeled this peptide strongly on Western blots more consistently than did TM#6 (see, for instance, Fig. 8). D3-16 also labeled a peptide with apparent molecular mass of 43 kD, which was labeled by the anti-tropomyosin antibody TM#6, as well, and which will be re-

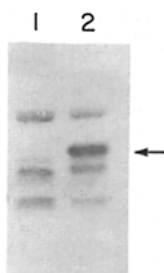


Figure 2. Western blot of total protein from tsNY68-infected muscle cells (lane 1) and normal muscle cells (lane 2). The blot was probed with TM#6 and HRP-coupled goat anti-rabbit antibody. The main observation is that the 37-kD peptide (arrow) present in noninfected muscle cells and absent from infected muscle cells is labeled with this antibody.

ferred to as tropomyosin 1 (Fig. 4). Tropomyosin 1 was distinct from alpha actin, which was not labeled by D3-16 in Western blots containing striated muscle myofibrillar protein (Fig. 4, lane 1). Western blots using D3-16 showed clearly that tropomyosin 2 was greatly decreased in tsNY68-infected cells (Figs. 4 B and 8 C).

Characterization of Tropomyosin 2 Expression

Cellular Specificity. We were interested in determining the cellular specificity of tropomyosin 2, its relationship to other muscle tropomyosins, and its distribution within muscle cells. To accomplish these goals, we used Western blotting and immunofluorescence techniques to compare the reactivity of D3-16 with that of TM#6 and with that of a mouse monoclonal antibody, CH 1, that reacts only with alpha and beta skeletal tropomyosins (31; kindly provided by Dr. Jim J.-C. Lin, Department of Biology, University of Iowa, Iowa City, IA).

The first issue we addressed was whether tropomyosin 2 truly was present only in normal striated muscle cells in culture or whether it was additionally found in fibroblasts. This was important because even though both infected and noninfected myotube cultures were treated with cytosine arabinoside for 48 h to decrease the number of fibroblasts, tropomyosins from residual fibroblasts might still have contributed to the many isoforms found in myotube cultures. In that case, the difference between normal and transformed cultures in the level of tropomyosin 2 might have been due to differing numbers of fibroblasts. Fig. 3 is a representative Coomassie Blue-stained SDS-polyacrylamide gel of total cell protein from fibroblast cultures (lane 2), tsNY68-infected myotubes (lane 3) and noninfected myotubes (lane 4), both grown at 42°C. Pectoral muscle myofibrils, which contain alpha, but little beta, tropomyosin were included to mark the position of that peptide (lane 5). Alpha-actin is also a major component of the pectoral myofibrils. Chicken gizzard tropomyosins at ~35 and 43 kD are also presented for comparison. As expected, the cultured cell protein preparations contained numerous polypeptides and especially prominent actin bands. However, fibroblasts did not have either a major peptide that

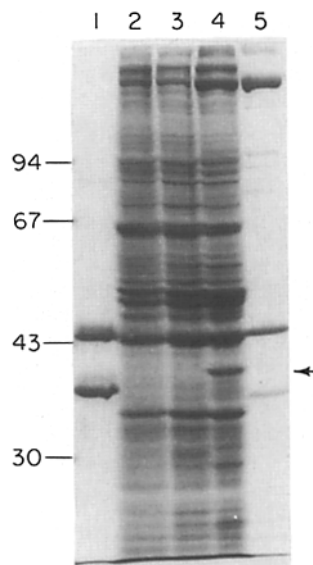


Figure 3. SDS-polyacrylamide gel comparing chicken gizzard tropomyosins (lane 1), total fibroblast protein (lane 2), total protein from tsNY68-infected muscle cells (lane 3), total protein from noninfected cultured muscle cells (lane 4), and pectoral myofibrils (lane 5). Tropomyosin 2 (arrow), prominent in cultured muscle cells, is markedly decreased in the other preparations. Pectoral myofibrils generally had small amounts of the 37-kD peptide. Positions of molecular mass standards are indicated on the left.

