Physiological significance of troponin T binding domains in striated muscle tropomyosin

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Jagatheesan, Ganapathy, Sudarsan Rajan, Natalia Petrashevskaya, Arnold Schwartz, Greg Boivin, Grace Arteaga, Pieter P. de Tombe, R. John Solaro, and David F. Wieczorek. Physiological significance of troponin T binding domains in striated muscle tropomyosin. Am J Physiol Heart Circ Physiol 287: H1484–H1494, 2004. First published June 10, 2004; 10.1152/ajpheart.01112.2003.—Striated muscle tropomyosin (TM) plays an essential role in sarcomeric contraction and relaxation through its regulated movement on the thin filament. Previous work in our laboratory established that α- and β-TM isoforms elicit physiological differences in sarcomeric performance. To address the significance of isoform-specific troponin T binding domains in this present work we replaced α-TM amino acids 175–190 and 258–284 with the β-TM regions and expressed this chimeric protein in the hearts of transgenic mice. Hearts that express this chimeric protein exhibit significant decreases in rates of contraction and relaxation when assessed by ex vivo work-performing cardiac analyses. There are increases in time to peak pressure and in half-time to relaxation. These hearts respond appropriately to β-adrenergic stimulation but do not attain control rates of contraction or relaxation. With increased expression of the transgene, 70% of the mice die by 5 mo of age without exhibiting gross pathological changes in the heart. Myofilaments from these mice have no differences in Ca2+ sensitivity of percent maximum force, but there is a decrease in maximum tension development. Our data are the first to demonstrate that the troponin T binding regions of specific TM isoforms can alter sarcomeric performance without changing the Ca2+ sensitivity of the myofilaments.

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age, there is a significant decrease in the maximal rate of relaxation, and this is coupled with an increased RT 1/2. By 4 mo of age, these mice exhibit dramatic changes in rates of both contraction and relaxation, coupled with a decrease in systolic pressure. Although there are no gross morphological or pathological changes in the hearts of 4-mo-old mice, with increased expression of the transgene 70% of the mice die by 5 mo of age. The hearts of the 4-mo-old TG mice respond appropriately to β-adrenergic stimulation, although they do not attain control rates of contraction or relaxation. In addition, whereas there are no differences in the Ca^{2+} sensitivity of force that is achieved by the myofilaments, there is a decrease in maximum developed tension. These results clearly demonstrate for the first time the physiological importance of the α- and β-TM isoform-specific TnT binding domains.

**EXPERIMENTAL PROCEDURES**

All animal procedures were conducted in conformance with the “Guiding Principles for Research Involving Animals and Human Beings” of the American Physiological Society.

**Generation of chimera α-β-TM-2 TG mice.** To generate the α-β-TM-2 TG mouse, we fused portions of the wild-type, muscle-specific, murine α-TM and β-TM cDNAs to make the transgene. These cDNAs were previously described (10, 12). A spliced overlap construct. In the initial step, the wild-type mouse α-TM cDNA was amplified with a forward PCR primer corresponding to amino acids 1–7 with an engineered SalI site: 5′-GAACGCGTCTAGAGCACGATGGACGCCATCAAG-3′. The reverse primer contains 21 nucleotides coding for amino acids 167–174 of α-TM and 48 nucleotides coding for amino acids 175–190 of β-TM: 5′-ACATTTACTCTCCAGCCACGCAGGCTCGCTCTCAGCCACCACGCTGCTGACGCT-3′. The size of this amplified fragment is 0.58 kb.

The second step for generation of the transgene used the chimera α-β-TM-1 cDNA as the template (6). This cDNA contains amino acids 1–257 of α-TM ligated to amino acids 258–284 and the 3′-untranslated region (UTR) of β-TM. The SV40 polyadenylation and termination region is fused 3′ of the β-TM sequences. The forward primer contains 48 nucleotides corresponding to codons 175–190 of β-TM and 21 nucleotides coding for amino acids 191–197 of α-TM: 5′-GAGCTGAGCCTGGAAGAGAGACCCAGAGGTGGCTGAGAGGAAATGGCCGAGTCCGGCTCTTCTCTTCTCTGACCAGTCCAGCAGCTT-3′. The reverse primer contains pBS KS+ plasmid sequence encompassing HindIII, NotI, and SacI sites immediately following SV40 termination sequences: 5′-TTGGAAGCTTACCCGCGGTGCGCCGCGCTCCT-3′. The size of this amplified fragment is 0.88 kb.

The third step for generation of the transgene used the two amplified fragments from steps 1 and 2 as a template, with an overlapping region encoding β-TM codons 175–190. This template encodes α-TM amino acids 1–174, β-TM amino acids 175–190, α-TM amino acids 191–257, and β-TM amino acids 258–284 + 3′-UTR sequence, followed by SV40 polyadenylation and termination sequences. The forward primer for this PCR amplification is 5′-GACGCGGTCTAGAAGCAGGACGCTGGAAGAGAGACCCAGAGGTGGCTGAGAGGAAATGGCCGAGTCCGGCTCTTCTCTTCTCTGACCAGTCCAGCAGCTT-3′. The reverse primer is 5′-TTGGAAGCTTACCCGCGGTGCGCCGCGCTCCT-3′. This size of this amplified fragment is 1.34 kb. Nucleotide sequencing and comparison with the published sequences verified the amplified sequences.

