Altered Expression of Tropomodulin in Cardiomyocytes Disrupts the Sarcomeric Structure of Myofibrils

Mark A. Sussman, Susanna Baqué, Chang-Sub Uhm, Mathew P. Daniels, Robert L. Price, David Simpson, Louis Terracio, Larry Kedes

Abstract—Tropomodulin is a tropomyosin-binding protein that terminates “pointed-end” actin filament polymerization. To test the hypothesis that regulation of tropomodulin:actin filament stoichiometry is critical for maintenance of actin filament length, tropomodulin levels were altered in cells by infection with recombinant adenoviral expression vectors, which produce either sense or antisense tropomodulin mRNA. Neonatal rat cardiomyocytes were infected, and sarcomeric actin filament organization was examined. Confocal microscopy indicated that overexpression of tropomodulin protein shortened actin filaments and caused myofibril degeneration. In contrast, decreased tropomodulin content resulted in the formation of abnormally long actin filament bundles. Despite changes in myofibril structure caused by altered tropomodulin expression, total protein turnover of the cardiomyocytes was unaffected. Biochemical analyses of infected cardiomyocytes indicated that changes in actin distribution, rather than altered actin content, accounted for myofibril reorganization. Ultrastructural analysis showed thin-filament disarray and revealed the presence of leptomeres after tropomodulin overexpression. Tropomodulin-mediated effects constitute a novel mechanism to control actin filaments, and our findings demonstrate that regulated tropomodulin expression is necessary to maintain stabilized actin filament structures in cardiac muscle cells. (Circ Res. 1998;82:94-105.)

Key Words: tropomodulin ■ actin ■ sarcomere ■ myofibril ■ cardiac, heart

Accurate control of thin-filament length in sarcomeres is critical for proper function of the contractile apparatus. Uniformity among actin filaments depends on consistent length specification and effective termination of polymerization. Tropomodulin has been implicated as the regulatory agent for inhibiting slowly growing (pointed) end elongation of actin filaments. Located at or near the ends of thin filaments in skeletal muscle at a calculated stoichiometry of 1.2 to 1.6 tropomodulin molecules per actin filament, tropomodulin is situated in the appropriate place at the right concentration to regulate thin filaments.1 Circumstantial evidence suggests that tropomodulin:actin filament stoichiometry is critical for maintaining actin filament structure. First, the stoichiometry of tropomodulins per actin filament is comparable between skeletal muscle and the erythrocyte cytoskeleton, despite vastly different actin organization in the two cell types.1 Second, tropomodulin releases tropomyosin from association with actin filaments in vitro when added in molar excess.2 Third, tropomyosin-coated actin filaments are stabilized by interaction with tropomodulin when both are present in equivalent stoichiometric levels.3 The importance of sarcomeric protein stoichiometry for muscle function and the regulation of sarcomeric organization is suggested from studies that found mutations of either α-tropomyosin or cardiac troponin T as the basis for the characteristic myofibrillar disorganization observed in familial hypertrophic cardiomyopathy.4

Tropomodulin is a component of the thin-filament complex in cardiac muscle where, as in skeletal muscle, the protein is localized within the region of actin filament pointed ends.3,6 Inhibition of tropomodulin binding to actin by microinjection of anti-tropomodulin antibody into cardiomyocytes results in actin filament elongation.7 Inherent difficulties of cardiomyocyte microinjection such as decreased cell survival8 and the potential for myofibril damage resulting from physical injection trauma prompted the novel approach used in the present study to demonstrate the role of tropomodulin in maintenance of thin-filament organization. Instead of inhibiting tropomodulin function with antibodies, tropomodulin expression levels were altered using recombinant adenoviral vectors expressing either sense (Ad+Tmod) or antisense (Ad−Tmod) tropomodulin cDNA. This approach allows for either high-level expression of the transfected gene or downregulation of the endogenous mRNA while minimizing ex vivo manipulation of cardiomyocytes by microinjection. The present study...
demonstrates that altered tropomodulin expression affects myofibril structure and is the first to describe the effect of tropomodulin overexpression, examining both structural and biochemical consequences for thin filaments. We conclude that changes in tropomodulin expression profoundly disrupt normal thin-filament organization and lead to myofibril degeneration. These findings are consistent with a role for tropomodulin in the maintenance of myofibril organization and with the necessity of regulated tropomodulin expression.

Materials and Methods

Cell Culture
All media, sera, and culture reagents were purchased from Gibco Laboratories. 10T1/2 fibroblast cells were propagated in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin. Human embryonic kidney cells expressing adenoviral protein (293 cells) were cultured in the same medium. Rat cardiomyocytes were isolated from 2- to 3-day-old pups as previously described. Cardiomyocyte cultures were preplated to enrich the cardiomyocyte population and deplete the population of fibroblasts, which would then constitute <3% of the total cell population when used in experiments, except where noted. Cardiomyocytes were plated onto glass coverslips coated with laminin (Sigma Chemical Co; catalog No. L 2020, diluted in PBS and used at a concentration of 50 μg/mL) and cultured in Eagle’s MEM, without L-glutamine, supplemented with 5% of the total cell population when used in experiments, except where noted. Cardiomyocytes were plated onto glass coverslips coated with laminin (Sigma Chemical Co; catalog No. L 2020, diluted in PBS and used at a concentration of 50 μg/mL) and cultured in Eagle’s MEM, without L-glutamine, supplemented with 2% fetal bovine serum and penicillin/streptomycin.