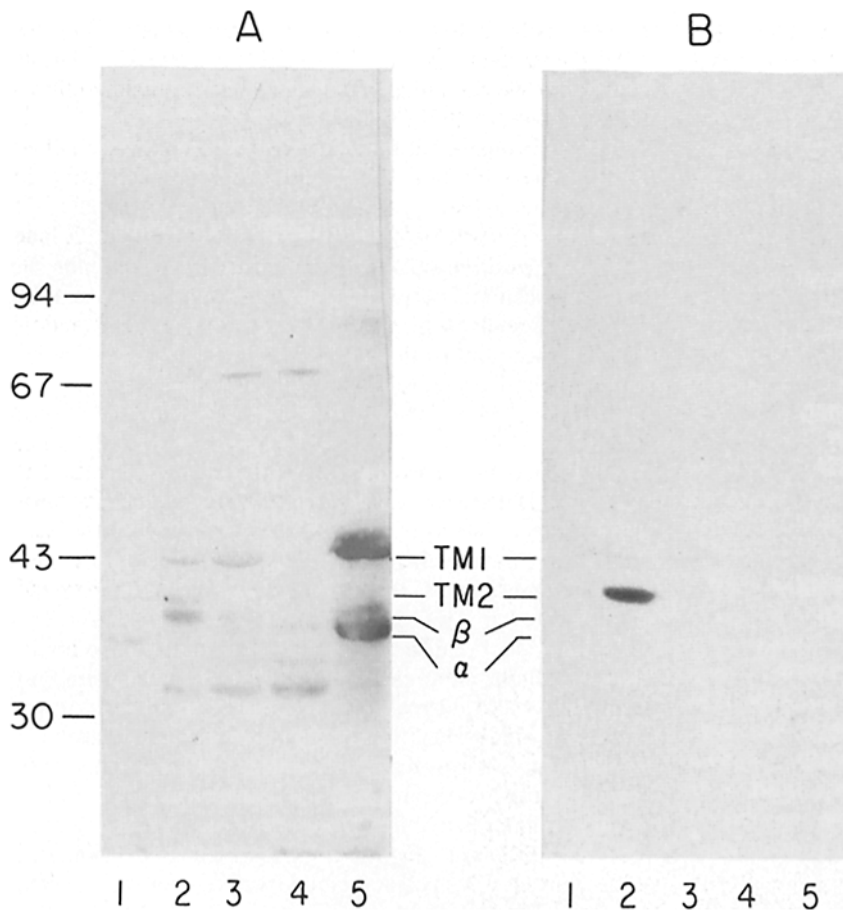


Figure 4. Western blots comparing reactivity with TM#6 (A) and D3-16 (B). Proteins from pectoral myofibrils (lane 1), noninfected muscle cells (lane 2), tsNY68-infected muscle cells (lane 3), fibroblasts (lane 4), and chicken gizzard tropomyosins (lane 5) were first resolved by SDS-PAGE. TM#6 stains most tropomyosin isoforms and shows the relative paucity of tropomyosin 2 in the other preparations. D3-16 does not label fibroblast proteins or chicken gizzard tropomyosins, but strongly reacts with tropomyosin 2. D3-16 also labels weakly tropomyosin 2 in pectoral myofibrils. Positions of tropomyosin 1, which also reacts somewhat with D3-16, and of alpha (α) and beta (β) tropomyosins are also given. Positions of molecular mass standards are indicated on the left.