The chimeric α-β-TM-2 cDNA was ligated at the 5′ end to the 5.5-kb murine cardiac-specific α-myosin heavy chain (α-MHC) promoter at the SalI site and cloned into pBS KS+ vector. The resultant 6.8-kb DNA construct was endonuclease restricted with KpnI and HindIII to release the transgene, purified, and microinjected into the male pronuclei of fertilized ova to generate transgenic mice as described previously (10). TG mice were produced from FVB/N strain mice. PCR was used to amplify DNA isolated from ear tissue and identify mice carrying the transgene. Founder mice were identified by use of 5′ and 3′ primers corresponding to the α-MHC 5′-UTR and α-TM, respectively. 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 enzyme-digested DNA was carried out on 0.8% agarose gels and transferred to nitrocellulose membranes. The Southern blots were probed with a 32P-radiolabeled β-TM 3′-UTR fragment corresponding to the 3′ end of the transgene. Quantification of the transgene was done on an Imagequant PhosphorImager (Molecular Dynamics; Sunnyvale, CA).

**RNA and protein analyses.** RNA was isolated from murine hearts with TriReagent (Molecular Research Center, Cincinnati, OH). Total RNA (10 μg) from TG and NTG mouse ventricles was electrophoresed on 1% formaldehyde gels and transferred to nitrocellulose membranes. Hybridization was conducted with 32P-radiolabeled cDNA fragments from the α-TM 3′-UTR, the β-TM 3′-UTR, and GAPDH. TM message levels were analyzed on the Imagequant PhosphorImager and normalized to GAPDH expression levels. Levels of nascent alpha- and beta-tropomyosin were measured as described previously (10). Equal amounts of protein (25 μg) were run simultaneously on two 10% SDS-PAGE gels. One gel was stained with Coomassie blue to ensure equal loading, and proteins from the other gel were transferred to nitrocellulose for Western blot analyses. Filters were probed with a striated muscle TM-specific CH1 antibody (Sigma; St. Louis, MO) at a 1:5,000 dilution for 2 h at room temperature. After washing was complete, an anti-mouse IgG antibody conjugated with peroxidase was incubated with the blot for 1 h at a dilution of 1:5,000. The blots were developed with an ECL kit (Amersham).

Two-dimensional gel electrophoresis was performed on myofibrillar protein preparations from NTG and TG hearts according to the method of O’Farrell (13). In brief, isoelectric focusing was carried out with 25 μg of the protein sample denatured in 300 μl of rehydration buffer [8 M urea, 2 mM tributyolphosphine, 2% (3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and 0.2% Bio-Lytes (4.7–5.9) (Bio-Rad)]. Each sample was used to hydrate a 17-cm ReadyStrip pH 4.7 (Bio-Rad) for 12 h. Isoelectric focusing was performed in three stages of applied potential difference: 250 V for 15 min, 10,000 V for 1 h, followed by 8,000 V for up to 6 h, until 40,000 V were achieved. Focused strips were then soaked in equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, and 20% glycerol) containing 130 mM DTT for 10 min followed by equilibration buffer containing 135 mM iodoacetamide for another 10 min. Strips were then applied to 10% acrylamide gels for SDS-PAGE, followed by transblotting onto polyvinylidene difluoride membranes. Western blot analysis with the striated muscle TM-specific CH1 antibody (Sigma) was conducted at a 1:5,000 dilution. Binding of the primary antibody was detected by peroxidase-conjugated secondary antibody (1:5,000) and enhanced by chemiluminescence (Amersham).

**Histological analysis.** Hearts were removed from 2- to 6.5-mo-old mice and immediately fixed in 10% neutral buffered formalin for 48 h. The hearts were dehydrated through a gradient of alcohols and xylene, followed by embedding in paraffin. Sections were cut at 5 μm thickness and stained with hematoxylin-eosin.

**Isolated anterograde perfused heart preparation.** Control and TG age-matched mice of either sex were anesthetized with 100 mg/kg sodium nembutal and injected with 1.5 units of heparin to prevent intracardiac blood coagulation. Hearts were removed via midsternal incision, immediately suspended on a 20-gauge steel cannula, and connected to the perfusion apparatus (6). Retrograde perfusion with modified Krebs-Henseleit buffer (in mM: 118 NaCl, 2.5 CaCl2, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 0.5 Na3EDTA, 25 NaHCO3, and 11
glucose) was started to clear the coronary vasculature, and a PE-50 catheter was inserted intraventricularly for pressure measurements. The perfusion fluid, oxygenation, and temperature were constant throughout the experiment.

