Tropomyosin Dephosphorylation Results in Compensated Cardiac Hypertrophy*

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Phosphorylation of tropomyosin (Tm) has been shown to vary in mouse models of cardiac hypertrophy. Little is known about the in vivo role of Tm phosphorylation. This study examines the consequences of Tm dephosphorylation in the murine heart. Transgenic (TG) mice were generated with cardiac specific consequences of Tm dephosphorylation in the murine heart. Little is known about tropomyosin phosphorylation. This study examines the role of Tm phosphorylation. This study examines the role of Tm phosphorylation.

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‡ The abbreviations used are: Tm, tropomyosin; Tn, troponin; TG, transgenic; NTG, non-transgenic; SERCA2a, sarcoplasmic reticulum Ca2+ ATPase 2a; PLN, phospholamban; TAC, transaortic constriction; CNa, calcium; LV, left ventricular; LVID, LV internal dimension; LVIDd, LV diastolic dimension; LVIDs, LV systolic dimension; LVOT, LV outflow tract; LVAV, LV anterior wall dimension; LVPW, LV posterior wall dimension.

Background: Changes in phosphorylation status of sarcomeric proteins allows rapid alteration of cardiac function.

Results: Tropomyosin dephosphorylation results in myocyte hypertrophy with increases in SERCA2a (sarcoplasmic reticulum Ca2+ ATPase 2a) expression and phospholamban phosphorylation but without functional changes.

Conclusion: Tropomyosin phosphorylation can influence calcium regulatory proteins and cardiac remodeling in response to stress.

Significance: This is the first report detailing that altering tropomyosin phosphorylation affects calcium handling proteins.

of the sarcomere. Upon binding of Ca2+ to the troponin complex, a conformational change occurs that allows the Tm filament to move away from the myosin-head binding site on the sarcomeric actin filament. Previous and recently published studies show striated muscle α-Tm is phosphorylated at one site, the penultimate amino acid, serine 283, by several potential kinases including tropomyosin kinase, protein kinase A, and protein kinase C (PKC) (1–8). During fetal development, 70% of cardiac α-Tm in rat hearts is phosphorylated, which decreases to ∼30% post-natally (9). In vitro studies investigating the functional role of Tm phosphorylation indicate that low phosphorylation levels decrease the ability of α-Tm to polymerize in a head-to-tail fashion; conversely, increasing phosphorylation enhances the interaction between the C- and N-terminal ends of adjoining Tm molecules. Additionally, changes in α-Tm phosphorylation status seem to alter sarcomeric function, as shown by differential function of the actin-activated myosin S1-ATPases (4, 10). Taken together, these in vitro data suggest that altering phosphorylation status affects the ability of Tm to cooperatively activate the thin filament upon binding of Ca2+ to troponin (Tn).

In recent years in vivo studies performed on animal models indicate that changes in the phosphorylation status of sarcomeric proteins such as troponin I (TnI), myosin binding protein C (MyBPC), and the regulatory myosin light chain result in alterations in Ca2+ sensitivity of the myofilament and changes in cardiac function and may play a role in the development of cardiac disease (11–15). Investigation of a dilated cardiomyopathy transgenic (TG) mouse model bearing a human α-Tm mutation (E54K) shows that phosphorylation levels of Tm decrease relative to non-transgenic (NTG) littermates (16, 17). Additionally, phosphorylation is increased in familial hypertrophic cardiomyopathy α-Tm N175D mice generated by this
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laboratory indicating a link between striated muscle Tm phosphor- ylation, sarcomeric function, and cardiac disease (18). To investigate the in vivo effect of decreased or ablated Tm phosphorylation, we substituted serine 283 with an alanine (S283A), removing the phosphorylation site and effectively inhibiting the ability of α-Tm to be phosphorylated. Several TG mouse lines expressing this α-Tm S283A mutation were generated and analyzed. These TG hearts show no changes in functional parameters when investigated by echocardiography, myofilament Ca²⁺-tension relations, or in studies of work-performing heart during β-adrenergic stimulus. However, these animals do have sex-specific differences in heart morphology likely due to the cardioprotective effects of estrogen that have been described previously (19, 20). Male TG mice show a hypertrophic phenotype as measured by echocardiography and supported by cardiomyocyte cross-sectional area measurements, whereas female mice do not. Male TG mice also show significant modifications in proteins controlling Ca²⁺ fluxes such as increases in the expression of the sarcoplasmic Ca²⁺ ATPase (SERCA2a) and phosphorylation of phospholamban (PLN). Thus, phosphorylation of α-Tm may be part of a signaling cascade that results in changes in Ca²⁺ handling protein levels and may explain the tight regulation of α-Tm phosphorylation levels. Additionally, when male TG animals are subject to pressure overload via transaortic constriction (TAC), they exhibit a significant increase in hypertrophy as well as functional defects including a striking decrease in fractional shortening compared with NTG litter mates. This is the first investigation to show that alterations in the phosphorylation status of a thin filament protein, namely α-Tm, can cause a moderate hypertrophic response and increase SERCA2a expression and PLN phosphorylation. Taken with our previous findings in cardiomyopathy models, these results firmly establish that α-Tm phosphorylation is necessary for an appropriate response during cardiac disease.