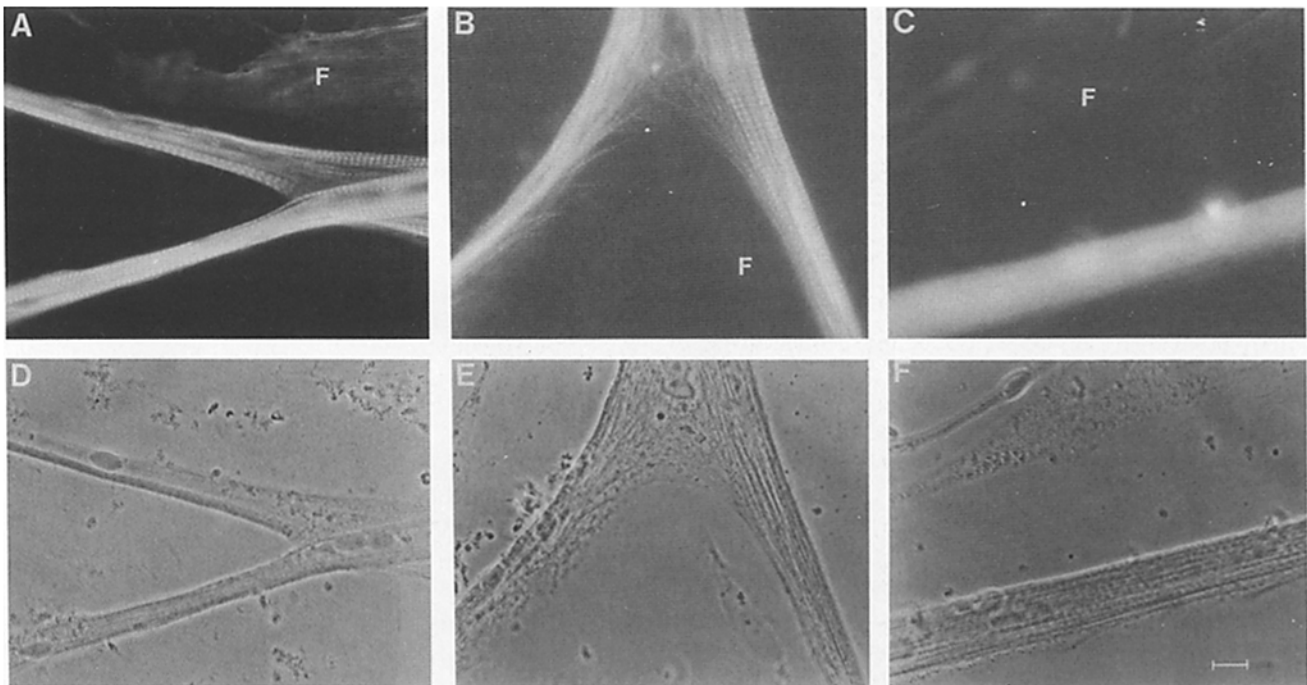


Figure 5. Immunofluorescence of uninfected chick myotubes labeled with TM#6 (A and D), CH 1 (B and E), and D3-16 (C and F). The top 3 micrographs are fluorescent images, and the bottom three are phase micrographs of the same fields. Both TM#6 and CH 1 label myotubes in a striated fashion. TM#6 labels fibroblasts (F) as well. D3-16 labels myotubes uniformly and, like CH 1, does not label fibroblasts. Bar, 10 μ M.

comigrated with tropomyosin 2 or a form that comigrated with tropomyosin 1 present in both noninfected and infected myotubes.

A Western blot prepared from a similar gel is shown in Fig. 4. The position of alpha-tropomyosin is specified by TM#6 reactivity with pectoral myofibrils, and the position of tropomyosin 2 by D3-16 reactivity with cultured muscle. As judged by reactivity with both TM#6 and D3-16, it is clear that fibroblasts did not contain any immunoreactive tropomyosin 2. They did contain, however, two tropomyosin isoforms migrating just above and below the 43-kD myotube form, but these did not stain well with TM#6 and did not stain at all with D3-16 (Fig. 4).

These results were consistent with those obtained from immunofluorescence studies. Normal myotubes from both chick and rat embryos stained diffusely with D3-16, but both types of fibroblast failed to stain (Figs. 5 and 6). Infected chick myotubes also did not stain (data not shown). Taken together, these experiments suggest that the 37-kD peptide missing from tsNY68-infected muscle cell cultures is not

present in fibroblasts. This also demonstrates that differences between normal and tsNY68-infected myotube cultures could not have been related to the presence of residual fibroblasts in these cultures.

We also compared myotube tropomyosins to those purified from chicken gizzard. The major tropomyosins found in chicken gizzard had apparent molecular masses of 35 and 43 kD, clearly different from that of tropomyosin 2 (Fig. 3, lane 1). These peptides were labeled by TM#6 (Fig. 4 A, lane 5), but not by D3-16 (Fig. 4 B, lane 5). Thus, D3-16 recognizes an epitope that is not present in smooth muscle tropomyosins.

