Tropomyosin 3 expression leads to hypercontractility and attenuates myofilament length-dependent Ca$^{2+}$ activation

KATHY PIEPLES,1 GRACE ARTEAGA,5 R. JOHN SOLARO,5 INGRID GRUPP,2 JOHN N. LORENZ,3 GREG P. BOIVIN,4 GANAPATHY JAGATHEESAN,1 ERIN LABITZKE,1 PIETER P. DETOMBE,5 JOHN P. KONHILAS,5 THOMAS C. IRVING,6 AND DAVID F. WIECZOREK1

1Department of Molecular Genetics, Biochemistry, and Microbiology, 2Department of Pharmacology and Cell Biophysics, 3Department of Molecular and Cellular Physiology, 4Department of Pathology and Laboratory Medicine, College of Medicine, University of Cincinnati, Cincinnati, Ohio 45267-0529; 5Department of Physiology and Biophysics, College of Medicine, University of Illinois, Chicago, 60612; and 6Center for Synchrotron Radiation Research and Instrumentation and Department of Biological, Chemical, and Physical Sciences, Illinois Institute of Technology, Chicago, Illinois 60616

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Pieples, Kathy, Grace Arteaga, R. John Solaro, Ingrid Grupp, John N. Lorenz, Greg P. Boivin, Ganapathy Jagatheesan, Erin Labitzke, Pieter P. deTombe, John P. Konhils, Thomas C. Irving, and David F. Wieczorek. Tropomyosin 3 expression leads to hypercontractility and attenuates myofilament length-dependent Ca$^{2+}$ activation. Am J Physiol Heart Circ Physiol 283: H1344–H1353, 2002. First published June 13, 2002; 10.1152/ajpheart.00351.2002.—Tropomyosin (TM), an integral component of the thin filament, is encoded by three striated muscle isoforms: α-TM, β-TM, and TPM 3. Although the α-TM and β-TM isoforms are well characterized, less is known about the function of the TPM 3 isoform, which is predominantly found in the slow-twitch musculature of mammals. To determine its functional significance, we ectopically expressed this isoform in the hearts of transgenic mice. We generated six transgenic mouse lines that produce varying levels of TPM 3 message with ectopic TPM 3 protein accounting for 40–60% of the total striated muscle tropomyosin. The transgenic mice have normal life spans and exhibit no morphological abnormalities in their sarcomeres or hearts. However, there are significant functional alterations in cardiac performance. Physiological assessment of these mice by using closed-chest analyses and a work-performing model reveals a hyperdynamic effect on systolic and diastolic function. Analysis of detergent-extracted fiber bundles demonstrates a decreased sensitivity to Ca$^{2+}$ in force generation and a decrease in length-dependent Ca$^{2+}$ activation with no detectable change in interfilament spacing as determined by using X-ray diffraction. Our data are the first to demonstrate that TM isoforms can affect sarcomeric performance by decreasing sensitivity to Ca$^{2+}$ and influencing the length-dependent Ca$^{2+}$ activation.

heart; cardiac muscle; thin filament regulation

TROPOMYOSIN (TM), an actin binding protein and a major component of the sarcomeric thin filament, forms coiled-coil dimers that assemble in a head-to-tail fashion in the major groove of actin. Numerous muscle proteins, including TM, have multiple isoforms that are expressed with developmental and tissue specificity. The TM gene family is composed of four genes [α-TM, β-TM, TPM 3 (γ-TM), and TPM 4 (δ-TM)], which through alternative splicing, the use of alternative promoters, and differential processing encode multiple striated muscle, smooth muscle, and nonmuscle specific transcripts (12, 17, 38). There are three primary striated muscle TM isoforms, α-TM, β-TM, and TPM 3, which are highly homologous and thought to exhibit unique physiological properties (28). In the mouse, α-TM is expressed mainly in the heart and also in slow- and fast-twitch musculature, β-TM is expressed predominantly in the developing heart and slow-twitch musculature, and TPM 3 is found only in slow-twitch musculature (27, 31).

TM, along with the troponin complex, regulates the Ca$^{2+}$-sensitive reaction of cross bridges with actin in striated musculature. The Ca$^{2+}$ signal is transmitted through the binding of Ca$^{2+}$ to troponin C (TnC), one of three proteins that comprise the troponin complex. The exact mechanism by which TM and troponin work is unclear; however, it is thought to include steric, allosteric, and cooperative elements (13, 18, 36). Current models propose that there are three distinct states that are dependent on the location of TM on the thin filament (23). The “blocked state” is when the TM blocks the myosin-actin interaction in the absence of Ca$^{2+}$. In the “closed state,” cross bridges are weakly bound when the position of TM has changed due to the binding of Ca$^{2+}$ to TnC. The “open state” is achieved following the transition of weak cross bridges into strong, force-generating cross bridges. Strong cross-bridge interactions promote additional TM movement and lead
to the stability of the TM in the open position. This activates the thin filament, stimulating muscle contraction and increasing cooperativity along the length of the myofibril (reviewed in Ref. 8).