Preparation and Characterization of Recombinant Adenoviral Expression Vectors
A full-length tropomodulin cDNA derived from mouse cardiac tissue was inserted into the EcoRI site of pAC-CMV.12 Tropomodulin cDNA fragment orientation was determined by restriction digest and sequence analysis (data not shown). The pAC-CMV vector containing tropomodulin cDNA in either sense or antisense orientation was cotransfected into human embryonic kidney 293 cells together with pJM-17.12,13 After homologous recombination, plaques were isolated, amplified, and characterized. Viral titer was estimated by plaque assay using serial dilutions of harvested viral pools to determine the number of virus particles per milliliter. Infected cells were cultured for 48 hours before use in experiments, unless otherwise noted. Infected efficiency was ~80%, as measured by microscopic analysis of transgene expression and/or phenotypic changes in infected cells.

Infection of Cardiomyocyte Cultures
Cardiomyocytes were cultured for 5 days to allow for development of extensive functional myofibril structure, as evidenced by rhythmic spontaneous contractions observed using phase microscopy. After wash with PBS (pH 7.2) to remove dead cells and debris, supernatant containing recombinant adenovirus was added to achieve an MOI of 500:1. Ad-Tmod was added to the cardiomyocyte cultures, and the cultures were incubated for 48 hours before use in experiments, unless otherwise noted. Infected efficiency was ~80%, as measured by microscopic analysis of transgene expression and/or phenotypic changes in infected cells.

Northern Blot Analysis
Cardiomyocytes cultured for 5 days were infected with Ad+Tmod or Ad−Tmod. Total RNA was extracted from the cells 3 days later using Trizol reagent (GIBCO-BRL), as directed by the manufacturer. Cellular RNA and molecular size standards for single-stranded RNA (0.24- to 9.5-kb RNA ladder, GIBCO-BRL catalog No. 15620-016) were separated by formamide gel electrophoresis and transferred to charged nitrocellulose membranes (Hybond N+, Amersham) by overnight capillary blotting with 5X SSC. The blot was washed with PBS (pH 7.2) to remove dead cells and debris, supernatant containing recombinant adenovirus was added to achieve an MOI of approximately one viral particle per cell. Infected cells were cultured for 48 hours before use in experiments, unless otherwise noted. Infected efficiency was ~80%, as measured by microscopic analysis of transgene expression and/or phenotypic changes in infected cells.

Protein Gel Electrophoresis and Western Blot Analysis
Cardiomyocytes cultured on laminin-coated 100-mm plastic Petri dishes were washed twice with PBS and solubilized in sample buffer containing 8 mol/L urea. After sonication to shear chromosomal DNA, the samples were boiled for 5 minutes, cooled to room temperature, and separated by SDS-PAGE on 10% gel. Gels loaded with 10 to 15 μg of lysate per lane were stained with Coomasie brilliant blue dye (Sigma) to visualize proteins. Molecular weight standards (GIBCO-BRL) were loaded with each gel. For immunoblots, lysates loaded at 10 to 15 μg per lane were separated by SDS-PAGE and transferred to nitrocellulose. The blots were then incubated with 5% nonfat dry milk in PBS (pH 7.4) for 1 hour, followed by incubation with specific antibodies. Blots were washed with PBS (pH 7.2) to remove unbound antibodies and then incubated with secondary antibodies labeled with horseradish peroxidase. Blots were washed with PBS (pH 7.2) to remove unbound antibodies and then incubated with secondary antibodies labeled with horseradish peroxidase. Blots were washed with PBS (pH 7.2) to remove unbound antibodies and then incubated with secondary antibodies labeled with horseradish peroxidase. Blots were washed with PBS (pH 7.2) to remove unbound antibodies and then incubated with secondary antibodies labeled with horseradish peroxidase. Blots were washed with PBS (pH 7.2) to remove unbound antibodies and then incubated with secondary antibodies labeled with horseradish peroxidase. Blots were washed with PBS (pH 7.2) to remove unbound antibodies and then incubated with secondary antibodies labeled with horseradish peroxidase.
generated in rabbits against purified human erythrocyte tropomodulin and was used at a concentration of 4 μg/mL, as previously described, with overnight incubation. Bound antibody was detected using horseradish peroxidase–conjugated anti-rabbit IgG, followed by enhanced chemiluminescence reaction as directed by the manufacturer (all purchased from Amersham Life Sciences). Kodak X-Omat AR film was exposed to the blot for 10 to 30 seconds.

**Immunofluorescence Localization and I-Band Measurement**

Immunofluorescence was performed using standard procedures. Polyclonal rabbit anti-tropomodulin antibody was affinity-purified and preadsorbed (as described in Reference 2; see immunoblot protocol) and used at a concentration of 5 μg/mL. Monoclonal mouse anti–α-actinin antibody was used at 2.5 μg/mL (Sigma Immunochemicals). β-Gal was detected using anti-β-gal antibody (GIBCO-BRL, catalog No. 19929–017), and actin was labeled by either anti-actin antibody (C4, provided by Dr James Lessard, The Children’s Research Foundation, Cincinnati, Ohio) or rhodamine-conjugated phalloidin (Sigma; catalog No. P 5157, used at 2.5 μg/mL). Secondary FITC-conjugated or TRITC-conjugated anti-rabbit IgG antibody was used at 1:250 dilution (Boehringer-Mannheim Biochemicals). Cardiomyocytes were mounted in Vectashield antifade medium (Vector Laboratories) and viewed using a Zeiss LSM-1 or Molecular Dynamics CLSM CLS10 confocal microscope. Plan Neofluor ×40 (numerical aperture, 0.75) and ×63 (numerical aperture, 1.40) oil immersion objectives were used to view cells. Image analysis was performed using either the standard system operating software provided with a Zeiss LSM microscope (version 2.08) or with the Molecular Dynamics CLS10 (ImageSpace version 3.2).