Differences between Tropomyosin 2 and Myofibrillar Tropomyosins

We were next interested in determining if tropomyosin 2 is one of the previously recognized striated muscle tropomyosin isoforms. Two forms of muscle tropomyosin have been well characterized. Myofibrillar beta tropomyosin has an apparent molecular mass of ~ 36 kD and is present in slow and

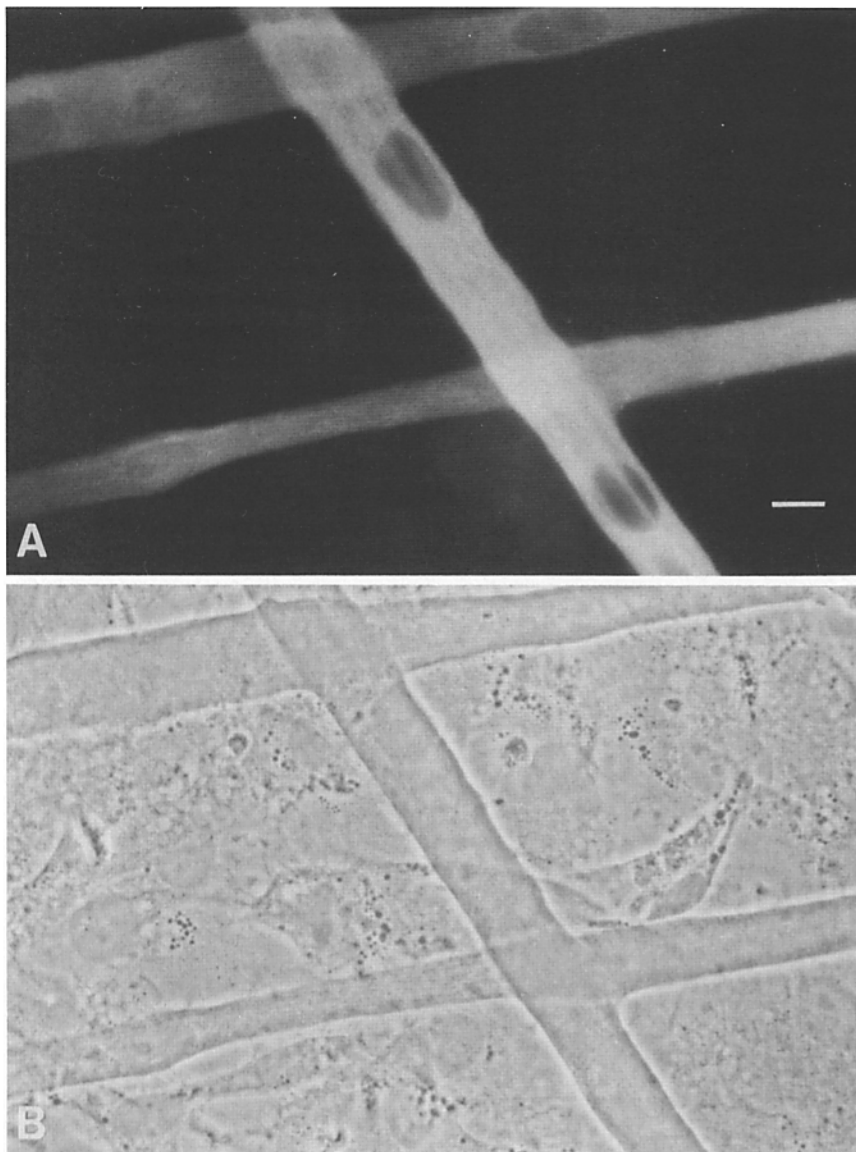


Figure 6. Immunofluorescence of rat myotube culture labeled with D3-16. Fluorescence (A) and phase (B) images are shown. D3-16 labels these myotubes uniformly, but again does not label fibroblasts that are present throughout the culture. Bar, 10 μ M.

mixed muscle types and in cultured myotubes (2). Myofibrillar alpha tropomyosin has an apparent molecular mass of 34 kD and is present in fast and slow muscle and in cultured myotubes. Montarras et al. (38) studied the expression of alpha and beta tropomyosins in myotubes formed from myoblasts transformed at 37°C with tsNY68 and shifted to 42°C. They found that expression of alpha-tropomyosin began soon after shift to the nonpermissive temperature, but that beta-tropomyosin expression did not peak until 48 h later. West and Boettiger (54) reported that synthesis of alpha and beta tropomyosin in RSV tsLA24-infected myotubes decreased ~40% after a 29-h shift from nonpermissive to permissive temperatures.

Experiments with pectoral muscle myofibrils demonstrated, on the basis of molecular weights and reactivity with D3-16 (Figs. 3 and 4), that alpha tropomyosin and tropomyosin 2 are different. However, because of the similarity in molecular weights between beta tropomyosin and tropomyosin 2 and because of the previously reported effects of transformation on muscle tropomyosin expression, it was important to determine whether the deficient 37-kD polypeptide was beta tropomyosin.