EXPERIMENTAL PROCEDURES

Generation of S283A α-Tm TG Mice—Mouse striated muscle α-Tm cDNA was subjected to QuikChange II site-directed mutagenesis (Agilent Technologies) utilizing the primer 5′-CAC GCT CTC AAC GAT ATG ACT GCC ATA TAA GTT TCT TTG CTT CAC-3′ mutating the penultimate serine to an alanine. The mutation was verified through sequencing of the TG mouse DNA verified the sequence of the transgene. Primers specific for the transgene are forward 5′-GCC CAC ACC AGA AAT GAC AGA-3′ and reverse 5′-TCC AGT TCA TCT TCA GTG CCC-3′. GAPDH is used as an internal control, and primers are as follows: GAPDH forward 5′-AGC GAG CTC AGG ACA TTC TGG-3′ and GAPDH reverse 5′-CTC CTA ACC ACG CTC CTA GCA-3′.

Transgenic Protein Quantification and Western Blot Analyses—Myofibrillar proteins were extracted from NTG and TG male mouse ventricles as previously described (22). 30 μg of the myofibrillar protein preparations were separated on a 10% SDS-PAGE gel. The Tm band was excised from the gel, reduced, alkylated, and subject to a trypsin digest. Recovered peptides were desalted with a μ-C18 ZipTip and spotted onto a matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) target plate. All spectra were acquired in reflector positive ion mode on an ABSciex 4800 MALDI-TOF/TOF instrument. The percentage TG protein was calculated after normalization and subtraction of background contributions.

Western blot analyses on myofibrillar protein preparations (4 μg) from 3-month male NTG and TG hearts were conducted using the Tm-specific antibody CH1 (Sigma), Tm Ser-283 phosphorylation-specific antibody generated for this laboratory (YenZyme), and sarcomeric α-actin antibody 5c5 (Sigma) as a loading control. To confirm the mutant Tm was properly assembled into the sarcomere, cytoplasmic protein fractions (4 μg) isolated from 3-month male NTG and TG male mice, were examined by Western blot analyses.

Whole ventricular homogenates from 3-month-old male NTG and TG mice were utilized to visualize Ca²⁺ handling protein expression levels. Western blots were used to visualize sarcoplasmic ATPase 2a (SERCA2a) (AbCam), TnI (Cell Signaling), pTnI23/24 (Cell Signaling), PLN (Thermo Scientific), phosphorylated serine 16 PLN (PLN Ser-16) (Badrilla), phosphorylated PLN threonine 17 (PLN Thr-17) (Badrilla), and calcineurin (CnA) (Sigma). Sarcomeric actin (Sigma) was used as a loading control.