After a short period of stabilization on retrograde perfusion, the pulmonary vein was cannulated and perfusion of the heart was switched from retrograde to anterograde. Anterograde work-performing perfusion was initiated at a workload of 250 mmHg ml⁻¹ min⁻¹, which was achieved with a venous return of 5 ml/min and an aortic pressure of 50 mmHg. The β-adrenoagonist isoproterenol (Sigma) was added to the Krebs-Henseleit solution entering the heart with a pressure of 50 mmHg. The signals were digitized and analyzed by Biobench software. Heart rate, left ventricular pressure (LVP), and mean coronary perfusion pressure were continuously monitored. The pressure curve was used to calculate the rate of pressure development (+dP/dt) and decline (−dP/dt), time to peak pressure (TPP), and RT1/2. TPP and RT1/2 were normalized with respect to peak LVP because they are dependent on the extent of pressure development. Pressure loading was carried out in working heart preparations after stabilization of the heart at basal loading conditions. Preload (venous return) was kept constant at 5 ml/min, and maximum afterload (aortic resistance) values were generated at this set preload. Aortic pressure was decreased with a custom-mode micrometer, and +dP/dt and −dP/dt were measured at different cardiac afterloads. Cardiac work at different workloads was calculated and expressed as milligrams of mercury times milliliter per minute. Frank-Starling curves were generated by linear regression analysis. For the regression analysis, the y-intercepts and slopes were calculated by Prism (GraphPad software, version 2.0). Isometric tension was plotted as a function of the pCa (−log[Ca();++]). The Hill equation was fitted to the Hill equation as described previously (23) by applying nonlinear least-squares regression analysis with Prism software (GraphPad version 2.0). Isometric tensions measured at submaximally activating [Ca+++] were expressed as a percentage of the maximum tension. Half-maximally activating [Ca+++] (pCa0) were determined from individual Hill fits of each pCa-tension relation and then averaged. Data for which measurements were made in the same fiber bundle at different sarcomere lengths were analyzed individually with Student’s paired t-test. When three or more groups were analyzed, a one-way repeated-measures ANOVA was used with a Newman-Keuls multiple-comparison test. The criterion for statistical significance was set at P < 0.05. Experimental values are expressed as means ± SE.

RESULTS

Generation of chimera α-β-TM-2 TG mice. To determine the relationship between TM isoform-specific TnT binding regions and contractile behavior in striated muscle, we developed a TG mouse model to overexpress a chimeric α-β-TM protein. The chimeric cDNA (α-β-TM-2) encoded the following amino acids: α-TM 1–174, β-TM 175–190, α-TM 191–257, and β-TM 258–284 + 3’-UTR. The transgenic construct was generated by ligating the murine TM sequences into an expression vector regulated by the murine α-MHC promoter, which directs cardiac-specific expression (Fig. 1). A SV40 polyadenylation and termination cassette was located 3’ of the TM cDNA to ensure correct transcript processing. Founder mice were identified by PCR and confirmed by Southern blot analysis. Four lines of TG mice were produced, and quantification demonstrated that copy numbers of the transgene range from two to eight copies in the four mouse lines: line 74, four copies; line 78, two copies; line 95, two copies; line 97, eight copies.

Chimera TM transcript and protein expression in TG mice. We measured TM expression of Northern blot analysis after RNA isolation from hearts of TG and NTG littermates. RNA from each TG line was isolated and probed with 32P-radiolabeled 3’-UTR sequences of α-TM and β-TM cDNAs; these nucleotide regions are specific to the particular striated muscle isoforms and do not cross-react with other striated muscle isoforms (17). As seen in Fig. 2A, endogenous α-TM is expressed in all hearts and in the skeletal muscle control. When β-TM 3’-UTR sequence is used as a probe, the expression of chimeric α-β-TM-2 is restricted to TG hearts; this probe also hybridizes to control skeletal muscle RNA, but it is not detected in NTG cardiac RNA. For quantification, TM levels are normalized against GAPDH expression in the hearts to account for any loading differences. A PhosphoImager quantitative analysis shows that the percentage of α-β-TM-2 RNA to endogenous α-TM RNA is as follows: 64 ± 2.6 vs. 36 ± 2.6 for line 74, 38 ± 4.6 vs. 62 ± 4.6 for

![Fig. 1. Chimera α-β-TM-2 construct. Chimera α-β-TM-2 tropomyosin (TM) cDNA coding for amino acids (aa) 1–190 of α-TM, 175–190 of β-TM, 191–257 of α-TM, and 258–284 and the 3’- untranslated region (UTR) of β-TM was PCR synthesized with the spliced overlap extension method (see EXPERIMENTAL PROCEDURES). This chimera TM cDNA was linked to the α-myosin heavy chain (MHC) promoter at the 5’ end and to the SV40 polyadenylation/termination cassette at the 3’ end.](http://ajpheart.physiology.org/)

http://ajpheart.physiology.org/ by 10.220.32.246 on June 10, 2017
Western blots showed no differences between TG and NTG hearts.