Functional differences between the α-TM and β-TM striated muscle TM isoforms have been demonstrated in both in vitro and in vivo (26, 35). The exchange of α-TM for β-TM increases the ability of strong crossbridge binding to activate the thin filament, and the force developed by β-TM myofilaments is more sensitive to calcium (29). This difference in Ca\(^{2+}\) sensitivity is more pronounced when the myofilaments are phosphorylated by cAMP-dependent protein kinase, indicating a role for TM in the modulation of myofilament activation by phosphorylation on troponin. In cardiomyocytes expressing β-TM, maximum rates of contraction and relaxation are significantly reduced, and ATPase activity of myofibrils is more sensitive to Ca\(^{2+}\) (39). Furthermore, expression of β-TM in transgenic (TG) mouse hearts leads to prolongation in relaxation rate and a reduction in time to half relaxation (R\(_{1/2}\)) (26).

Although much information has been obtained for physiological differences between the α- and β-TM striated muscle isoforms, less is known about the striated muscle isoforms, less is known about the striated muscle isoforms. Recent work in our laboratory (31) cloned and characterized this isoform in the mouse. The TPM 3 gene, approximately 42 kb in length, is composed of 13 exons with its primary transcripts differentially processed to produce >11 mRNA isoforms (4, 5). Most of these isoforms are expressed in developmental and tissue-specific patterns in neurons; however, one of these isoforms is striated muscle specific with expression restricted to slow-twitch musculature (31). Unlike, α-TM and β-TM, the striated muscle isoform of TPM 3 is not expressed endogenously in murine cardiac tissue, but it is expressed in the adult human heart (31, 32).

In this study, we sought to determine the functional significance of the striated muscle isoform of TPM 3 by its overexpression in murine hearts. Murine hearts possess a uniform myofiber composition with a well-defined complement of sarcomeric proteins; thus the effects of ectopic expression of TPM 3 in the absence of other myofibrillar protein changes are readily ascertained. Six TG mouse lines were generated, which express varying levels of the TPM 3 RNA. Results show that the TPM 3 transgene is expressed at high levels, with the TPM 3 protein accounting for 40–60% of the cardiac muscle TM. Morphological analyses employing both light and electron microscopy demonstrate there are no structural changes in the heart or in the sarcomeres that are associated with the increased TPM 3 expression. However, extensive physiological analyses of these TG hearts using in situ and in vitro measurements reveal myocardial contraction and relaxation parameters are altered; there are increased rates of relaxation (–dP/dt) and contraction (+dP/dt). Also, measurement of force generated by skinned fiber bundles demonstrates a decreased sensitivity to Ca\(^{2+}\) and a decrease in the length dependence of myofilament Ca\(^{2+}\) activation. This functional phenotype is unique to the TPM 3 mice and differs from wild-type mice and β-TM TG mice. This is the first report demonstrating that a novel functional property is associated with TPM 3 expression, namely increased cardiac performance associated with perturbations in Ca\(^{2+}\) sensitivity and activation.

**EXPERIMENTAL PROCEDURES**

**Construction of the α-myosin heavy chain/TPM 3 TG construct and production of TG mice.** The TPM 3 cDNA was generated from murine skeletal muscle by using PCR primers corresponding to the human TPM 3 sequence (4, 31). The forward primer begins at the translational start position and maintains the highly conserved length of the striated muscle TM. Its nucleotide sequence is 5′-ATGGAGGCCCATCAA-GAAA-3′. The reverse primer includes the 3′-untranslated region (3′-UTR) of the cDNA and is 5′-TTTCCAGCAGCT-TAACAT-3′. The 1.1-kb TPM 3 cDNA fragment was ligated into the HindIII/SalI sites of a vector containing the α-myosin heavy chain (α-MyHC) promoter and its 5′-UTR. The 600-bp human growth hormone (hGH) polyadenylation signal was added to ensure correct transcript processing of the transgene (gift from Dr. Jeff Robbins). The 7.2-kb fragment containing the α-MyHC promoter, the TPM 3 cDNA, and the hGH polyadenylation signal was released from the vector by NotI digestion (Fig. 1). The resulting linear DNA fragment was isolated on a low-melting point agarose gel, followed by purification in a cesium chloride gradient. The resulting DNA was suspended in 5 mM Tris-HCl, pH 7.4, 0.1 mM EDTA at a final concentration of 2 μg/ml. Single-cell embryos derived from superovulated FVB/N mouse strain females were used in the microinjection. Purified DNA was microinjected into the pronuclei, and the surviving embryos were implanted into pseudopregnant foster mothers.