Two-dimensional Isoelectric Focusing-PAGE—Two-dimensional isoelectric focusing-PAGE was performed on mouse hearts as previously described with modifications (17). 3 μg of myofibrillar preparations were resolved on a 24-cm 4.0–5.0 immobilized pH gradient isoelectric focusing strip. After isoelectric focusing, the samples were resolved in the second dimension on a 10% SDS-PAGE gel and transferred to nitrocellulose membrane for Western blotting. Tm muscle-specific antibody CH1 was used to visualize both the unphosphorylated Tm and phosphorylated Tm species. Percentage of Tm phosphorylation was calculated as: (phosphorylated Tm)/(phosphorylated Tm + nonphosphorylated Tm) × 100, where the values for the two protein species have been determined using Image Quant v5.1.

Histopathological Analyses and Cardiomyocyte Cross-sectional Area Analyses—Male mouse hearts at 3, 6, and 9 months were analyzed. Heart weight to body weight ratios were calculated to evaluate for the presence of cardiac hypertrophy. For histological analyses, the hearts were stained with hematoxylin/eosin or Masson’s Trichrome and evaluated for the presence of necrosis, fibrosis, myocyte disarray, and calcification. Images were taken on a Nikon SM2–2T dissecting microscope and an Olympus BX4C compound microscope.

3 H. N. Sheikh and D. F. Wieczorek, unpublished information.

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To quantify changes in cardiomyocyte cross-sectional area, tissue sections were stained with wheat germ agglutinin from *Triticum vulgaris* conjugated with Texas Red (Sigma) to visualize cardiomyocyte membranes. DAPI was used to stain the nuclei of cardiomyocytes. Randomized images of the left ventricular free wall were taken using a fluorescence camera mounted on a Zeiss Axiom, and the cardiomyocyte cross-sectional area was measured using ImageJ (NIH).

Quantitative Real-time PCR Analyses—RNA isolated from 3-month-old NTG and TG male mouse ventricular tissue was isolated using TRIzol reagent (Invitrogen). Real-time RT-PCR was performed using an Opticon 2 real time RT-PCR machine (MJ Research). Each sample was measured in triplicate, and each experiment was repeated twice. Target mRNA was normalized to GAPDH expression as described by Pfaffl (23).

Echocardiographic and Pressure Overload Measurements—Echocardiographic measurements were performed utilizing a 30-MHz high resolution transducer (Vevo 770 high resolution imaging system) after anesthetization of 3-month-old mice as previously described (24). Echocardiographic dimensions and thicknesses were taken from two-dimensional guided M-Mode from the parasternal long axis view in triplicate on NTG and TG 12–16-week-old mice. Fractional shortening (in %) was obtained by the formula 100 × (LVIDd − LVIDs)/LVIDd, where LVIDd and LVIDs are left ventricular (LV) internal dimensions in diastole and systole, respectively. The relative wall thickness indices were calculated by the formula (LVAW + LVPW)/LVIDd, where LVAW and LVPW indicate anterior and posterior wall thicknesses, respectively, and LVIDd is the LV diastolic internal dimension. The LV outflow tract (LVOT) diameter (D) was measured to calculate the LVOT cross-sectional area (LVOT CSA = πD/2)². The velocity-time integral (VTI, in cm) was calculated by integrating the Doppler velocities in the LVOT. The product LVOT CSA × VTI is the LV stroke volume, which multiplied by heart rate gives us the cardiac output (ml/min). 12–16-Week-old male mice of both genotypes were subject to TAC or sham operation as previously described (25). Echocardiographic measurements were taken in M-Mode. Because only male TG mice exhibit changes in echocardiographic measurements, only male animals are used in this study. Pressure gradients across the constriction were measured using Doppler echocardiography as previously described (25). Two weeks post-surgery, mice were again subjected to echocardiography and sacrificed.

Calcineurin/Protein Phosphatase Activity Assay—Calcineurin activity (CnA), also known as protein phosphatase 2B, was measured using a calcineurin/protein phosphatase 2B activity kit (Calbiochem). Cardiac homogenates from 3-month-old male NTG and TG hearts were used. CnA activity is measured as the rate of dephosphorylation of a synthetic peptide in the presence and absence of EGTA, okadaic acid, and EGTA with okadaic acid. Phosphate release was measured by the colorimetric Green Reagent (Calbiochem).