Because the SDS-PAGE and Western blot analyses are unable to discriminate between the endogenous α-TM and the exogenous chimeric α-β-TM-2 proteins, we used two-dimensional gel electrophoresis followed by Western blot analysis to resolve the different TM species. Under reduced-reduced electrophoretic conditions, cardiac myofibrillar proteins from NTG mice display a single spot, labeled α-TM, that is reactive with the TM-specific antibody (Fig. 4). This result is in agreement with previous studies on TM composition in cardiac musculature, which show that α-TM is the predominant TM isoform in the heart (11). In TG mice, the results demonstrate that both endogenous α-TM and chimeric α-β-TM-2 protein isoforms are present in the cardiac musculature. This result is in agreement with the previous finding that both of the associated mRNA species are produced in the TG hearts. A PhosphorImager quantitative analysis shows that the percentage of α-β-TM-2 protein to endogenous α-TM protein is as follows: 34.6 ± 1.8% for line 74, 26.4 ± 3.5% vs. 73.6 ± 3.3% for line 95, and 39.4 ± 2.8% vs. 60.6 ± 2.8% for line 97 (Fig. 4B).

To negate the possibility that an unexpected mutation or deletion of the α-β-TM-2 DNA sequences occurred during transgenesis, we conducted PCR analysis of TG mouse tail DNA. With oligonucleotide primers corresponding to the α-MHC and β-TM 3′-UTR sequences, a PCR fragment was

Fig. 2. A: RNA expression in transgenic (TG) mice. Ten micrograms of total RNA (pooled from 5 mice from each line) were electrophoresed, transferred to nitrocellulose membrane, and probed with 32P-radiolabeled β-TM 3′-UTR, α-TM 3′-UTR, or GAPDH. B: graphic representation of the levels of chimera α-β-TM-2 and endogenous α-TM message in various lines of TG mice. Levels from each TG line were compared with the nontransgenic (NTG) level, which was set at 100%, and limb skeletal muscle (SKM). Three sets of experiments were averaged and normalized against GAPDH expression.

line 78, 41 ± 5.2 vs. 59 ± 5.2 for line 95, and 60 ± 4.5 vs. 40 ± 4.5 for line 97 (Fig. 2B). The varied expression of the chimeric mRNA correlates with the copy number of the transgene in the different lines.

To examine the production of exogenous chimeric α-β-TM-2 protein in the hearts of TG mice, we isolated and compared myofibrillar protein fractions from TG and NTG hearts. These proteins were run on 10% SDS-polyacrylamide gels and either stained with Coomassie Blue or subjected to Western blot analysis. Coomassie Blue staining of myofibrillar proteins showed that equal amounts of TM are present in all TG and NTG lanes (Fig. 3A); myofibrillar preparations from the hearts of β-TM TG mice have increased levels of β-TM and a concomitant decrease in α-TM levels. Proteins in the skeletal muscle serve as a control for α- and β-TM. These results also show that there are no gross differences in the expression of other cardiac contractile proteins between the TG and NTG mice. Under these conditions, endogenous α-TM and the chimeric α-β-TM-2 isoforms are not separated electrophoretically and their isoforms cannot be distinguished. To enhance the sensitivity of the assay, we conducted a Western blot analysis with a striated muscle TM-specific antibody (Fig. 3B). This antibody (CH1) recognizes TM striated muscle isoforms with equal affinity (data not shown). Results from the Western blot analysis, however, detected only a single band in the TG myofibrillar protein samples even though the chimeric α-β-TM-2 protein has one additional negative charge associated with its structure. Quantification of total TM protein levels from the Coomassie Blue-stained SDS-PAGE gels and the
generated, amplified, cloned, and sequenced. The results show there are no mutations or deletions in the α/β-TM-2 nucleotide sequence (or the corresponding amino acid sequence) in the integrated transgene construct.

**Histological analysis.** To determine whether histological or pathological alterations occurred after expression of the TM transgene, we examined over 15 TG mice from three distinct lines. Mice ranged in age from 2 to 7 mo. Hearts from TG and NTG controls were isolated, weighed, sectioned, and stained with hematoxylin and eosin. Results show that at 3 mo of age there are no gross phenotypic alterations in any of the TG lines. There are no differences in the percentage heart weight-to-body weight ratios (mg/g × 100) in any of the TG lines. Interestingly, mice that express the highest level of chimeric α/β-TM-2 protein (line 97) start to die by 3 mo (Fig. 5). By 5 mo, 70% of these mice are dead. TG mice producing lower chimeric protein levels (lines 78 and 95) also show an increased mortality but not as dramatic as line 97 mice. We are currently investigating why line 74, which exhibits a large amount of chimeric protein, does not have an increased level of mortality. Hearts from the line 97 TG mice do not show an increased percentage heart-to-body weight ratio or any signs of concentric or dilated cardiac hypertrophy. Surprisingly, histological analyses of these TG hearts at 4.5 mo did not demonstrate any gross pathological changes. Histopathological analyses of these mice do not demonstrate signs of classic cardiac failure, such as pleural effusions or peripheral edema. Also, an examination of the cardiac conduction system by electrocardiographic analysis did not show alterations in any of the four lines (data not shown). The sudden death of the α/β-TM-2 chimera mice without any overt pathology or gross hypertrophy is reminiscent of patients with familial hypertrophic cardiomyopathy (FHC)-encoding TnT mutations (20).