Mice carrying the transgene were identified by using PCR. DNA was isolated from ear tissue and PCR primers designed to the α-MyHC 5′-UTR and TPM 3 amino acid coding se-

![Fig. 1. Cardiac-specific tropomyosin (TM) isoform TPM 3 construct. TPM 3 cDNA including the coding region and the 3′-untranslated region (3′-UTR) was ligated to the α-myosin heavy chain (α-MyHC) promoter and a human growth hormone (hGH) poly A signal. Construct was released with NotI digestion and injected into male pronuclei to generate the transgenic (TG) mice.](http://ajpheart.physiology.org/10.1152/ajpheart.00237.2002)
quences were used to amplify a 234-bp fragment from mice carrying the transgene. Lines were established by breeding positive founder TG mice to nontransgenic (NTG) littermates.

**Genomic Southern blot analysis.** To confirm the presence of the transgene and to determine the number of copies integrated into each line, genomic DNA from tails was digested overnight with EcoRI, and electrophoresis was carried out on 0.8% agarose gels. The Southern blots were probed with a 32P-radiolabeled hGH fragment specific to the 3' end of the transgene. Quantification of the transgene was done on Imagequant phosphorimager version 5.1 (Molecular Dynamics; Sunnyvale, CA).

**Northern blot analysis.** RNA was isolated from murine hearts using TriReagent (Molecular Research Center; Cincinnati, OH). Total RNA (15 μg) from each line was electrophoresed on 1% formaldehyde gels and transferred to nylon cinnati, OH). Total RNA (15 μg) from each line was electrophoresed on 1% formaldehyde gels and transferred to nylon membranes. Hybridization was performed with 32P-radiolabeled α-TM 3'-UTR, TPM 3'UTR, and GAPDH probes. Blots were washed in 2× SSC and 0.05% SDS for 20 min at 50°C, followed by 0.1× SSC and 0.1% SDS for 30 min at 60°C. TM message levels were analyzed on Imagequant phosphorimager version 5.1 and normalized to GAPDH expression levels. Results are presented as a percentage of the highest expressing line.

**Western blot analysis.** Whole tissue and myofibrillar homogenates were prepared as previously described (24). Protein samples were run simultaneously on two 3.4 M urea, 8% SDS-PAGE gels. One gel was stained with Coomassie blue to ensure equal loading, and the other was transferred to nitrocellulose for Western blot analyses. Filters were probed with a striated muscle, TM-specific troponin T antibody (Sigma; St. Louis, MO) at a 1:10,000 dilution for 2 h at room temperature (19). After washing was completed, an anti-mouse IgG antibody conjugated with peroxidase (Roche; Indianapolis, IN) was incubated with the blot for 1 h at a dilution of 1:1,000. Detection was carried out with the Super Signal West Pico Chemiluminescent Substrate Detection System (Pierce; Rockford, IL). Band intensity was analyzed on Imagequant phosphorimagener Version 5.1. Results are presented as means ± SE.

**Histological examination.** For light microscopy, hearts were removed from 10- to 12-mo-old mice and immediately fixed in 10% neutral buffered formalin. Sections were cut at 5-μm thickness and stained with hematoxylin-eosin. For electron microscopy, hearts were fixed in 2% glutaraldehyde and then transferred to cacodylate buffer. Heart tissue was postfixed in osmium tetroxide. Sections (70–80 Å) were stained with lead acetate and examined on a Hitachi H6000 microscope.

**Working heart preparations.** Perfusion and analysis of mouse hearts were done as previously described (10, 11). Briefly, mice were anesthetized with pentobarbital sodium intraperitoneally, and the hearts were removed and cannulated. Five TG mice and four NTG age- and sex-matched littermates were examined. Cardiac performance was monitored by a six-channel P7 Grass polygraph. Intraventricular pressure, aortic pressure, and heart rate recordings were digitized via a TL-1 DMA interface board (Axon Instruments; Foster City, CA). Rates of +dP/dt, −dP/dt, time to peak pressure (TPP), and RTs were derived. Isoproterenol was added to the perfusion fluid close to the heart at increasing concentrations (8 × 10−11 to 8 × 10−8 M) with a multiprise, microperfusion pump (model 600, Harvard Apparatus). Individual points were recorded and summarized as means ± SD.

**Closed-chest preparations.** Closed-chest analysis was carried out as previously described (20). Six TG and six NTG littermates were examined. After anesthesia (Inactin, 100 μg/g body wt and ketamine, 50 μg/g body wt intraperioneally), a 1.4-Pr Millar MIKRO-TIP transducer (SPR-671, Millar Instruments; Houston, TX) was placed in the right carotid artery and threaded down into the ascending aorta and into the left ventricle to record the heart's performance. A cannula was placed into the right femoral vein to deliver dobutamine using a syringe pump, and a catheter that was connected to a pressure transducer was placed in the right femoral artery to monitor blood pressure. Dobutamine was delivered to challenge the heart. Measurements are recorded and analyzed on a MacLab 4/s data acquisition system (AD-Instruments; Mountain View, CA).