Measurements of Ca²⁺-dependent Activation of Tension—Fiber bundles from papillary muscles of 5-month-old male NTG and TG hearts were detergent-extracted in high relaxing buffer as described previously (26) and mounted between a force transducer and a micromanipulator. The sarcomeric length was adjusted to 2.0 and 2.2 μm using laser diffraction patterns, and isometric tension was measured. Fiber bundles were then subjected to sequential Ca²⁺ solutions (pCa), and isometric tension was again measured. All experiments were carried out at 22 °C.

**Isolated Work-performing Heart Model**—Three-month-old male NTG and TG were anesthetized and treated with heparin to prevent microthrombi as previously described (27). The aorta was cannulated, preserving the aortic valve and the coronary artery. To measure intraventricular systolic and diastolic pressures, an intraventricular catheter was inserted into the left ventricle. A cannula was also inserted into the left pulmonary vein, allowing the direction of the perfusate to be switched from retrograde (Langendorff) to anteriograde (working). COBE pressure transducers were utilized to measure aortic pressure, atrial pressure, and left ventricular pressure and were recorded using a Grass polygraph and digital acquisition system.

Statistics—All statistics are presented as the mean ± S.E. Where appropriate, paired and unpaired t tests, analysis of variance with Bonferroni correction, and analysis of variance with repeated measures were used to detect significance. Significance was set at p < 0.05.

**RESULTS**

**Generation of α-Tm S283A TG Mice**—To determine the functional significance of Tm phosphorylation, we generated TG mice in which the Tm phosphorylation site (serine residue 283) was replaced with a non-phosphorylatable alanine residue (S283A). The transgene construct used to generate α-Tm S283A TG mice is shown in Fig. 1A. Multiple TG lines were generated and studied. Line 2 has the highest TG mRNA expression and the second highest copy number of all transgenic animals generated (17 copies) determined by genomic Southern blot analysis. Forward and reverse sequencing of the construct indicates no mutations or deletions in the transgene.

**Cardiac α-Tm S283A Protein Expression and Phosphorylation in Transgenic Mice**—Often, mutations in Tm isoforms lead to differential migration on SDS-PAGE gels (16, 18, 22, 28). However, because serine is only 16 daltons larger than alanine and has nearly an identical isoelectric point, expression levels of TG and endogenous protein cannot be separated using traditional methods. Instead, myofibrillar protein preparations of age-matched NTG and TG mouse hearts as well as recombinantly expressed NTG or TG protein are resolved by a combination of SDS-PAGE and MALDI-TOF analyses (Fig. 1B). The ratio of serine containing peptides (endogenous Tm) and alanine containing peptides (TG Tm) is calculated after normalization and background subtraction. As an additional control, the peptides corresponding to both the serine and alanine profiles are further fractionated to ensure that the proper tryptic peptide is being analyzed. Line 2 has ~93.7% TG protein expression, Line 25 has ~86% TG protein expression, and Line 97 has ~88% TG protein expression (Fig. 1C), with a concomitant decrease in NTG protein, maintaining total Tm levels at 100%. Investigation of the cytosolic fraction shows no significant accumulation of either endogenous or TG Tm, indicating that the TG protein is properly incorporated into the myofibril (data not shown). Additionally, myofibrillar protein prepara-
tions run on an SDS-PAGE gel show that all myofibrillar proteins are present in the proper ratio in all three TG lines, indicating that the myofibrils are being properly assembled and there is no change in total Tm levels (data not shown).

*Tm Phosphorylation in NTG and α-Tm S283A Mouse Hearts*—To study Tm phosphorylation in TG mice, it was necessary to establish the basal level of Tm phosphorylation in NTG hearts using two-dimensional isoelectric focusing-PAGE. Results show that an unphosphorylated and a single-phosphorylated species of Tm appears in NTG heart samples (Fig. 2A). Upon calf intestinal phosphatase treatment, the phosphorylated Tm protein species is lost. These results are in agreement with previously published studies that identify Ser-283 as the phosphorylation site in striated muscle Tm (1, 8, 9, 17). Further analysis shows a trend of decreasing Tm phosphorylation from 6 weeks to 5 months of age with an average of ~30%. At 15 months of age, animals show a significant increase in Tm phosphorylation, indicating a possible return to fetal gene programs due to senescence (Fig. 2B) (29–31).