Confocal images were recorded using a Sony or Codonics dye sublimation printer. Average I-band widths were calculated after 25 individual measurements of sarcomeric actin per field. Measurements were taken using Imagespace software on a Silicon Graphics Indy platform from magnified images (Fig 4B, 4D, and 4F) displayed on a Silicon Graphics 20-inch high-resolution monitor. The Molecular Dynamics 2010 confocal system is capable of resolving structures as little as 200 nm apart, and measurements can be made using Imagespace software to within a reproducibility of ±10 nm. Significance values were calculated using Student's t test.

**Nondenaturing Gel Analysis**

After infection, cardiomyocytes cultured in 100-mm dishes were washed once in PBS and scraped into 1.0 mL of ice-cold buffer containing 15 mmol/L HEPES (pH 7.0), 145 mmol/L NaCl, 0.1 mmol/L MgCl₂, 10 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl fluoride, and various protease inhibitors (aprotinin, chymostatin, leupeptin, N-α-p-tosyl-L-lysine chloromethyl ketone, and pepstatin, each at 20 μg/mL). Cells were disrupted by repeated pipetting, aliquoted, and stored at −20°C until use in experiments. Intact myofibrils were not visible in these lysates by differential interference contrast microscopy (data not shown). Nondenaturing gel electrophoresis was performed as described, with some modifications. Samples were loaded onto slab gels consisting of a 4.2% separating gel with a 3% stack and electrophoresed at 50 V for 72 hours at 2°C using 40 mmol/L Tris-acetate, pH 7.4, for the running buffer. Proteins were transferred to nitrocellulose for immunoblot analysis (described above) using mouse monoclonal anti-actin antibody (C4, provided by Dr James Lessard). Actin immunoreactivity was visualized by chemiluminescence followed by laser densitometric analysis (Ultrascan XL and GelScan XL software, Pharmacia) to determine distribution of actin label.

Relative levels of G-actin and F-actin were determined by immunoblot analysis of SDS-PAGE–separated cardiomyocyte lysates. Anti–actin antibody (clone C4, Boehringer-Mannheim Biochemicals) was used at a concentration of 2.0 μg/mL. Total actin and G-actin content of cardiomyocytes was also estimated using the Fluoroperter actin assay kit as directed by the manufacturer with slight modifications (Molecular Probes).

**Protein Turnover Analysis**

Freshly isolated myocytes were plated onto aligned collagen gels prepared after the methods of Simpson et al in 35-mm culture dishes at a density of 800 000 cells per dish. The cells were cultured for 3 days in DMEM (GIBCO supplemented with 8.0% horse serum (Flow Laboratories), 5.0% newborn bovine serum (GIBCO), and cytosine arabinoside (10 μg/mL). On day 3 of culture, the cells were rinsed in serum-free medium and cultured overnight in methionine-deficient DMEM supplemented with 2.0% newborn bovine serum and 5 μCi/mL [35S]methionine (ICN Biomedicals). On day 4 of culture, the cells were rinsed three times in serum-free medium and infected with β-gal, Ad–Tmod, or Ad–T–mod at an MOI of 50 for 2 hours. Additional control cultures that were identically treated but not infected were included. Preliminary experiments assessed the nonspecific effects of viral infection at various MOIs using the β-gal viral construct. The MOI used in the tropomodulin experiments was determined by titrating the MOI of the β-gal viral construct until total protein turnover and the total cellular concentration of actin and myosin heavy chain were identical in control uninfected cultures and cultures infected with β-gal. All infection procedures were carried out in DMEM supplemented with 2.0% newborn bovine serum. At various times after infection, the replicate cultures were rinsed and transferred to serum-defined chase medium (DMEM/F12/PC-1 [1:1:1] supplemented with 2 mmol/mL unlabeled methionine, 3 mmol/mL glutamine, 100 U/mL fungizone, 100 U/mL streptomycin, 100 U/mL penicillin, and 10 μg/mL cytosine arabinoside; PC-1 medium was purchased through Hyco Biomedical Corp.). This formulation matches, as closely as possible, conditions previously used to study turnover of actin and myosin heavy chain in neonatal cardiomyocytes. Replicate cultures were rinsed three times in ice-cold serum-free DMEM, extracted in 250 μL of gel loading buffer (62.5 mmol/L Tris–HCl, pH 6.8, plus 5.0% β-mercaptoethanol, 10.0% glycerol, and 8.0% SDS), and boiled for 10 minutes. Samples were stored at −80°C until analyzed. The relative amount of protein-bound radioactivity remaining in the different treatment groups was determined by liquid scintillation counting. Cell extracts were boiled for 5 minutes, and equal volumes of extract were removed and counted in a Packard 1500 Tri Carb liquid scintillation counter (Packard Instruments). The data from the replicate cultures of each treatment group were averaged and expressed as a percentage of the protein-bound radioactivity remaining with respect to time 0 (immediately after infection). An independent t test was used to assess the effects of the different viral constructs.