Comparisons between myofibrillar tropomyosins and tropomyosin 2 were again made by Western blot analysis and by immunofluorescence using the different antibodies. Myofibrils from ALD and PLD muscles and total protein from noninfected and infected myotubes were first resolved by SDS-PAGE and stained with Coomassie Blue (Fig. 7). These preparations contained relatively high levels of contaminating proteins because of the difficulty in obtaining pure myofibrillar preparations (compared with pectoral myofibrils, as seen in Fig. 3). Myofibrils from both PLD and ALD had major bands at 34 and 36 kD (lanes 1 and 2). In addition, ALD myofibrils had a band at ~37 kD, as did noninfected cultured muscle cells. The 37-kD band was clearly resolved from that migrating at 36 kD. Thus, on the basis of apparent molecular weights tropomyosin 2 is different from beta tropomyosin.

The difference between tropomyosin 2 and beta tropomyosin was confirmed by Western blotting of proteins prepared from similar SDS gels are presented in Fig. 8. The 34- and 36-kD bands in ALD, PLD, and normal cultured muscle were labeled by both TM#6 (Fig. 8 A) and CH 1 (Fig. 8 B) and must, therefore, correspond to alpha and beta myofibrillar tropomyosin. Staining of beta tropomyosin appeared heavier in PLD than in ALD. CH 1 did not label tropomyosin 2 in normal cultured muscle cells, though (Fig. 8 C). Furthermore, D3-16 did not label any proteins present in PLD myofibrils, although it lightly labeled a 37-kD band in some preparations of ALD myofibrils.

Immunofluorescence of fixed and permeabilized cells also revealed differences in the staining patterns of the three antibodies and emphasized the difference between myofibrillar tropomyosins and tropomyosin 2. Both TM#6 and CH 1 stained cultured myotubes in a striated fashion. D3-16 stained chick and rat myotubes brightly, but uniformly (Figs. 6 and 7).

Differences were also observed when the antibodies were used to label myofibrils isolated from ALD muscle, which contained both alpha and beta tropomyosins, or from pectoral muscle. TM#6 and CH 1 labeled the myofibrils in a striated fashion. D3-16, on the other hand, labeled diffusely (in

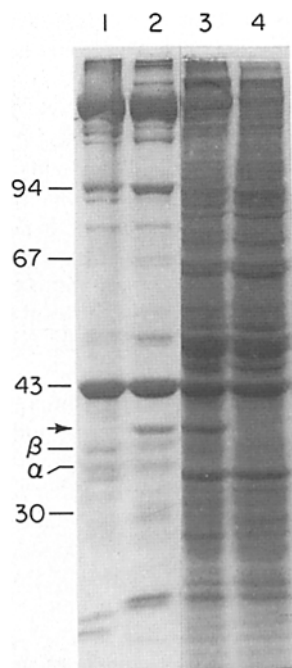


Figure 7. Coomassie Blue-stained SDS-polyacrylamide gel of proteins from PLD myofibrils (lane 1), ALD myofibrils (lane 2), noninfected cultured muscle cells (lane 3) and tsNY68-infected muscle cells (lane 4). Tropomyosin 2 (arrow) is missing from PLD myofibrils, but is present in ALD myofibrils. It is distinct from beta (β) and alpha (α) tropomyosins present in both types of myofibrils. Positions of molecular mass standards are given on the left.

the case of ALD myofibrils) or not at all (Fig. 9). These results strongly support the idea that tropomyosin 2 is not myofibrillar.

Other Differences between Normal and Infected Muscle Cells

On occasion, there were decreased amounts of beta tropomyosin (Fig. 8 A, lanes 2 and 3), as observed previously (54), as well as of tropomyosin 2, in the infected muscle cells. In addition, tropomyosin 1 was sometimes present in increased amounts in the transformed cells (Fig. 8 A). CH 1 labeling (Fig. 8 B) suggested that alpha tropomyosin may have been decreased in transformed cells also, but this was not observed consistently. Also, a peptide with the approximate molecular mass of myosin was decreased in most experiments (Figs. 1 and 3).