**Force and X-ray diffraction measurements of skinned fiber bundle preparations.** Mechanical experiments in which we determined relations between free Ca2+ and tension and interfilament spacing were done as previously described (1, 14, 29). The mice were anesthetized with ethyl ether, and their hearts were removed and incubated in a cold high-relaxing solution (53 mM KCl, 10 mM EGTA, 20 mM MOPS, 1 mM free Mg2+, 5 mM MgATP2−, 12 mM creatine phospho- tase, and 10 IU/ml creatine phosphokinase and protease inhibitors), adjusted to pH 7.0. Left ventricular papillary muscles were removed, and bundles of fibers were prepared (4–5 mm in length and 150–200 μm in width). Fiber bundles were attached to a micromanipulator and a force transducer with cellulose-acetate glue and extracted in high-relaxing solution containing 1% Triton X-100. Sarcomere lengths (SLs) were set at 1.9 and 2.3 μm as determined by laser diffraction patterns. The fiber bundles were first bathed in a low-relaxing solution (high-relaxing solution with 0.1 mM instead of 10 mM EGTA). Force was then measured in high-relaxing solution containing varying levels of free Ca2+.

We compared interfilament spacing as a function of SL in skinned fiber bundles from NTG and TPM 3 TG mice by using the BioCAT undulator based beamline at the Advanced Photon Source, Argonne National Laboratory as previously described (14). For the X-ray studies, the fiber bundles were mounted between a force transducer and a servo motor in a small trough that allowed simultaneous collection of the X-ray patterns and determination of SL by laser diffraction. Fiber bundles were dissected from the left ventricle and demembranated overnight with 1% Triton X-100 in standard relaxing solution (10 mM EGTA; 180 mM ionic strength). All solutions contained the following: 10 mM KCl, 100 mM NaCl, 2.5 mM MgCl2, 1 mM CaCl2, 1 mM MgATP2−, 0.1 mM Mg2+, 1 mM GTP, 4 U/ml creatine phosphokinase. The free Mg2+ and Mg-ATP concentrations were calculated at 1 and 5 mM, respectively. Experiments were carried out in a relaxing solution at 15°C. Fiber bundles were 2 to 3 mm in length and between 100 to 200 μm in width. Fiber length was systematically lengthened or shortened to collect at least 10–12 (at least every 50 nm) consecutive data points describing the relationship between SL and interfilament lattice spacing. An additional two to four data points were then collected at various SLs back along the curve to check for reproducibility or hysteresis. The level of passive tension in the lattice was recorded when the X-ray exposure was taken. Low-angle X-ray diffraction patterns were collected on a CCD-based X-ray detector. Spacings between the 1,0 and 1,1 equatorial reflections in the diffraction pattern were converted to d0 lattice spacings using Bragg’s Law. Data analysis was as previously described (14). The differences among groups at each SL were analyzed with a one-way ANOVA followed by Student’s t-test with a post hoc Bonferroni cor-
rection to assess differences among mean values. All data are shown as means ± SE.

RESULTS

Generation of TPM 3 TG mice. To elucidate relations between sarcomeric TM isoforms and contractile behavior in striated muscle, we developed a TG mouse model to overexpress the striated muscle-specific TPM 3 isoform in the heart. The transgene construct was generated by ligating the murine TPM 3 cDNA (containing the entire amino acid coding region and the 3′-UTR) into an expression vector regulated by the murine α-MyHC promoter, which directs cardiac-specific expression (Fig. 1). A hGH polyadenylation sequence is located 3′ of the TPM 3 cDNA to ensure correct transcript processing. Founder mice were identified by PCR and confirmed by Southern blot analysis. Six lines of TG mice were produced with copy numbers ranging from 2 to 60.

TPM3 transcript and protein expression in the TG mice. We measured TM expression by using Northern blot analysis following RNA isolation from hearts of TG and NTG littermates. RNA from each TG line was isolated and probed with 32P-radiolabeled 3′-UTR sequences from endogenous α-TM and the TPM 3 transgene construct; these nucleotide regions are specific to the particular striated muscle isoform and do not cross react with other striated muscle TM isoforms (31). As seen in Fig. 2A, endogenous α-TM is expressed in all hearts, whereas TPM3 expression is restricted to TG hearts. For quantification, TPM 3 transcript levels are normalized against GAPDH expression in the hearts to account for any loading differences. Maximum expression of the transgene occurs in line 89; this quantified value was set at 100%. Expression of the transgene varies from ~40% in line 71 to 100% in line 89 (Fig. 2B).