**Physiological analyses of α/β-TM-2 chimera TG mice.** To obtain a better understanding of the physiological contribution of the α/β-TM-2 protein to sarcomeric function, we conducted analyses of cardiac performance utilizing isolated working-heart preparations and skinned fiber bundles. TG animals from line 97 were studied, with confirmation of the results in mice from lines 74, 78, and 95. Previous studies demonstrated that overexpression of wild-type α-TM in TG mice does not lead to alterations in morphology or physiological performance of the heart (18, 23). Also, overexpression of the striated muscle β-TM isoform in the hearts leads to a decrease in the rate of relaxation and an increase in RT1/2 (10), in part because of an increase in the myofilament sensitivity to Ca2+. In recent work, we showed (6) that expression of a chimeric TM protein (α/β-TM-1) that contains the COOH-terminal β-TM TnT binding domain (amino acids 1–257 from α-TM and amino acids 258–284 from β-TM) results in decreases in the rates of both contraction and relaxation. In the current study, we exchanged both TnT binding regions (amino acids 175–190 and 258–284) in the α-TM protein for their counterparts in the β-TM molecule to ascertain the importance of these regions in sarcomeric function.

We used an isolated anterograde-perfused heart preparation to ascertain a functional analysis of the chimeric α/β-TM-2 hearts at both 3 and 4 mo of age. By 3 mo of age, the TG hearts showed a significant decrease in the maximum rate of relaxation (−dP/dt) from 3,369 ± 115 mmHg/s in the wild-type hearts to 2,649 ± 235 mmHg/s in the TG hearts (P < 0.05); there is also a concomitant increase in RT1/2 [0.52 ± 0.033 vs. 0.74 ± 0.064 ms/mmHg for NTG vs. TG, (P < 0.05)] (Table 4).
Table 1. Cardiovascular and contractile parameters of 3- and 4-mo-old α/β-TM-2 TG and NTG hearts in isolated heart preparations

<table>
<thead>
<tr>
<th>Parameters</th>
<th>3 Mo Old</th>
<th>4 Mo Old</th>
<th>P value</th>
<th>P value</th>
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<tr>
<td></td>
<td>NTG (n = 5)</td>
<td>TG (n = 6)</td>
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<td></td>
<td>4 Mo Old</td>
<td>4 Mo Old</td>
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<tr>
<td>Systolic pressure, mmHg</td>
<td>96.9 ± 4.3</td>
<td>94.7 ± 3.96</td>
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<td>Diastolic pressure, mmHg</td>
<td>-12.2 ± 2.55</td>
<td>-14.02 ± 2.7</td>
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<td>-14.4 ± 1.4</td>
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<td>-9.8 ± 1.6</td>
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<td>End-diastolic pressure, mmHg</td>
<td>4.7 ± 1.86</td>
<td>5.1 ± 1.23</td>
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<td>6.9 ± 3.5</td>
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<td>6.1 ± 3.6</td>
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<td>Maximal +dP/dt, mmHg/s</td>
<td>3.864 ± 0.114</td>
<td>3.635 ± 238</td>
<td>0.44</td>
<td>4.014 ± 179</td>
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<td>2.568 ± 204</td>
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<tr>
<td>Maximal -dP/dt, mmHg/s</td>
<td>3.369 ± 115</td>
<td>2.649 ± 235*</td>
<td>0.03</td>
<td>3.528 ± 168</td>
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<td>2.180 ± 184</td>
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<tr>
<td>Heart rate, beats/min</td>
<td>314 ± 21</td>
<td>340 ± 19</td>
<td>0.35</td>
<td>309 ± 17</td>
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<td>328 ± 21</td>
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<tr>
<td>TTP, ms/mmHg</td>
<td>0.43 ± 0.016</td>
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<td>0.74 ± 0.11</td>
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<td></td>
<td>0.52 ± 0.033</td>
<td>0.74 ± 0.064*</td>
<td>0.018</td>
<td>0.54 ± 0.02</td>
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<td>0.85 ± 0.06</td>
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Values are means ± SE; n, no. of mice. TG, transgenic; NTG, nontransgenic; +dP/dt, rate of pressure development; -dP/dt, rate of relaxation; TTP, time to peak pressure; RT1/2, time to half-relaxation. *P < 0.05; †P < 0.01; ‡P < 0.001, NTG vs. TG.

1. Fig. 6). However, by 4 mo of age, the TG hearts exhibited severe alterations in both baseline systolic and diastolic cardiac function, compared with wild-type control mice. Under identical conditions of workload, the TG hearts produced systolic pressures that were significantly lower than the control hearts [111.9 ± 4.8 mmHg in NTG hearts compared with 77.5 ± 5.5 mmHg in TG hearts (P < 0.001); Table 1]. The maximal rate of pressure development for contraction (+dP/dt) was significantly decreased [4,014 ± 179 mmHg/s in controls vs. 2,568 ± 204 mmHg/s in TG hearts (P < 0.001); Table 1, Fig. 6]. Coupled with this decrease in contractility, there was also a decrease in −dP/dt [3,528 ± 168 mmHg/s vs. 2,180 ± 184 mmHg/s in NTG vs. TG, respectively (P < 0.001)].