Discussion

Absence of Tropomyosin 2 in Cells Unable to Cluster AChRs

In a previous paper, we reported on properties of chick skeletal muscle cells infected with a temperature-sensitive mutant of RSV (3). In those experiments, myoblasts were infected at the permissive temperature and were unable to fuse as long as they were kept at this temperature. When the cells were shifted to the nonpermissive temperature, they formed myotubes that were then unable to cluster AChRs, although they resembled normal cells in many other respects. Muscle cells infected with td107A, a transformation-defective RSV that lacks the *src* gene, were still able to cluster their AChRs. Thus, we hypothesized that the presence of pp60^{src} somehow interfered with the clustering process.

We have identified a major deficit in the infected cells: greatly decreased amounts of a 37-kD protein that is labeled by a polyclonal anti-tropomyosin antiserum. Its reactivity

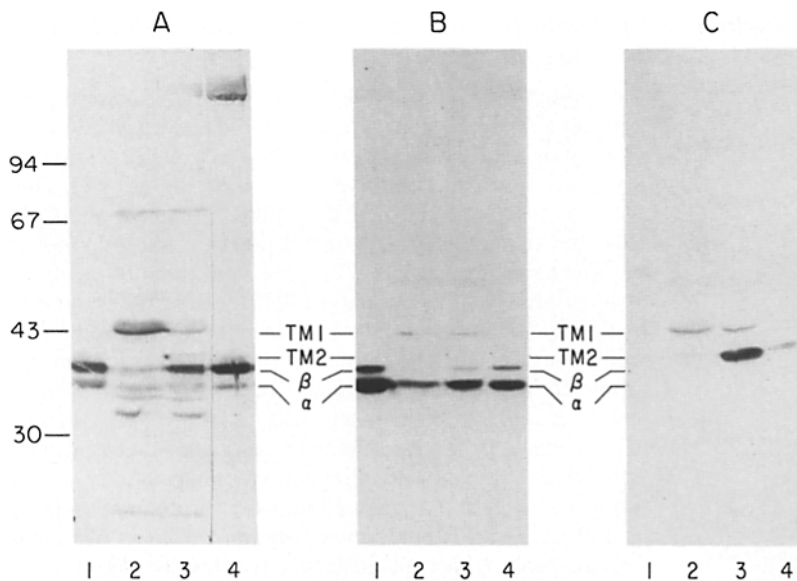


Figure 8. Western blots comparing reactivity with TM#6 (A), CH 1 (B), and D3-16 (C). Proteins from PLD myofibrils (lane 1), tsNY68-infected myotubes (lane 2), noninfected cultured muscle cells (lane 3) and ALD myofibrils (lane 4) were resolved by SDS-PAGE. As before, TM#6 stains several tropomyosin isoforms including the predominant alpha (α) and beta (β) forms in the myofibrils. In this figure, TM#6 does not label tropomyosin 2 strongly. CH 1 stains mainly alpha and beta tropomyosins, but it stains alpha more intensely than it does beta, even though both are present in roughly equal amounts as judged by Coomassie Blue staining (Fig. 7). D3-16 labels tropomyosin 1 and tropomyosin 2 which is absent from PLD myofibrils, but present in small amounts in the ALD myofibril preparations (probably due to contamination with cytoplasmic or cytoskeletal material). Positions of molecular weight standards are given on the left.

with this antiserum suggests that it is a tropomyosin isoform, and we have referred to it tentatively as tropomyosin 2. The resemblance between the 37-kD peptide and the class of tropomyosins was further indicated by Western blot analysis of two-dimensional gels using TM#6. A highly acidic protein, similar in isoelectric point to the tropomyosins, was absent from lysates of the infected myotubes (data not shown).

The possibility that tropomyosin 2 is a tropomyosin-like protein is also supported by the observation that RSV-transformed fibroblasts are known to be missing a tropomyosin isoform (26, 27, 32, 34). That particular form, however, must be different from the one we have studied, which is not present in fibroblasts. Amino acid analysis of the 37-kD peptide will assist in confirming its molecular identity.