To determine the phosphorylation status of Tm in TG myofibrillar preparations, we generated a Tm Ser-283 phosphorylation-specific antibody. As seen in Fig. 2, C and D, there is a clear decrease in the phosphorylation status of Tm in the TG myofibrillar preparations compared with the NTG preparations. As TG Line 2 had the greatest decrease in phosphorylation and exhibited the same phenotype in comparison to the other TG lines, we chose to focus on Line 2 TG mice. When considering the phosphorylation status of these S283A TG mice, it is important to remember that endogenous Tm in NTG mice is phosphorylated at 30%. Line 2 has ~5-fold less (or 80% less) phosphorylation than NTG littermates, corresponding to 6% endogenous Tm available for phosphorylation in this line. We believe Line 25 has more endogenous Tm phosphorylation because of its lower level of transgene expression. These results suggest that most, if not all, of the endogenous Tm in the TG mice is being phosphorylated.

**Gravimetrics and Cardiac Morphology of α-Tm S283A TG Hearts**—Morphological analyses of the left ventricular wall shows a very mild increase in cardiomyocyte disarray and disorganization as indicated by centrally located nuclei and partial loss of the typical cobblestone shape of the cardiomyocyte (Fig. 3A). Staining the membrane with wheat germ agglutinin and measurement of cross-sectional area shows a significant increase in TG cardiomyocyte area (445.5 ± 17.4 μm² versus...
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FIGURE 2. A, two-dimensional isoelectric focusing-PAGE gels show that Tm has one phosphorylation site indicated by the arrow (upper panel) that can be removed after calf intestinal phosphatase treatment (lower panel). Cardiac myofibrillar protein preparations were probed with the CH1 striated muscle Tm antibody. B, percent of total Tm phosphorylated in NTG mice was measured using two-dimensional isoelectric focusing on myofibrillar preparations taken at 1.5, 3, 5, and 15 months of age. C, shown is Western blot analysis of in α-Tm phosphorylation (pTm) from hearts at 3 months of age. n = 3. D, shown is quantification of phosphorylation levels of α-Tm found in panel C.

686.9 ± 66.9 μm² p < 0.05, NTG and TG, respectively (Fig. 3B). Gravimetric analysis was performed on TG animals from 1 month to 9 months of age. Interestingly, results show no changes in heart weight to body weight ratios, likely due to the moderate nature of this hypertrophy (Fig. 3C). There are no differences in the survival of NTG and TG mice.

Cardiac Function of α-Tm S283A TG Hearts—To determine whether the relationship between Ca²⁺ concentration and force-tension development is altered in myofilaments at the sarcomeric level in TG hearts with significantly decreased phosphorylation of Tm, we analyzed skinned fiber bundles from the papillary muscle of 5-month-old hearts. No significant changes in absolute tension or normalized tension in NTG versus TG mice were found (Table 1, Fig. 4, A and B). Additionally, there are no significant differences in pCa₅₀ or the Hill coefficient (n_H), a measure of the cooperative activation of the thin filament of the sarcomere. The work-performing heart model was utilized to determine ex vivo functional effects of the decrease in Tm phosphorylation status. These measurements were performed in mice at 3 months of age. At basal levels, there are no changes in contraction and relaxation parameters in TG hearts. Additionally, when isoproterenol is administered to determine whether the β-adrenergic response is impaired, there are no significant differences in contraction and relaxation (Fig. 4, C and D).

To assess whether decreasing the phosphorylation level of α-Tm has an effect on in vivo cardiac function, we performed echocardiographic analysis on 3-month-old NTG and TG mice. There are no physiological changes in heart function between the NTG and TG mice as shown by fractional shortening, cardiac output, or ejection fraction (Table 2). However, there are sex-specific differences in cardiac morphology. Male TG animals show significant increases in LV mass, LV anterior wall thickness, LV posterior wall thickness, and LV relative wall thickness index, indicating that TG mice have a hypertrophic phenotype without attendant functional defects. Female TG mice show no changes when compared with female or male NTG hearts. Differences in the development of cardiac hypertrophy between sexes have been previously noted (20, 32). Thus, the increase in cardiomyocyte area and left ventricular hypertrophy with no change in heart weight to body weight ratio or female cardiac enlargement demonstrates the moderate nature of this hypertrophic phenotype.