To compare contractile protein profiles from TPM3 TG mice with controls, we isolated myofibrillar proteins from the hearts of these mice. These proteins were subject to SDS-PAGE gels and stained with Coomassie blue to visualize protein content (Fig. 3A). Results show there are no gross changes in the expression levels of cardiac contractile myofilament proteins between NTG and TPM3 TG mice. Under these conditions, endogenous α-TM and TPM3 isoforms are not separated electrophoretically, and their isoforms cannot be distinguished. To facilitate separation and iden-
tification of muscle TM isoforms, which have similar molecular masses (~36 kDa), SDS-PAGE gels were modified to include 3.4 M urea. A Western blot employing the modified urea/SDS-PAGE conditions was conducted using a striated muscle TM-specific antibody (Fig. 3B). This antibody recognizes the three striated muscle TM isoforms with the same affinity (data not shown). The NTG samples only contain the α-TM protein, whereas both α-TM and TPM 3 protein is expressed in all six TG lines. The levels of α-TM protein in the TG mouse hearts does not decrease significantly from the control NTG levels; this result is in agreement with the RNA analysis where α-TM transcript levels are similar between TG and NTG mice. Quantification of the Western blot TM bands shows that TPM 3 protein accounts for 40–60% of the total striated muscle TM protein in all of the lines (Fig. 3C).

Analysis of the results from Fig. 3A indicate there are no quantitative differences in cardiac troponin T (TnT) content in NTG versus TG hearts. To confirm this result, Western blot analysis was conducted on cardiac myofibrillar proteins by using a cardiac-specific TnT antibody (Fig. 3D). Binding of the cardiac TnT antibody is specific for TnT produced in the heart from either TG or NTG control mice and does not react with skeletal muscle TnT. Results indicate there are no differences in the incorporation of cardiac TnT into the cardiac myofibrils of NTG and TPM3 TG hearts. Also, there is only a single conserved amino acid difference (E272D) between α-TM and TPM3 in the TnT binding site located in the carboxyl region of TM (amino acids 258–284), which supports the likelihood of cardiac TnT binding to the TPM 3 isoform. Thus expression of TPM3 in the heart does not adversely affect the ability of cardiac TnT to incorporate into myofibrils and bind to this TM isoform.

Histological and physiological studies. None of the founder mice or their progeny demonstrates any gross phenotypic alterations or reduced viability. Over 30 TG mice ranging in age from 2 mo to 1 yr were examined for morphological changes. Hematoxylin and eosin-stained heart sections show no signs of hypertrophy,
fibrosis, thrombi, necrosis, or any other pathological condition in any of the six lines examined (data not shown). Electronmicrographs also show no disruption of normal sarcomeric structure. Furthermore, no differences in percent heart-to-body weight ratios from the TG to the NTG mice are observed. Thus incorporation of TPM3 into the cardiac myofibers does not lead to morphological or pathological alterations in the sarcomere.

We pursued several lines of experiments to address the physiological significance of expression of TPM 3 in the regulation of cardiac contractility. TG animals from TPM3 TG line 56 were studied with different approaches, including in situ cardiac performance (closed-chest preparations), in vitro characteristics where contractile and relaxation parameters can be studied with different preloads and afterloads (isolated-working heart preparations), and ultimately, cardiac mechanical performance using skinned fiber analysis. Mice from TG line 89 were also examined to confirm the results. Previous studies have shown that overexpression of wild-type α-TM in TG mice does not lead to alterations in morphology or physiological performance of the heart (33, 39).

To evaluate cardiac performance in the whole animal, closed-chest preparations were done. Six TG and six littermate controls were examined for in situ cardiac performance. Both the basal contraction (dP/dtₘₐₓ) and relaxation (dP/dtₘᵢₙ) rates of the TPM 3 TG hearts were accelerated (Table 1). In addition, heart rate and left ventricular systolic pressure were both elevated in the TG mice. These results indicate the functional performance of the TPM 3 hearts exhibits a hyperdynamic phenotype. A normal dobutamine response curve is seen in both sets of animals (data not shown).

Table 1. In situ cardiac performance of TPM 3 overexpression (TG) vs. littermate controls (NTG)

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<th>NTG</th>
<th>TG</th>
<th>P Value</th>
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<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>380.4 ± 14.1</td>
<td>434.3 ± 16.7</td>
<td>0.03</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>71.22 ± 1.65</td>
<td>73.5 ± 2.4</td>
<td>0.45</td>
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<tr>
<td>Systolic LVP, mmHg</td>
<td>87.6 ± 1.1</td>
<td>94.1 ± 3.1</td>
<td>0.03</td>
</tr>
<tr>
<td>dP/dtₘₐₓ, mmHg/s</td>
<td>6,914.7 ± 272.6</td>
<td>8,296.2 ± 414.7</td>
<td>0.02</td>
</tr>
<tr>
<td>dP/dtₘᵢₙ, mmHg/s</td>
<td>-7,368.3 ± 367.0</td>
<td>-9,482 ± 292.2</td>
<td>0.001</td>
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Values are means ± SE; n, number of hearts. TG, transgenic; NTG, nontransgenic; HR, heart rate; MAP, mean arterial pressure; LVP, left ventricular pressure; dP/dt, rate of pressure development.