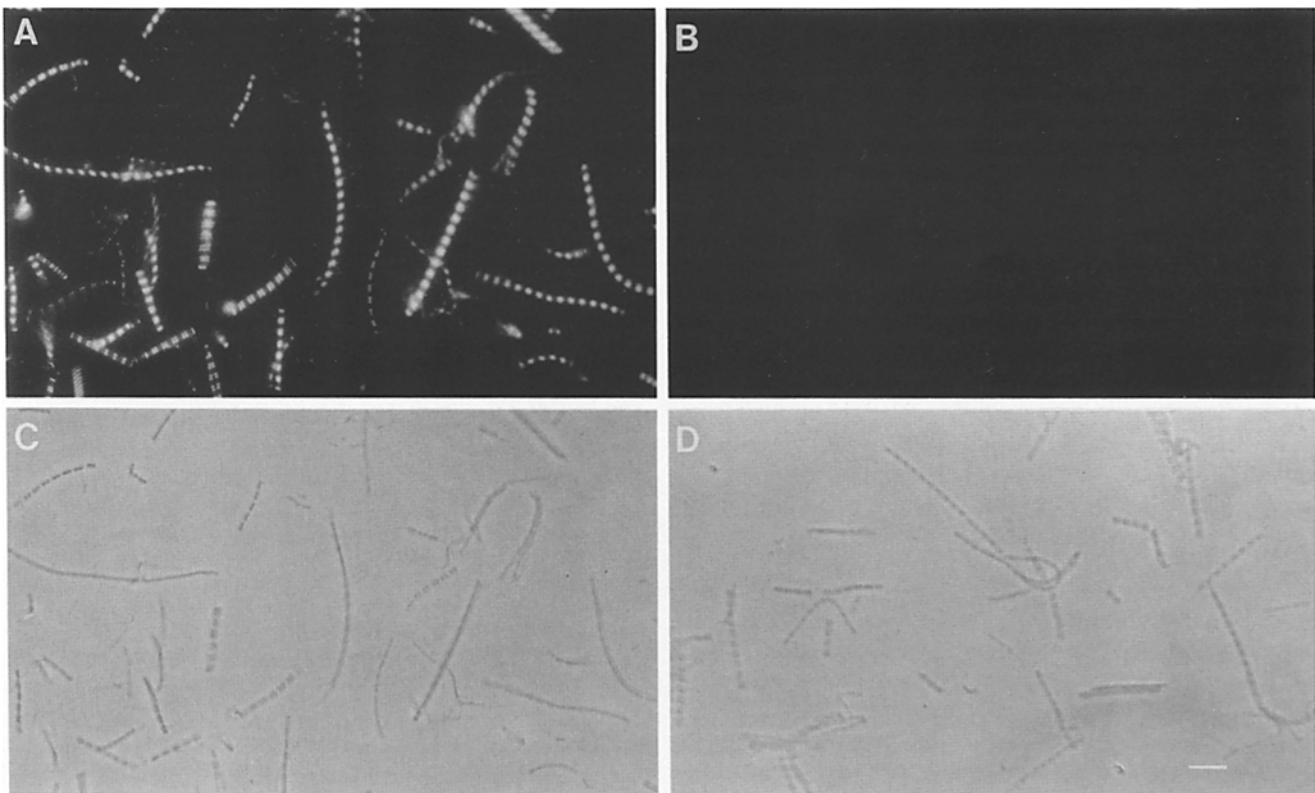


Figure 9. Immunofluorescence of pectoral myofibrils stained with TM#6 (A and C) and D3-16 (B and D). Fluorescence (A and B) and phase (C and D) images of the same fields are given. TM#6 stains the myofibrils in an obviously striated fashion, but D3-16 stains the myofibrils weakly at best. Bar, 5 μ M.

We do not yet know how the presence of pp60^{src} controls the amount of tropomyosin 2 in tsNY68-infected myotubes. The presence of pp60^{src} in other kinds of infected cells is also known to affect synthesis of particular proteins. These include fibronectin (1), collagen isoforms (1, 15), caldesmon (41) and alpha-actin (55). As mentioned above, tropomyosin isoforms have also been shown to change in transformed fibroblasts (see also reference 14). Thus, there is certainly precedent for the type of alteration we have observed. It is not clear how any of these effects are related to pp60^{src}'s activity as a tyrosine protein kinase.