Gene Expression and Protein Changes in α-Tm S283A TG Hearts—Given that histological and echocardiographic analyses indicate that TG mice exhibit a moderate hypertrophic phenotype at 3 months of age, altered gene expression was determined in α-Tm S283A TG hearts. Real time RT-PCR analysis of the RNA isolated from ventricular tissue indicate a trend toward an increase in β-MHC, brain natriuretic peptide (BNP) and atrial natriuretic peptide (ANP) without significant statistical increases (Fig. 5A).

Genes involved in cardiomyocyte Ca²⁺ handling were also examined. Interestingly, there are no changes in gene expression of SERCA2a, the L-type Ca²⁺ channel, NCX, PLN or RyR2 (Fig. 5B). However, there is a significant increase (p < 0.05) in the gene expression of MCI1P, a protein involved in modulating CnA activity in vivo (33).

To determine whether real time RT-PCR levels of Ca²⁺ handling genes correlate with corresponding protein expression in the TG myocardium, protein expression from whole hearts was determined by Western blot analysis (Fig. 5, C and D). Results indicate that the phosphorylation site at PLN Ser-16 and SERCA2α protein expression are increased by >30% over NTG levels. There are no changes in total PLN, phosphorylation at PLN Thr-17, TnI, or phosphorylation at TnI 23/24 or CnA. The lack of increased SERCA2α gene expression by real time RT-PCR analysis compared with the significant increase in protein expression suggests that increased protein stability or translation may be operative. MCI1P gene expression is utilized as a marker of alterations in CnA protein expression or activity. Although MCI1P gene expression is increased, there is no increase in CnA expression. However, it is possible that CnA activity may be altered without altering protein expression.

Calcineurin Activity Assay—As MCI1P can be both a facilitator and an inhibitor of CnA activity, it is necessary to determine whether changes in mic1p gene expression alters CnA activity, as CnA is an important regulator in the Ca²⁺ handling process. A CnA/ protein phosphatase 2B activity assay was performed (Calbiochem) on whole heart preparations from 3-month-old mouse hearts. There are no significant changes in CnA activity (Fig. 5E).

Cardiac Function in α-TM S283A Pressure Overload Mouse Hearts—To determine the effect of decreased α-TM phosphorylation during cardiac stress and disease, 12–16-week-old TG mouse hearts were subject to TAC along with NTG littermates and sham-operated animals from both genotypes.

Animals were subject to echocardiography before surgery as well as 2 weeks after TAC and were then sacrificed for histology and gravimetric studies. NTG and TG TAC-operated animals
show significantly increased pressure gradients at 2 weeks, indicating the efficacy of the pressure overload model (Fig. 6A). LVIDd, LVIDs (LVID systole), and percent fractional shortening are the only parameters that show significant alterations in function between NTG TAC- and TG TAC-operated animals (Fig. 6B and D). Interestingly, although NTG TAC operated hearts have a greater pressure gradient, TG TAC-operated hearts are the only group that experiences a significant decrease in percent fractional shortening (Fig. 6D). NTG sham, TG sham, and NTG TAC all have fractional shortening at 33%, whereas the TG TAC-operated group has fractional shortening at 24%, indicating impairment in cardiac function in that group. These data indicate that significantly decreasing the phosphorylation status of α-Tm impairs the ability of the myocardium to properly respond to acute stress.

Gravimetrics and Cardiac Morphology in α-TM S283A TAC and Sham-operated Animals—NTG TAC operated animals have a significant increase in heart weight to body weight when compared with NTG sham-operated animals (p < 0.0001) (Fig. 6E). Additionally, TG TAC-operated animals show an increase in heart weight to body weight ratios compared with TG sham-operated animals (p < 0.05).