The relative concentrations of actin and myosin heavy chain were quantitatively analyzed by SDS-PAGE and laser densitometry. Protein bands corresponding to myosin heavy chain (206 KD) and actin (43 KD) were identified by calculating the relative mobility of proteins on SDS gels relative to molecular mass markers. Equal volumes of radiolabeled cell extract from each replicate culture were separated on a 10.0% SDS-polyacrylamide slab gel, fixed, and stained with Coomassie brilliant blue overnight. Destained gels were scanned with an LKB Ultrascan XL laser densitometer. Protein bands of interest were scanned three times, and the average area under each band was determined in arbitrary units of optical density and averaged. Results from different treatment groups were expressed as a percentage of optical density observed in cultures at time 0 (immediately after infection). Independent t tests assessed the effects of the different viral constructs.

Relative amounts of biosynthetically labeled actin and myosin heavy chain were determined by separating equal volumes of cell extract on a 10.0% slab gel as described and processing the gels for autoradiography with fluorographic enhancement. Gels were soaked in DMSO for two 45-minute dehydration incubations on a shaking table, followed by a 3-hour incubation in DMSO supplemented with 20.0% (wt/wt) 2,5-diphenyloxazole. Gels were rehydrated overnight, dried, and analyzed by standard fluorographic methods. Film exposure was varied to obtain data in the linear range of the autoradiograph. Data from replicate cultures in each treatment group was...
expressed in arbitrary units of optical density and averaged. Once again, the data from the different treatment groups were expressed as a percentage of optical density observed in cultures at time 500 (immediately after infection).

Electron Microscopy
Cultures were fixed for 15 minutes at room temperature in 2.5% glutaraldehyde and 0.12 mol/L sodium cacodylate buffer, pH 7.4, and then for 45 minutes in the same fixative with the addition of 0.08% tannic acid. The cultures were postfixed in OsO4, stained en bloc with uranyl acetate, and further processed as described.21 After embedding, two or three typical areas were selected on the basis of the shape and density of the cells. Thin sections were obtained parallel to the surface of the coverslip. Some sections were further stained with uranyl acetate and lead citrate. The 0.08-μm sections were observed with a JEOL 1200EX II electron microscope at 80 kV, and the 0.25-μm sections were viewed with a JEOL 200CX electron microscope at 160 and 200 kV.

Results
Recombinant Adenoviral Expression Vectors Increase or Decrease Tropomodulin Levels
Tropomodulin mRNA production by Ad+Tmod was confirmed by extraction of total RNA from adenovirus-infected and control cardiomyocytes. Adenovirally encoded tropomodulin mRNA size was identical to mRNA detected in 10T1/2 cells after Ad1Tmod infection (data not shown). Endogenous tropomodulin mRNA level was insignificant compared with the amount of tropomodulin mRNA cells after Ad1Tmod infection (Fig 1A). Endogenous tropomodulin mRNA production in control cells was undetectable with a short (30-minute) exposure, but a longer (48-hour) exposure showed a major transcript at 5.0 kb, two lesser transcripts at 3.0 and 2.0 kb, and a very faint 1.3-kb signal (Fig 1B). Multiple
tropomodulin mRNA transcripts in heart tissue are thought to originate from a single coding region with alternate 3’ termination sites in the untranslated region. Endogenous tropomodulin mRNA was undetectable by Northern blot analysis of Ad−Tmod−infected cardiomyocytes (data not shown).

Tropomodulin protein content of infected cardiomyocytes was altered by infection with recombinant adenoviruses. Since tropomodulin has an apparent mobility close to actin (Mr, 43,000 kD) and is not a major protein constituent of the cardiomyocyte, it cannot be clearly identified in a Coomassie brilliant blue–stained gel (Fig 1C). Patterns of Coomassie-stained bands were comparable between uninfected lysates and cultures infected with β-gal, Ad+Tmod, or Ad−Tmod throughout the 94 hours following the initiation of infection. Similarly prepared gels were prepared for immunoblot analysis of tropomodulin expression by transfer to nitrocellulose. The region of the blots corresponding to the Mr of tropomodulin was excised and labeled with anti-tropomodulin antibody. Immunoblots (Fig 1D) showed consistent levels of endogenous tropomodulin immunoreactivity (Fig 1D, control and β-gal–infected) throughout the culture period until 94 hours after infection, which was probably due to the proliferation of noncardiomyocytes. Greatly increased tropomodulin levels were evident after Ad+Tmod infection (Fig 1D, Ad+Tmod). Additional immunoreactive bands that could function as dominant negative mutations and thus affect tropomodulin function (data not shown) were also present, although these aberrant species accounted for <10% of the total expressed transgene protein (determined by densitometric scans). The relatively minor aberrant protein component could stem from either breakdown of overproduced tropomodulin protein and/or the low-level production of longer virus-encoded tropomodulin mRNAs. Tropomodulin immunoreactivity in Ad−Tmod lysates was decreased (Fig 1D, Ad−Tmod) compared with that in control cells, consistent with the expectation that expression of antisense tropomodulin mRNA would inhibit protein production. The tropomodulin protein level apparent in Ad+Tmod−infected cells in Fig 1D, Ad+Tmod infection after 3 days (F) or 4 days (G) is shown. Cells in various stages of myofibrillar degeneration show heterogeneity of actin filament organization. Control uninfected cells show no evidence of myofibrillar disarray after this time in culture (not shown). H through M, Coincident localization of α-actinin (H) and phaloidin (I) reactivity in control cells is shown. Loss of α-actinin sarcomeric organization (J) closely follows disruption of phaloidin reactivity (K) after tropomodulin overexpression. Extended actin filament bundles formed by lowering tropomodulin expression level show a similar pattern of overall labeling by either α-actinin antibody (L) or phaloidin (M). However, the α-actinin labeling associated with extended actin filament bundles retains some periodicity. Bar shown in panel A corresponds to 7.2 μm (A, B, and D), 3.6 μm (G), 5 μm (E), or 10 μm (Fand G). Panel H through M are all shown at the same magnification, with the bar in panel M equal to 5 μm.