To study the correlation between in vivo results and TPM 3 expression at the sarcomere level, a series of experiments was carried out using skinned fiber preparations at two different SL to obtain the following information: 1) the change in myofilament Ca²⁺ sensitivity, 2) the effect on the myofilament cooperativity as determined by Hill coefficients, 3) the effect on SL-dependent Ca²⁺ activation and interfilament spacing, and 4) tension development.

In the first set of experiments using fiber bundles, we compared the Ca²⁺-force relations obtained from NTG and TPM 3 TG hearts. As illustrated in Fig. 4A, Ca²⁺ sensitivity measured as –log of free [Ca²⁺] required for half-maximum activation (pCa₅₀) at the short SL (1.9 μm) does not show a significant difference (NTG, pCa₅₀ 5.75 ± 0.01, TPM 3 TG, pCa₅₀ 5.68 ± 0.01). However, at relatively long SL (2.3 μm), the pCa₅₀ for NTG is 5.98 ± 0.03, whereas for TPM 3 TG the pCa₅₀ is 5.80 ± 0.02, demonstrating a decrease in Ca²⁺ sensitivity at the long SL (P < 0.05). Interestingly, the pCa₅₀ difference (ΔpCa₅₀ between a SL of 1.9 μm and 2.3 μm) was 0.23 for the NTG group and 0.12 for the TG group (P < 0.05). Thus there was significant attenuation of length-dependent Ca²⁺ activation of the TPM 3 TG fiber bundles compared with controls. The Hill coefficients (n) between the NTG and the TPM 3 TG mice were not significantly different at the short SL of 1.9 μm (NTGₙ = 4.81, TPM 3 TGₙ = 3.68) or at the long SL of 2.3 μm (NTGₙ = 3.91, TPM 3 TGₙ = 4.06). There was no significant difference in the maximum steady-state tension development between control and TPM 3 TG skinned fiber bundles (Fig. 4B). For the NTG fibers at SL 1.9 μm, the maximum tension was 59.79 ± 11.92 mN/mm², and at SL 2.3 μm the tension was 80.60 ± 11.85 mN/mm². In the TPM 3 TG groups, maximum tension at SL 1.9 μm was 61.50 ± 10.93 mN/mm², and at SL 2.3 μm the tension was 72.01 ± 13.96 mN/mm².

To test whether the alteration in length-dependent activation was associated with a change in radial distances between the cross bridges and thin filament, we measured interfilament spacing of the skinned fiber bundles over a wide range of SL by using synchrotron X-ray diffraction. As illustrated in Fig. 5, as SL was increased from 1.8 to 2.4 μm, lattice spacing and thus interfilament spacing decreased as expected. However, the change in lattice spacing with changes in SL was the same for the NTG controls and the TPM3 TG fiber bundles.
RESULTS

Results of experiments reported here provide new and important insights into structure-function relations of TM and their influence on cardiac function. TM has three main striated muscle isoforms: α-TM, β-TM, and TPM 3. We have employed a transgenic approach to determine the functional significance of these three highly homologous isoforms. Our data are the first to demonstrate that TM isoform switching from α-TM to TPM 3 is associated with a decrease in myofilament Ca
2
+ sensitivity and an attenuation of length-dependent activation. Moreover, the change in myofilament activation correlates with hyperdynamic ventricular function. Previous studies indicated that specific thin filament alterations are able to modify length-dependent activation but not in the case of TM isoform switching. We reported that specific and complete switching of slow skeletal troponin I (ssTnI) for cardiac troponin I (cTnI) attenuated length-dependent activation (1). In this case, cardiac myofilaments with ssTnI were significantly more sensitive to Ca
2
+ than controls. Switching of β-TM for α-TM in TG mice also increased myofilament Ca
2
+ sensitivity but had no effect on length-dependent activation (39).

Mechanisms for length-dependent activation that may be modified by switching of TPM 3 for α-TM include alterations in interfilament spacing as well as modulation of the cooperative feedback of strong cross bridges on myofilament activation. As reported in Fig. 5, results from our experiments using X-ray diffraction show that skinned fiber bundles from TPM 3 and control hearts both demonstrate the same change in interfilament spacing as SL is changed. This result indicates that the response of myofilament activation to the change in SL and interfilament spacing must be different in TPM 3 containing myofilaments versus controls.