Other parameters of cardiac function (TTP and RT1/2) were derived from intraventricular pressure tracings and normalized to peak pressure and half-relaxation pressure, respectively. As shown in Table 1, the TTP of the chimeric mouse was significantly longer than that of control mice [0.43 ± 0.02 vs. 0.74 ± 0.11 ms/mmHg for NTG vs. TG (P < 0.001)]. Also, RT1/2 was longer in the TG hearts [0.54 ± 0.02 vs. 0.85 ± 0.06 ms/mmHg for NTG vs. TG (P < 0.001)].

The Frank-Starling relationship, reflecting dependence between increased minute work and the rate of pressure development and decline were examined. The NTG and TG hearts revealed a positive correlation between workload and functional parameters of cardiac performance (Fig. 7). At age 3 mo, the y-intercepts for −dP/dt were significantly different between NTG and TG hearts, indicating that the TG myocardium worked at a lower lusitropic (“diastolic”) state. The contractile reserve of the TG hearts diminished between 3 and 4 mo. At 4 mo of age, the y-intercepts of the TG hearts for +dP/dt and −dP/dt were significantly different, indicating decreased contractile and lusitropic properties. The slopes of the regression curves at both ages were not different, indicating that the maximal −dP/dt and +dP/dt were sensitive to length-dependent regulation to an extent similar to that in the NTG hearts.
Frank-Starling left ventricular function curves (+dP/dt and −dP/dt vs. cardiac work) in wild-type and TG mouse hearts at 3 and 4 mo are shown. Hearts were examined under constant preload (venous return 5 ml/min) and at various afterloads. The relations between increases in +dP/dt and −dP/dt and cardiac work are given by regression lines. At 3 mo: y = 2,513 (±168) + 4.6 (±0.8)x (+dP/dt, NTG heart), y = 2,143 (±86) + 4.9 (±0.43)x (+dP/dt, TG heart); y = 2,265 (±1.8) + 3.4 (±0.54)x (−dP/dt, NTG heart), y = 1,424 (±133) + 3.5 (±0.67)x (−dP/dt, TG heart). At 4 mo: y = 2,347 (±247) + 5.6 (±0.71)x (+dP/dt, NTG heart), y = 1,499 (±112) + 4.1 (±0.56)x (+dP/dt, TG heart); y = 2,603 (±112) + 2.4 (±1.56)x (−dP/dt, NTG heart), y = 1,493 (±105) + 2.5 (±0.53)x (−dP/dt, TG heart), n = 4 for 3-mo age group for both NTG and TG mice; n = 6 for 4-mo age group for both NTG and TG mice.

Response to β-adrenergic stimulation. Because there was a significant decrease in the rates of both contraction and relaxation, we checked the isoproterenol response of these TG hearts to see whether this may be due to blunting of the β-adrenergic response. The reduced contractile and lusitropic performance by α/β-TM-2 TG hearts was assessed during stimulation with isoproterenol, a β-adrenergic agonist that augments muscle contraction and relaxation by cAMP/PKA-dependent phosphorylation by increasing the rate of Ca²⁺ cycling (Fig. 8). The 3-mo-old TG hearts responded to increased concentrations of isoproterenol by attaining control rates of contraction and relaxation. Although the 4-mo-old TG hearts responded to the β-adrenergic agonist appropriately, they were unable to reach control levels of contraction or relaxation. Thus, in the isolated heart preparations, the response of both contractile (+dP/dt, TPP) and relaxation (−dP/dt, RT½) parameters was similar in the TG and NTG hearts even though the functional differences remained after isoproterenol administration at multiple concentrations. These results show that these TG hearts respond appropriately to isoproterenol but maximum response is still below the NTG levels, indicating that the defect is not due to an altered adrenergic response mechanism.

Ca²⁺-force measurements in skinned fiber bundles. Studies were implemented to examine the correlation between the physiological results from the whole heart and chimera α/β-TM-2 (TG lines 97 and 74) left ventricular fiber bundles of 4-mo-old mice. Measurements were made at both short and long sarcomeric lengths. In the first set of experiments, we compared the pCa-percent maximum force relations obtained from control α-TM versus α/β-TM-2 (TG lines 97 and 74) left ventricular fiber bundles for a specific sarcomere length: at 1.9 μm, the pCa 50 is 5.63 ± 0.02 for NTG (n = 10). At 2.3 μm, the pCa 50 is 5.74 ± 0.02 for TG and 5.74 ± 0.01 for NTG (n = 10). At 1.9 μm, the Hill coefficients of the Hill equation were similar for both TG and NTG hearts, with Hill coefficients of 2.4 (±0.2) for NTG and 2.3 (±0.1) for TG. At 2.3 μm, the Hill coefficients of the Hill equation were similar for both TG and NTG hearts, with Hill coefficients of 2.5 (±0.1) for NTG and 2.4 (±0.1) for TG.
coefficient (nH) value of the chimeric TM (2.7 ± 0.1) was similar to the control value (2.6 ± 0.1); at 2.3 μm also, the nH value of the chimeric TM (3.1 ± 0.3) was similar to the NTG value (2.9 ± 0.2), indicating that the measure of cooperativity is similar between TG and NTG myofilaments. Also, cross-bridge-dependent myofilament activation, as determined from measurements of the relation between MgATP concentration and tension at pCa 9.0, is not altered by the chimeric protein (data not shown).