**Figure 2.** Myofibril structure is altered by changes in tropomodulin expression. Confocal micrographs of cardiomyocytes labeled with anti-tropomodulin antibody detected by FITC-conjugated secondary antibody (green) and FITC-phalloidin (red) show actin filaments (A through C), anti-myosin antibody and FITC-phalloidin (D and E), anti-α-actinin antibody (H, J, and L), and FITC-phalloidin (F, G, I, K, and M). Areas of coincidence between labels in color micrographs appear yellow. A, Intracellular organization of normal cardiomyocytes consists of a well-organized myofibrillar array. Tropomodulin immunoreactivity is present in the A band of the sarcomere; actin filaments are concentrated in the center of the I band. B, Loss of myofibrillar density, variation in concentrations of actin along myofibrils, and lack of myofibrillar directional alignment after Ad+Tmod infection are shown. Tropomodulin immunoreactivity is widespread, as evidenced by cytoplasmic labeling. Immunoreactivity is reduced where nuclei are located in the cell center. C, Abnormally long actin filament bundles form after Ad−Tmod infection. Central regions of myofibril organization are arranged into elongated actin filament bundles (at long arrows) with non-sarcomeric actin also present along the periphery (at short arrows). D, Sarcomeric myosin disruption after Ad+Tmod infection with diffuse cytoplasmic myosin immunoreactivity is shown. E, Myosin immunoreactivity is diminished or absent from extended actin filament bundles in Ad−Tmod−infected cells. F and G, Progression of myofibrillar degeneration induced by Ad+Tmod infection after 3 days (F) or 4 days (G) is shown. H through M, Coincident localization of α-actinin (H) and phaloidin (I) reactivity in control cells is shown. Loss of α-actinin sarcomeric organization (J) closely follows disruption of phaloidin reactivity (K) after tropomodulin overexpression. Extended actin filament bundles formed by lowering tropomodulin expression level show a similar pattern of overall labeling by either α-actinin antibody (L) or phaloidin (M). However, the α-actinin labeling associated with extended actin filament bundles retains some periodicity. Bar shown in panel A corresponds to 7.2 μm (A, B, and D), 3.6 μm (G), 5 μm (E), or 10 μm (F and G). Panel H through M are all shown at the same magnification, with the bar in panel M equal to 5 μm.
thin-filament complexes bordered by tropomodulin immunoreactivity. Actin filament staining with phalloidin was strongest at the center of the I band, as demonstrated by localization relative to tropomodulin at the distal ends of actin filaments. Control experiments performed with β-gal adenovirus demonstrated that myofibril architecture is not overtly affected by processes of adenoviral infection or expression of an exogenous protein product (data not shown). The well-developed myofibril organization of cultured cardiomyocytes was lost after infection with Ad+Tmod. Tropomodulin immunoreactivity was present throughout the cytoplasm of Ad+Tmod–infected cells, and there was a profound loss of myofibrillar density (Fig 2B). Extensive areas devoid of myofibrils were apparent, and remaining myofibrils were generally not aligned. In contrast to the loss of myofibril structure following Ad+Tmod infection, cardiomyocytes infected with the Ad−Tmod exhibited unusually long actin filament bundles (Fig 2C). Central areas of the cell showed well-developed myofibril organization with associated tropomodulin immunoreactivity, as could be expected from immunoblot analyses. Striated myofibrils did not extend throughout the cell but underwent a structural transition to elongated actin filament bundles, which lacked normal sarcomeric organization. Extended actin filament bundles were usually seen in peripheral regions, where they followed cell contours and formed protrusions. Actin filament structures comparable to those observed in Ad−Tmod–infected cardiomyocytes were never observed in uninfected or β-gal–infected cardiomyocytes.

Changes in tropomodulin expression disrupted the organization of sarcomere-associated myofibrillar proteins. Sarcomeric myosin distribution was lost after Ad+Tmod infection (Fig 2D), indicating loss of thick filament organization. This was accompanied by an increase in diffuse cytoplasmic myosin immunoreactivity. Little or no myosin immunoreactivity was present along the extended actin filament structures observed after Ad−Tmod infection (Fig 2E). Sarcomeric α-actinin reactivity appeared coincident with phalloidin labeling in control cells (Fig 2H and 2I, respectively). Areas within cardiomyocytes where sarcomeric α-actinin organization was lost after tropomodulin overexpression (Fig 2J) showed comparable disruption of actin filament organization (Fig 2K). The extended actin filament structures observed under conditions of decreased tropomodulin were also labeled by α-actinin antibody (Fig 2L and 2M). The primary difference between myosin and α-actinin redistribution following changes in tropomodulin expression was that myosin immunoreactivity was lost from areas where sarcomeric actin filament organization was disrupted, whereas α-actinin immunoreactivity remained, regardless of sarcomeric integrity, as long as actin filament bundles were present. Various stages of myofibrillar degeneration were observed after infection: from regions of relatively well-organized actin filaments (Fig 2F) to virtual elimination of actin filaments except for random punctate staining (Fig 2G). Myofibrils in Ad+Tmod–infected cardiomyocytes degenerated further with prolonged culture, during which time tropomodulin immunoreactivity remained strong (data not shown).
Tropomodulin Overexpression Decreases I-Band Width