We also do not know why the amounts of tropomyosin 2 remain low even when the cells are maintained at the non-permissive temperature. In most cases, effects on particular proteins are temperature-sensitive when cells are infected with ts-mutants. It is possible that, in our experiments, pp60^{src} had an effect at the permissive temperature that was not reversible when the temperature was elevated. This difference may reflect differences in the response to transformation between terminally differentiated nonreplicative cells, such as multinucleated muscle cells, and cells that remain able to multiply. This idea is supported by the observation of West and Boettiger (54) that tsLA24-RSV infected myotubes maintained for several days at 42°C still lack well-developed myofibrils. In our experiments as well, most noninfected myotubes were elongated and striated, with well-organized myofibrils and stress fibers. However, transformed myotubes seldom had striations and often appeared very broad and flattened. Also, Miskin et al. (36) reported elevated plasminogen activator levels in tsNY68-infected myotubes kept at 42°C compared with fibroblasts treated similarly. These results support the present observation that there are differences, even at 42°C, between normal and infected myotubes in the types or levels of proteins synthesized.

Properties of Tropomyosin 2

We used a monoclonal antibody generated against gel-eluted tropomyosin 2 to study its properties. In particular, tropomyosin 2 was shown to be absent from rat and chick fibroblasts and to be distinct from smooth muscle tropomyosins. Western blot analysis and immunocytochemical studies indicate that tropomyosin 2 is not predominantly myofibrillar. The small amount of labeling of ALD myofibrils probably reflects contamination from cytoplasmic or cytoskeletal tropomyosin 2. Our experiments distinguish especially between the 36-kD myofibrillar beta-tropomyosin recognized by TM#6 and CH 1 antibodies and the 37-kD non-myofibrillar tropomyosin 2 recognized by TM#6 and D3-16.

If tropomyosin 2 is non-myofibrillar, what might its location be within muscle cells? Preliminary experiments involving the immunoprecipitation of [³²P]orthophosphate labeled infected and noninfected myotubes with TM#6 followed by SDS-PAGE and autoradiography revealed two phosphoproteins from both infected and noninfected cells at 34 and 36 kD that did not comigrate with tropomyosin 2 (data not shown). These are presumably alpha and beta tropomyosin, which are both known to be phosphorylated (37, 38). This suggests that tropomyosin 2 resembles more closely the non-myofibrillar tropomyosins, which are not phosphorylated (25, 37). Thus, tropomyosin 2 may be a cytoskeletal molecule in muscle cells.

Tropomyosin 2 May Function as Part of a Sub-Cluster Cytoskeletal Network

We and others have found the 43-kD AChR-associated protein and a nonmuscle form of actin to be concentrated in the vicinity of AChR clusters and in the postsynaptic region of the adult neuromuscular junction (5, 8, 21, 42, 47). Our previous work showed that the initial formation of AChR clusters, but not the integrity of existing clusters, is disrupted by cytochalasin treatment (17). One interpretation of these results is that an essential part of AChR clustering is the formation of a network of actin filaments beneath clusters, which then is somehow stabilized against disruption by cytochalasins. In other cell types, tropomyosins are thought to form part of cytoskeletal networks, presumably by binding to actin filaments (14, 51). Under some circumstances, tropomyosins also appear to stabilize actin networks (4, 18). Thus, it might well be that tropomyosin 2 is an important component of the muscle's cytoskeleton, binding to actin filaments forming beneath nascent clusters. The role of the 43-kD protein would be to serve as a link between the AChR and actin. Evidence presented in this paper suggests that tropomyosin 2 is present throughout embryonic muscle fibers grown in cell culture. Recently, we have found that tropomyosin 2 is enriched in junctional regions of adult rat intercostal fibers. This would be consistent with a crucial role in AChR clustering. The presence of tropomyosin 2 in other regions of muscle cells may indicate that it is also involved in other processes in developing muscle that are dependent on cytoskeletal organization, such as the formation of contractile filaments (19), which, as was pointed out above, is defective in the transformed cells.

In conclusion, we have shown that RSV-infected muscle cells that are unable to cluster AChRs are missing a 37-kD peptide. The missing peptide is labeled by an anti-tropomyosin antibody and may prove to be a novel muscle cytoskeletal tropomyosin. Such a cytoskeletal component might mediate AChR clustering and may also play a role in other processes within muscle cells, such as the assembly of myofibrils. We are in the process of microinjecting our monoclonal anti-tropomyosin 2 antibody into normal myotubes to see if it has any effects on AChR clustering. We also intend to introduce tropomyosin 2 into tsNY68-infected myotubes to see if this allows them to cluster AChRs. These types of experiment may give us a more direct indication of the role of tropomyosin 2 in AChR clustering.

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