Interestingly, the results show that the maximum tension developed in myofilaments containing β-TM-2 is significantly depressed [28.0 ± 0.4 vs. 33.6 ± 0.5 mN/mm² for TG vs. NTG (n = 7); Fig. 9B].

**DISCUSSION**

The work presented here addresses the significance of TnT binding regions in different striated muscle TM isoforms. More specifically, we replaced the α-TM regions (amino acids 175–190 and amino acids 258–284) with the corresponding β-TM regions to ascertain whether this switch causes the α/β-TM-2 chimeric molecule to function similarly to either wild-type α- or β-TM proteins. The results of the experiments demonstrate that simultaneously switching both TnT binding domains between the α- and β-TM proteins causes decreases in myofilament tension and in the rates of both myocardial contraction and relaxation, without dramatically altering the myofilament sensitivity to Ca²⁺.

By combining results from this current work with those from our previous studies (6, 10, 14), we can summarize how α- and β-TM isoforms and their respective TnT binding regions determine maximum tension generation in myofilaments as a function of [Ca²⁺] (Fig. 10). Maximum tension falls to the same extent when pCa-tension relations of preparations containing endogenous α-TM are compared with those containing the full-length β-TM (14). This decrease in tension also occurs with the α/β-TM-1 chimera in which β-TM residues 258–284 replace those in α-TM (6) or an α/β-TM-2 chimera in which β-TM residues 175–190 and 258–284 replace those in α-TM. However, there are also large changes in myofilament sensitivity to Ca²⁺. With respect to myofilaments containing α-TM, the switch to β-TM increases Ca²⁺ sensitivity, the switch to α/β-TM-1 depresses Ca²⁺ sensitivity, and the switch to α/β-TM-2 has no effect on Ca²⁺ sensitivity. However, the α/β-TM-2-containing myofilaments do exhibit dramatically increased Ca²⁺ sensitivity compared with the α/β-TM-1 myofilaments. We have discussed previously the potential mechanisms that may account for the differences between alterations induced by the presence of β-TM (10, 14, 23) and the α/β-TM-1 chimera (6) compared with α-TM. One conclusion from
the present data is that β-TM residues 175–190 and 258–284 together cannot fully account for the increase in myofilament Ca$^{2+}$ sensitivity that occurs with isoform switching of the α-β-TM-1 chimeras. This charge change may be a key determinant in the regulation of Ca$^{2+}$ sensitivity and force production. In vitro studies have demonstrated that single amino acid substitutions in the COOH-terminal region at Ser283 with Ala, Lys, or Glu altered the binding of TM both to actin and to itself in end-to-end interactions (19). Natural differences in COOH-terminal amino acids, which occur in different isoforms of TM, also demonstrate differences in the strength of end-to-end interactions and determine the size of the cooperative unit [i.e., the number of actins controlled by a Tn-TM complex (7)]. Also, a recent study shows that exchanging the two charged amino acid residues that differ between α- and β-TM results in a decrease in myofilament Ca$^{2+}$ sensitivity without affecting tension development (3). Studies with recombinant proteins with alterations in the COOH terminus also indicate a correlation with actin affinity as well as myosin S-1-induced cooperative binding of TM to actin (4, 9).

Thus the mechanism for the decreased Ca$^{2+}$ sensitivity of α-β-TM-1 myofilaments compared with control myofilaments could involve a decrease in the strength of end-to-end TM-actin interactions as well as a decrease in TM binding to actins reacting with the myosin head in the force-generating state.

Compared with α-TM, myofilaments containing α-β-TM-1 exhibit a desensitized pCa-force relationship whereas α-β-TM-2 demonstrate a normal pCa-force relationship; the amino acids in the 175–190 region of β-TM must account for this difference. The amino acid 175–190 region of β-TM substantially increased myofilament sensitivity to Ca$^{2+}$ with respect to the α-β-TM-1 chimeric myofilaments. There are five amino acid differences between α- and β-TM in the 175–190 region: Asp175Glu, Ala179Ser, Leu185Val, Ser186Ala, and Gly188Ser. There are no significant differences in the charge or size of the amino acid side groups; however, there is a dramatic decrease in the hydrophobicity (a calculated −3.1 kcal/mol in this region). We suggest that these differences between the α- and β-TM in amino acid hydrophobicity, size, and charge affect the binding of TnT to TM, altering the ability of TnT to properly regulate TM movement during thin filament activation. The return to α-TM control levels in the pCa-force measurements with myofilaments containing chimera α-β-TM-2 protein where both TnT binding regions have been switched suggests that the two regions function synergistically to regulate Ca$^{2+}$

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**Fig. 9.** pCa-tension relations of skinned fiber bundle preparations from NTG control and chimeric α-β-TM-2 TG mouse hearts at sarcomere lengths of 1.9 and 2.3 μm. A: pCa-force relations of NTG and chimeric α-β-TM-2 TG skinned fiber bundles at sarcomere lengths of 1.9 and 2.3 μm. Data are normalized to % maximum force. The −log of free Ca$^{2+}$ concentration required for half-maximal activation (pCaso) in myofilaments at 1.9-μm sarcomere length are 5.63 ± 0.01 and 5.58 ± 0.01 for TG and NTG, respectively. The pCaso for 2.3-μm sarcomere length is 5.74 ± 0.02 and 5.74 ± 0.01 for TG and NTG, respectively. Neither of these values is significant at P < 0.05 (n = 10). B: Ca$^{2+}$-tension measurements in skinned fiber bundle preparations in NTG and α-β-TM-2 TG mouse hearts. Maximum developed tension is depressed in the TG vs. NTG myofilaments (28.0 ± 0.4 vs. 33.6 ± 0.5 mN/mm$^2$, respectively); measurements were conducted at a 2.3-μm sarcomere length (P < 0.05; n = 7).