Actin was labeled by anti-actin antibody in control and Ad+Tmod–infected cardiomyocytes to assess the effect of tropomodulin overexpression on I-band width (Fig 3). Five individual cells were examined in each culture condition with four to six separate measurements per cell. Average I-band width for Ad+Tmod–infected cells is probably shorter than estimates allow, since measurements could only be performed on I bands with discernible sarcomeric structure. I bands in some areas showing severe myofibril degeneration were not evaluated because of the lack of a clearly discernible structure (Fig 3E, upper region). I-band widths were estimated by identifying the maximal width (thus the longest region of the band) of labeled sarcomeric actin blocks between Z disks and intervening bare zones (narrow or wide gaps, respectively; see Fig 3B, 3D, and 3E). A total of 25 separate measurements were made for each group. Variation in I-band width was comparable within a single cell or between different cells, suggesting similar progression of I-band width degeneration throughout the culture. Estimated I-band width was greater in control cells (Fig 3A, 0.804±0.09 μm) than in either of the two separate samples infected with Ad+Tmod (Fig 3C and 3E, 0.626±0.10 or 0.512±0.11 μm). Differences between these groups were statistically significant (**P<.01 by Student’s t-test). Cardiomyocytes labeled with antibodies to either tropomyosin or troponin T gave results qualitatively similar to those with anti-actin antibodies (data not shown). This suggests that the components of the thin-filament complex remain associated until the filament is disrupted.

Relative mobilities of actin and actin filament complexes in cardiomyocytes after Ad+Tmod infection were determined by nondenaturing gel analyses. After separation on SDS gels, proteins were transferred to nitrocellulose, and blot strips were labeled with anti-actin antibody. Chemiluminescent signals were detected on film and quantified by laser scanning densitometric analysis. Increased mobilities of actin complexes through the gel (indicative of decreasing size) are evident after Ad+Tmod infection. Three regions that correspond to broadly overlapping peaks were defined on densitometric plots (Fig 4). Results of integration analysis on peak areas showed that Ad+Tmod infection decreased the amount of large actin complexes (from 56.5% in the control condition to 10.9% after overexpression) and increased the levels of smaller actin complexes (from 43.5% in the control condition to 89.1% after overexpression). Percent values for all three regions from both control and tropomodulin overexpression lysates are presented in the table at the bottom of Fig 4. Relative levels of G-actin or F-action did not differ significantly between control, β-gal, Ad+Tmod, or Ad−Tmod cardiomyocytes as determined by immunoblot analyses (data not shown). A more sensitive DNase-based fluorometric assay indicated that G-actin levels increased 21% after Ad+Tmod infection and decreased 13% after Ad−Tmod infection; differences in G-actin levels between the β-gal and Ad+Tmod or Ad−Tmod lysates were not statistically significant (**P≤.12 and .59, respectively).

Rate of Total Protein Turnover Is Not Affected by Infection With Recombinant Adenoviruses, but Decreased Tropomodulin Expression Causes Actin Accumulation

The state of intracellular biochemistry following disruption of tropomodulin levels was monitored by time-course experiments that followed total protein turnover by metabolic labeling. Altered tropomodulin expression has no significant effect on total protein turnover in the cardiomyocyte culture (Fig 5A). Furthermore, no significant impact on protein turnover was observed for over 1 week after infection with β-gal, Ad+Tmod, or Ad−Tmod. The loss of contractile proteins as a function of time in all cultures is associated with the very low serum concentrations used in the media in these experiments. Therefore, adenoviral infection does not overtly affect cellular protein metabolism, and altered tropomodulin expression affects myofibril organization by reorganizing sarcomeric proteins rather than by causing increased protein turnover. Additional analyses were performed to determine changes in cellular content and turnover of sarcomeric actin and myosin heavy chain (Fig 5B). No significant effect was observed after infection with either β-gal or Ad+Tmod, but Ad−Tmod infection led to accumulation of actin and a...
concomitant decrease in actin turnover. Thus, Ad+Tmod-induced myofibril degeneration is not caused by increased sarcomeric protein turnover. However, the specific decrease in actin turnover observed after Ad−Tmod infection may be related to the loss of sarcomeric actin structure and appearance of extended actin filament bundles (Fig 2).

Ultrastructural Analysis Reveals Details of Myofibril Disarray and Leptomere Formation

Control cardiomyocytes (19 cells from two different areas) showed parallel bundles of myofibrils with normal sarcomeric structure. The majority of myofibrils extending into the processes were normal, and only a single leptomere (see description below) was observed in one cell. A few myofibrils extending into myocyte processes had oblique irregularly spaced Z lines, particularly those close to the substrate-apposed cell surface. Typical views are shown in Fig 6A and 6B.

Cardiomyocytes from Ad+Tmod–infected cultures were subjectively categorized into three groups, depending on the extent of structural disruption. Of 24 cardiomyocytes from three different areas, 16 cells showed completely abnormal structure (abnormal), 3 cells had a mixture of normal and abnormal structure (mixed), and 5 cells had almost normal structure (normal). Abnormal myofibrillar structures included shortening of sarcomeres, loss of thick and thin filaments, decreased myofibril thickness, loss of banding patterns, clumping of Z-line material, and, most strikingly, the frequent appearance of leptomeres in the Ad+Tmod group. Leptomeres (described in Reference 24) were usually found at subsarcolemmal regions appearing as regularly spaced (150- to 160-nm) narrow dense bands with fuzzy attached material and an amorphous material between bands. Some leptomeres were associated with extended remnants of myofibrils having normal periodicity or with remnants composed of electron-dense
clumps of Z-line material in the vicinity of a few thin myofibrils. These Z-line clumps were not usually associated with leptomeres. Cardiomyocytes in mixed and normal groups had typical organized myofibrils that were found only within the first 10 to 15 thin sections from the substrate-apposed surface. The cytoplasm close to the dorsal surface of normal cells was virtually devoid of myofibrils. The abnormal myofibrils in the mixed and abnormal groups were scattered throughout the cytoplasm. Examples of Ad+Tmod–infected cells are shown in Fig 6C through 6E and Fig 7A and 7B).