Hematoxylin/eosin staining of TG sham-operated hearts show a mild increase in disorganization compared with NTG sham-operated hearts. Masson’s Trichrome staining of both NTG and TG sham-operated hearts show no significant increases in the deposition of fibrotic tissue in the left ventricular free wall (Fig. 6G, i and ii). NTG TAC- and TG TAC-operated heart sections stained with hematoxylin/eosin show cardiomyocyte disorganization and centrally located nuclei. Both NTG and TG TAC-operated hearts stained with Masson’s Trichrome show increases in fibrosis. Cardiomyocyte cross-sectional analyses demonstrate significant increases in TG sham and NTG TAC- and TG TAC-operated hearts (p < 0.001) compared with NTG sham mice (Fig. 6Gi and i and ii). TG TAC cardiomyocytes show greater increases in size compared with both TG sham and NTG TAC cardiomyocytes (p < 0.0001, p < 0.01) (Fig. 6F).

DISCUSSION

Post translational modifications, such as alterations in the phosphorylation status of sarcomeric and Z-disc proteins can result in altered cardiac contractility with progression to disease and death (11–14). This is the first in vivo study investigating the functional role of cardiac α-Tm phosphorylation. To address this, the single Tm phosphorylation site, serine 283, was changed to an alanine, and TG animals were generated for study. In vivo assessment of basal cardiac function of α-Tm S283A TG mice shows that the hearts exhibit a moderate compensated hypertrophic phenotype with an increase in myocyte size due to a stimulus initiated by decreased Tm phosphorylation. It is possible that the increase in cardiomyocyte size occurs in response to mechanical defects induced by autophagy or apoptosis, two cell death processes involved in the transition from compensated to decompensated hypertrophy (34, 35). However, the data suggest this compensatory response is an
attempt to normalize LV wall stress and preserve pump function, which may point toward the role that autophagy places in maintaining cell and tissue homeostasis (36). Increases in LV mass, LVAW, LVPW, relative wall thickness, and increases in contractile function assayed by skinned fiber preparations indicate that TG hearts are in a compensated or adaptive state of hypertrophy in response to compensatory activation of the thin filament. Thus, phosphorylation may not be a major modulator of cooperative spread of activation in the myofilament lattice and may be more significantly related to the actin filament independent of Tm head-to-tail interactions (38). NMR studies of the interaction between the N- and C-terminal dimers indicate that the last 2–5 amino acids at the C terminus are flexible (39). The fact that the very last C-terminal residues are mildly disordered may offer some explanation as to why loss of additional negative charges in the form of phosphorylated serine has no effect on cooperativity or Tm head-to-tail interactions. The phosphorylated Ser-283 may be in an area too flexible to allow significantly increases.

A study by Gaffin et al. (37) indicates that substitution of negatively charged amino acids at the C terminus causes a significant change in the distance between Tm monomer strands and possibly alterations in contiguous Tm molecule interactions. In vitro studies investigating the striated muscle Tm phosphorylation site indicate that changing the phosphorylation status of α-Tm alters the head-to-tail interaction between neighboring Tm molecules (4). Contrary to expectation, removing the phosphorylation of α-Tm at Ser-283 in an in vivo system does not result in any alterations in cooperative activation of the thin filament. Thus, phosphorylation may not be a major modulator of cooperative spread of activation in the myofilament lattice and may be more significantly related to the actin filament independent of Tm head-to-tail interactions (38). NMR studies of the interaction between the N- and C-terminal dimers indicate that the last 2–5 amino acids at the C terminus are flexible (39). The fact that the very last C-terminal residues are mildly disordered may offer some explanation as to why loss of additional negative charges in the form of phosphorylated serine has no effect on cooperativity or Tm head-to-tail interactions. The phosphorylated Ser-283 may be in an area too flexible to allow for strong interaction with residues in the N terminus of the subsequent Tm molecule.