What are the differences between TPM 3 and α-TM that could account for an alteration in cooperative feedback of strong cross bridges on thin filament activation? The feedback effects of strong cross bridges on myofilament activation appear certain to involve the flexibility of TM as well as its interactions with TnT, actin, and with contiguous TMs along the thin filament. Differences in hydrophobic amino acids between α-TM and TPM 3 may account for the functional effects of isoform switching. There are 26 amino acid differences between TPM 3 and α-TM, of which 15 are highly conservative. Of the remaining 11 amino acid differences, 10 are associated with the gain or loss of a hydrophobic amino acid (Ala
31
Gln, Ser
45
Ala, Glu
135
Leu, Ser
174
Gly, Gly
188
, Ser, Ser
186
Ala, Ala
191
Ser, Thr
199
Asn, Ser
229
Thr, and Ser
252
Thr), whereas just one polar change is found (Lys
29
Gln). Seven of the nonconservative amino acid differences between α-TM and TPM 3 are present after codon 185 in the TPM 3 molecule, a region that influences thin filament cooperativity and TnT binding. Our hypothesis is that these differences in hydrophobic amino acids alter the structure of TM, thereby altering the transition from myofilament “on” to “off” states as well as “off” to “on” states. Interestingly, functional differences between muscle and nonmuscle TM isoforms include modulation of myosin binding to actin. The difference in myosin binding is apparently due to both steric effects as well as induction of changes in actin structure by both TM and myosin binding (37). The importance of hydrophobic residues in this differential functional effect was pointed out by the work of Brown et al. (2), who suggested that the hydrophobic core consisting of seven alanine clusters is important in the flexibility of the TM that is key in its role in regulation. A mutation (V55A) involving this hydrophobic core has been linked to a penetrant form of hypertrophic cardiomyopathy associated with alterations in thin filament cTnC Ca
2
+ binding and thin filament activation.

Amino acid differences between α-TM and TPM 3 may also change the stoichiometry of dimer formation

Table 2. Cardiovascular and contractile parameters of TG and NTG mouse hearts in work-performing heart preparation

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<th>NTG</th>
<th>TG</th>
<th>P Value</th>
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<tbody>
<tr>
<td>n</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>25.94 ± 1.718</td>
<td>26.38 ± 0.9272</td>
<td>0.789</td>
</tr>
<tr>
<td>Heart weight, mg</td>
<td>178 ± 2.339</td>
<td>177 ± 5.128</td>
<td>0.964</td>
</tr>
<tr>
<td>Heart weight/body weight, mg/g</td>
<td>6.787 ± 0.5383</td>
<td>6.721 ± 0.1524</td>
<td>0.899</td>
</tr>
<tr>
<td>Mean aortic pressure, mmHg (after load)</td>
<td>50.35 ± 0.2157</td>
<td>49.85 ± 0.1475</td>
<td>0.088</td>
</tr>
<tr>
<td>Intraventricular pressure, mmHg</td>
<td>98.99 ± 1.883</td>
<td>105.21 ± 3.112</td>
<td>0.148</td>
</tr>
<tr>
<td>Systolic</td>
<td>3,190.6 ± 86.12</td>
<td>3,463 ± 121.02</td>
<td>0.028</td>
</tr>
<tr>
<td>Diastolic</td>
<td>3,930.6 ± 86.12</td>
<td>4,363 ± 121.02</td>
<td>0.028</td>
</tr>
<tr>
<td>End diastolic</td>
<td>3.82 ± 1.0246</td>
<td>5.47 ± 0.8945</td>
<td>0.263</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>375 ± 29.54</td>
<td>399.5 ± 11.43</td>
<td>0.061</td>
</tr>
<tr>
<td>Venous return, ml/min (preload)</td>
<td>5.03 ± 0.025</td>
<td>5.0 ± 0.0291</td>
<td>0.474</td>
</tr>
<tr>
<td>LV minute work, mmHg·ml⁻¹·min⁻¹</td>
<td>252.9 ± 1.592</td>
<td>248.9 ± 1.111</td>
<td>0.071</td>
</tr>
<tr>
<td>+dP/dt, mmHg/s</td>
<td>3,930.6 ± 86.12</td>
<td>4,363 ± 121.02</td>
<td>0.028</td>
</tr>
<tr>
<td>−dP/dt, mmHg/s</td>
<td>−3,207 ± 61.17</td>
<td>−3,969.2 ± 205.39</td>
<td>0.015</td>
</tr>
<tr>
<td>TPP, ms/mmHg</td>
<td>0.420 ± 0.0137</td>
<td>0.379 ± 0.0083</td>
<td>0.031</td>
</tr>
<tr>
<td>RT1/2, ms/mmHg</td>
<td>0.60 ± 0.0193</td>
<td>0.477 ± 0.0239</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of hearts. LV, left ventricular; +dP/dt, maximal rate of pressure development; −dP/dt, maximal rate of relaxation; TPP, time to peak pressure; RT1/2, half-time to relaxation.
and thereby alter myofilament activation by Ca\(^{2+}\). For example, TPM 3 may preferentially form dimers with β-TM in slow-twitch muscle to modulate its effects, but when expressed in the heart, TPM 3 forms dimers with α-TM leading to a unique “hypercontractile” phenotype. Amino acid analysis of the coiled-coil TM structure shows repetitive segments of seven amino acids of which the “first” and “fourth” (a and d positions) are hydrophobic and the “fifth” and “seventh” (e and g positions) are polar (24). Analysis of the α-helical repeat of the coiled-coil structure of TM shows two TPM 3 hydrophobic residues in the “fifth” and “seventh” polar position. These properties may change the stoichiometry of dimer formation. The one polar change observed in TPM 3 (Lys\(^{29}\)Gln) located in a “first” hydrophobic position might destabilize the coiled-coil structure and alter TM flexibility in this portion of the NH\(_2\)-terminus of TM because evidence shows that a determining factor affecting the stability of TM dimers is the hydrophobicity of the residues at the “first”/“fourth” coiled-coil interface (9). Furthermore, it is known that the NH\(_2\)- and COOH-terminal sequences of TM determine its actin affinity and cooperativity (25, 34). The basis for the slight but significant increase in heart rate present in the TPM 3 TG mice is unclear. The role that TM plays in the conduction system is obscure; TPM 3 is a protein that is normally found only in skeletal muscles and may substitute for α-TM in the specialized cells of the conduction system. Heart rate differences could be observed in the TPM 3 mice relative to control groups even with increased concentrations of isoproterenol. It is unclear whether these increased heart rates were a primary or secondary effect of the transgene being expressed in the generalized cardiomyocyte population. Interestingly, alterations in heart rate have been reported with cardiac-specific overexpression of another contractile protein, myosin binding protein C (40).