Nineteen cardiomyocytes infected with Ad−Tmod from two different areas were observed. Fifteen cells showed regions with abnormal myofibrils, and four cells appeared normal. Most of the myofibrils were well organized, at least in the central region of the cell. Abnormal myofibrils had normal sarcomeric structure near the cell center, which made a transition into aberrant structures closer to the cell periphery. These included the disorientation, fragmentation, and disappearance of Z lines, loss of thick filaments, and loss of sarcomeric structure. Aberrant myofibrils often terminated in long tapering actin filament bundles, which sometimes branched into smaller bundles that extended into peripheral projections of myocytes. Some aberrant myofibrils appeared twisted, with irregular sarcomeres and abnormal organization of thick and thin filaments. Examples of Ad−Tmod–infected cells are shown in Fig 6F through 6H and Fig 7C and 7D.

### Discussion

The present study shows that tropomodulin is intimately tied to cardiomyocyte intracellular organization, presumably by influencing actin filament structure and affecting myofibril stabilization. The role of tropomodulin in actin filament regulation may be critical in cardiac muscle, where, unlike skeletal muscle, there is no nebulin to act as a template for actin filaments. Although sarcomeric actin filament length in cardiomyocytes is more variable than in skeletal muscle, highly ordered I-Z-I brushes are rapidly and uniformly assembled by an unknown mechanism.

Neonatal rat cardiomyocytes form myofibrils within days after isolation and in vitro culture (Fig 2) and begin to beat within 2 to 3 days after plating. Spontaneous beating enhances the formation and organization of myofibrils. Cardiomyocytes used in the present study had well-developed sarcomeric architecture and functional myofibrils before the adenoviral infection. The process of adenoviral infection does not affect intracellular myofibrillar organization, so the observed effects are due to the expression of transfected sense or antisense tropomodulin cDNAs. Adenoviral infection does not compromise cardiomyocyte viability; all of the cells in each group can be infected together; and infected cells continue to contract spontaneously throughout the culture period (data not shown), unlike the tropomodulin study of Gregorio et al' in which antibody was microinjected into cardiomyocytes.
modulin levels. Ad+Tmod infection resulted in loss of synchronous beating as cells twitched or quivered at irregular intervals. In comparison, decreased expression of tropomodulin by Ad−Tmod infection caused loss of beating only in peripheral regions, where myofibrils transitioned into extended actin filament bundles.

I-band width reduction after Ad+Tmod infection (Fig 3) suggests that tropomodulin overabundance leads to progressive breakdown of thin filaments. Non-denaturing gel analyses (Fig 4) support this conclusion, with faster migration of actin complexes through the gel presumably due to smaller size rather than an altered charge of actin complexes. Cardiomyocyte protein composition was unaffected by infection, except for tropomodulin protein accumulation after Ad+Tmod infection (Fig 1, additional data not shown) and the increased actin after Ad−Tmod infection (Fig 5). Biochemical results (Fig 5) are consistent with the hypothesis that actin residing within myofibrils is physically sequestered from proteolytic events constitutively active in the cytoplasm. Interventions that promote myofibril breakdown accelerate protein turnover and deplete cardiomyocytes of actin and myosin heavy chain. Since Ad+Tmod infection did not lead to increased protein turnover, sarcomeric proteins may be held in multimeric complexes, which are unsuitable for formation of sarcomeric complexes. Conversely, interventions that stabilize or promote the assembly of actin monomers into myofibrils inhibit proteolytic processing of actin. This occurs for Ad−Tmod infection, where actin filaments become extended and nonsarcomeric. Collectively, these results indicate that loss of sarcomeric actin filaments following tropomodulin overexpression is likely due to the reorganization of myofibril components into smaller complexes rather than proteolytic effects or depolymerization of filaments to monomers.

Appropriate termination of thin filaments depends on a regulated tropomodulin/actin filament ratio. Insufficient levels of nascent tropomodulin protein synthesis resulting from Ad−Tmod infection caused dramatic increases in the length of actin filament bundles. Inhibition of tropomodulin-capping activity by microinjection of anti-tropomodulin antibody into chick cardiomyocytes produced similar results. Our results extend the observations of Gregorio et al by showing the transitions between preexistent striations and the new stress-fiber-like structure on the myofibrils. In the present study, conversion to stress-fiber-like bundles of actin filaments occurred preferentially at peripheral regions of the cardiomyocyte (Fig 2). Although nonsarcomeric actin is a normal component of developing myofibrils in isolated cardiomyocytes, myofibril structure in the present study was already well developed after 5 days in culture. Tropomodulin protein synthesis was inhibited by Ad−Tmod infection, and persistence of tropomodulin protein after infection with Ad−Tmod (Figs 1 and 2) was presumably due to residual protein synthesized and assembled into myofibrils before infection.