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**Fig. 10.** pCa-tension relations in skinned fiber bundle preparations for TM TG hearts. The various pCa-% maximum tension graphs were derived from distinct TG models: α-TM, NTG control; β-TM, overexpression of the complete β-TM isoforms (10, 14, 23); chimera 1, overexpression of the chimeric α-β-TM-1 TM protein encoding amino acids 1–257 of α-TM ligated to amino acids 258–284 of β-TM (6); chimera 2 (α-β-TM-2), TG model described in the current work. The data are normalized to % maximum tension at 2.3-μm sarcomere length, with maximum tension of the α-TM NTG control taken as 100%.
sensitivity, with the amino acid 175–190 region associated with increases in Ca$^{2+}$ sensitivity. This notion is supported by two facts: 1) FH mutations in this region (Asp175Asn and Glu180Gly) lead to increased sensitivity in the pCa-force measurements (1, 8, 12, 18); and 2) inclusion of the β-TM 175–190 amino acids in the α-/β-TM-1 construct increased the Ca$^{2+}$ sensitivity of the pCa-force measurements. Because the inclusion of the β-TM 175–190 region did not cause the pCa-force measurements to attain the Ca$^{2+}$ responsiveness of myofilaments containing the entire β-TM molecule, additional TM regions probably play a role in the determination of myofilament Ca$^{2+}$ sensitivity.

Our data showing that maximum tension was similarly depressed by both α-/β-TM-1 and α-/β-TM-2 myofilaments compared with α-TM controls indicates that the amino acid substitutions in the regions of TM comprising amino acids 258–284 are critical in this process, whereas the 175–190 region may not differentially affect interactions between TnT and TM with respect to tension. This hypothesis is further supported by the fact that myofilaments containing whole β-TM also display decreases in maximum tension development. Ongoing studies in which the internal TnT binding sites of TM are exchanged between α- and β-TM are directly addressing this hypothesis. Replacement of native TnT in cardiac myofilaments with a mutant truncated at the NH$_2$ terminus results in a depression in maximum tension with no change in cooperativity or Ca$^{2+}$ sensitivity (2). Using biochemical, biophysical, and structural approaches, Tobacman et al. (21) reported that the TnT tail domain consisting of amino acids 1–153 is able to suppress the actin-myosin interaction as well as determine the position of TM away from the groove formed by the actin helix. This study made the important observation that the blocking of the actin-myosin reaction by TM involves both TnI as well as TM. Because our data do not indicate that the interaction of the TM 175–190 region is critical for the regulation of tension, adjacent TM regions may play an essential role in this process.

An important question is whether the alterations in tension development or Ca$^{2+}$ sensitivity observed at the level of the myofilaments in hearts expressing β-TM (10, 14), α-/β-TM-1 (6), or α-/β-TM-2 correlate with the functional changes measured at the level of the whole heart, compared with α-TM control hearts. Previous studies comparing myofilament response to Ca$^{2+}$ and cardiac dynamics suggest that enhanced Ca$^{2+}$ sensitivity may be correlated with a slowing of relaxation and decreased Ca$^{2+}$ sensitivity correlated with an increased relaxation rate (10, 12, 14, 16, 18). Comparison of $-dP/dt$ between hearts expressing α-TM vs. β-TM or FHC α-TM175 or FHC α-TM180 fit with this idea, but there is also a slowing of relaxation in the hearts expressing α-/β-TM-1, which demon- strate a decrease in myofilament Ca$^{2+}$ sensitivity compared with those expressing α-TM, and in hearts expressing α-/β-TM-2, which have the same Ca$^{2+}$ sensitivity as α-TM myofilaments. This lack of correlation between Ca$^{2+}$ sensitivity and relaxation performance would indicate that in vivo and ex vivo cardiac function is more complex than a direct sarcomeric performance extrapolation.

In summary, our data indicate that amino acids 258–284 of TM comprise a region specialized for isoform-specific effects on maximum tension. This result supports the hypothesis that altered interactions of TM with TnT as well as altered near-neighbor end-to-end interactions of TM affect tension as well as cooperative interactions along the thin filament. On the other hand, amino acids 175–190, which also interact with TnT together with amino acids 258–284, cannot fully account for altered Ca$^{2+}$ sensitivity between α- and β-TM isoforms. This result fits with the complexity of the thin filament protein-protein interactions that determine the net Ca$^{2+}$ sensitivity of the myofilaments. We speculate that TM regions adjacent to amino acids 175–190 may interact with the TnT tail to regulate myofilament Ca$^{2+}$ sensitivity.

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