Surprisingly, the TPM 3 physiological phenotype was opposite of what we anticipated. The TM isoforms are highly homologous at the amino acid level: α-TM to β-TM: 86%; β-TM to TPM 3: 87%; and TPM 3 to α-TM: 91%. β-TM and TPM 3 are predominantly found in slow-twitch muscles, whereas α-TM is more prevalent in all types of striated muscle. From the amino acid homologies and expression profiles, we predicted that TPM 3-overexpressing mice would exhibit a physiological profile similar to β-TM TG mice but less pro-
nounced due to the higher homology with α-TM. However, in the working heart preparations and closed-chest preparations of TPM 3 mice, +dP/dt, and −dP/dt are increased, and TTP and the RT1/2 are decreased. In the β-TM mice, there is a decreased −dP/dt and an increased RT1/2. Also, in the β-TM mice, there are no changes in contraction parameters with the working-heart preparations, but there is a decrease in maximum shortening velocity of isolated myocytes (39). TPM 3 force/pCa analysis shows a rightward shift in the calcium sensitivity (decreased sensitivity) that was directly opposite the results of the β-TM phenotype (which exhibit a leftward shift or increased calcium sensitivity) (29). The opposite results in Ca2+ sensitivity correlate with an increase in −dP/dt. The apparent steeper slope in the pCa-maximum force curve for the TPM 3 at the 2.3-μm sarcomeric length may account for the increased +dP/dt even though there was a decreased sensitivity to Ca2+. To determine whether sarco(endo)plasmic reticulum Ca2+ ATPase and phospholamban contributed to this change in calcium response, we measured their protein levels by Western blot analysis. There are no significant differences in these protein levels between NTG and TG mice (data not shown). Although we cannot rule out that isoform changes in contractile or calcium regulatory proteins occurred in the TG mice, no gross quantitative alterations in the levels of the proteins were detected.

In summary, our data show that compared with controls, hearts of mice expressing TPM 3 demonstrate increased contractile and relaxation parameters, decreased myofilament Ca2+ sensitivity, and an attenuation of myofilament length-dependent Ca2+ activation. These unexpected physiological differences associated with TPM 3 expression may be due to unique properties of this isoform. It is possible that changing the hydrophobicity of amino acid residues alters the secondary structure of the TM, allowing for faster transitions from the “on” state to the “off” state of the myofilament structure, leading to increased basal myocardial function of the TPM 3 TG mice. As seen with α-TM point mutations associated with familial hypertrophic cardiomyopathy and dilated cardiomyopathy, it is possible that a few amino acid differences among the TM isoforms can drastically alter various functions (i.e., cooperativity, steric hindrance, blockage of myosin interactions, TM dimer formation, head-to-tail interactions, and interactions with actin and TnT) (16, 21). An important finding of this investigation is that the TG fiber bundles demonstrate an attenuated length-dependent activation. This result indicates an important role for TM structure relations in the variability of near-neighbor interactions along the thin filament of various muscle types (3). Our result also indicates that the mechanism of length-dependent activation involves structure-function relations of TM in addition to current theories that are based on changes in interfilament spacing (6, 7, 15, 22).

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