Disruption of myofibril structure by altered tropomodulin levels was extensive, affecting organization of α-actinin and myosin sarcomeric proteins. Tropomodulin-induced changes in α-actinin distribution (Fig 2) can be related to the loss of thin-filament integrity observed using fluorescence-tagged phalloidin. Tropomodulin overexpression resulted in apparent dissolution of thin filaments and breakdown of the regular sarcomeric distribution of α-actinin into punctate clusters, whereas inhibition of tropomodulin expression caused smearing of α-actinin along the length of myofibrils (Fig 2). Actin filament organization in early stages of myofibrillogenesis is also affected by inhibition of another capping protein, CapZ, which localizes at the Z disks of organized myofibrils. In contrast to the experimental design used for assessing CapZ disruption with developing myotubes, our experiments were performed using cardiomyocytes with fully formed mature myofibrils. Inhibition of
CapZ activity delayed the appearance of actin and α-actinin in a striated pattern, whereas alteration of tropomodulin expression caused reorganization or degeneration of preexisting myofibrillar structure.

Compelling evidence of actin reorganization is evident from the ultrastructural analysis of infected cardiomyocytes (Figs 6 and 7). The presence of leptomeres in cardiomyocytes after A×+ Tmod infection supports the idea that actin filaments are depolymerized. Treatment of cultured skeletal muscle fibers with cytochalasin D, an inhibitor of actin polymerization, also induces the formation of leptomeres.\(^\text{32}\) Immunocytochemical characterization of leptomeres has demonstrated that they consist primarily of actin and are not labeled by antibodies to desmin, vimentin, α-actinin, filamin, or vinculin.\(^\text{33}\) The functional significance of leptomeres remains unknown, but their appearance in a variety of myopathies and tumors is suggestive of pathological cytosplasmic inclusions. Leptomeres seem to form in cells whose myofibrillar development has been arrested or diverted at an early stage.\(^\text{33}\) Effects of actin filament reorganization were also evident in the redistribution of Z-disk-like material in electron micrographs (Figs 6C and 6E), consistent with the changes in α-actinin localization shown by immunofluorescence microscopy (Fig 2).

A dynamic interplay exists between tropomodulin expression and thin-filament organization in cardiomyocytes. Tropomodulin gene transcription or mRNA translation, or both, are presumably tightly regulated in cardiomyocytes during and after cell differentiation. A regulated pool of soluble tropomodulin may exist in equilibrium with the bound protein that limits actin filament elongation from the pointed end. Perturbation of the normal endogenous tropomodulin pool caused the myofibril reorganization described in the present study. Experiments in progress demonstrate that tropomodulin overexpression in hearts of transgenic mice causes myofibrillar degeneration and cardiomyopathy.\(^\text{35}\) Future studies will address the relationship between loss of thin-filament organization and the developing cardiomyopathy using these tropomodulin-overexpressing transgenic mice.

Acknowledgments

This study was supported by grants from the Greater Los Angeles and Ohio Affiliates of the American Heart Association (Dr Sussman), the National Institutes of Health, and funds provided by the Cigarette and Tobacco Surplus Fund of the State of California through the Tobacco-Related Disease Research Program (Dr Kedes), Dr Sussman is a recipient of an Initial Investigator Award from the Greater Los Angeles Affiliate of the American Heart Association and a Scientist Development Grant from the Ohio Affiliate of the American Heart Association. Dr Baquè is a recipient of a fellowship (FL/92–9) from the Generalitat de Catalunya. Thanks are extended to Tom Borg and Jeff Robbins for helpful discussions and to Jim Lesard for providing the anti-actin antibody used in this study. Karmell Kalibiyama and Pejman Motarjam provided expert technical assistance with the preparation of neonatal heart cell cultures. We are also indebted to Sara Welch, who provided invaluable assistance with Northern, SDS-PAGE, and nondenaturing gel analyses. We would also like to thank Janet Blanks and Michael Rudinbl of the Dehony Microscopy Core Facility for access to and assistance with the Zeiss confocal microscope, which was purchased with funds provided by the Seaver Foundation.

References

1. Fowler VM, Sussman MA, Miller PG, Flesher BE, Dansch MP. Tropomodulin is associated with the free (pointed) ends of the thin filaments in rat skeletal muscle. \(\text{J Cell Biol} \ 1993;120:411–420.\)
2. Fowler VM. Tropomodulin: a cytoskeletal protein that binds to the end of actin filaments and inhibits tropomyosin binding to actin. \(\text{J Cell Biol} \ 1990;111:471–482.\)
8. Shabita H, Thorburn J, Chien K. Microinjection of antibodies and expression vectors into living myocardial cells. \(\text{Circulation} \ 1992;85:2326–2346.\)
9. Bishopric NH, Simpson PC, Orndahl CP. Induction of the skeletal α-actin gene in α-actin-transfected myocytes. \(\text{J Clin Invest} \ 1987;80:1194–1199.\)
15. Towbin H, Stachelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. \(\text{Proc Natl Acad Sci U S A} \ 1979;76:4350–4354.\)
16. Fowler VM. Identification and purification of a novel M, 43,000 tropomyosin-binding protein from human erythrocyte membranes. \(\text{J Biol Chem} \ 1987;261:12792–12800.\)


Altered Expression of Tropomodulin in Cardiomyocytes Disrupts the Sarcomeric Structure of Myofibrils
Mark A. Sussman, Susanna Baqué, Chang-Sub Uhm, Mathew P. Daniels, Robert L. Price, David Simpson, Louis Terracio and Larry Kedes

Circ Res. 1998;82:94-105
doi: 10.1161/01.RES.82.1.94

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/82/1/94

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/