The lack of change in cardiac function, myofilament cooperativity, and Ca\(^{2+}\) sensitivity coupled with the development of compensated hypertrophy in the TG animals warranted an investigation into the possible mechanisms involved in the hypertrophic response. The gene expression profile of the α-Tm S283A TG found that mcip1 significantly increases. Mcip1 gene expression is utilized as a marker for CnA activity and has been alternatively shown as both a facilitator and inhibitor of CnA activity in vivo (33, 40, 41). Increases in CnA activity have been shown to induce cardiac hypertrophy in mouse models, and conversely, the development of a hypertrophic phenotype can be prevented via CnA inhibition (42, 43). Interestingly,
in the α-TM S283A TG animals, the increase of mcip1 mRNA did not result in changes of CnA expression or activity, indicating another effector downstream of Tm phosphorylation loss may be responsible for the hypertrophic phenotype.

As numerous studies have demonstrated the importance of Ca²⁺ in the modulation of cardiac hypertrophy, we examined whether alterations in expression of Ca²⁺ proteins occurred in TG mice. Although there are no changes in total TnI expression or TnI phosphorylation at amino acids 23 or 24, there are alterations in sarcoplasmic reticulum proteins. Increasing SERCA2a protein expression and/or activity can rescue multiple disease phenotypes and improve myofibrillar efficiency and contractile parameters both in human cardiomyocytes and rodent hearts (44–47). Conversely, in animal models of cardiac disease as well as human patients with heart failure, SERCA2a protein levels and activity often decrease (48, 49). Surprisingly, SERCA2a protein levels are increased in the α-Tm S283A TG hearts by ~30% over NTG levels. Additionally, the 30% increase in PLN phosphorylation at Ser-16 indicates that further restriction on SERCA2a activity has been released, as PLN in an unphosphorylated state results in inhibition of the pump (50–52). Similar to increasing SERCA2a expression and activity, phosphorylation of PLN Ser-16 results in a hypercontractile heart. However, there are no changes in cardiac contractility in the α-Tm S283A mice. Rather, normal cardiac function as measured by multiple methods is preserved in the TG hearts rather than enhanced. This indicates that the increase in SERCA2a protein levels as well as the increase in PLN Ser-16 phosphorylation may be necessary to maintain normal cardiac function in a heart in which Tm has largely been dephosphorylated. It is possible, therefore, that if SERCA2a activity was inhibited, a greater degree of hypertrophy and/or a progression to decompensated cardiomyopathy and heart failure would result.

Increased SERCA2a protein and activity levels are associated with physiological hypertrophy. In most exercise trained models, PLN protein levels are unchanged, although changes in PLN...
phosphorylation are seen (53, 54). This is similar to the results found with the α-Tm S283A mice. Additionally, in animals that have compensated or physiological hypertrophy due to exercise training, there are no changes in gene expression of common cardiomyopathy markers, identical to what is seen in the TG mice investigated here (55). Exercise training improves cardiomyocyte contractility and calcium handling and often improves disease in both animal and human models of cardiac disease (56–58). Although the mechanisms responsible for the development of physiological hypertrophy during exercise training are not well elucidated, we speculate that ablating the phosphorylation site of α-Tm results in a similar signaling cascade that occurs in response to exercise training. In a physiologically exercised heart, the signaling pathways that are activated result in improved cardiac function, whereas in response to Tm dephosphorylation, the TG hearts are able to function normally with a mild hypertrophic phenotype.

Increases in the level of SERCA2a appear to be responsible for cardiac dysfunction in the TG TAC-operated animals compared with NTG TAC-operated animals. Previous work indicates that SERCA2a up-regulation by ~20% did not result in increased energy consumption by the heart at basal levels (59). However, when those animals were subjected to pressure overload hypertrophy, SERCA2a overexpressing TG mice show significant decreases in contractile force and free energy, leading to increased morbidity. The adaptive response involving SERCA2a up-regulation and increased PLN Ser-16 phosphorylation ensures that Tm dephosphorylated hearts remain compensated in a physiological state and do not progress to cardiomyopathy and heart failure under normal conditions. However, this increased SERCA2a expression and PLN phosphorylation most likely leads to cardiac dysfunction and pathology after TAC operation. Animals were sacrificed 2 weeks after TAC, but we speculate, based on the increase in

![Echocardiographic analyses of NTG sham, TG sham, NTG TAC, and TG TAC hearts from 12–16-week-old mice. A, pressure gradients in NTG sham, TG sham, NTG TAC, and TG TAC hearts are shown. B and C, diastolic and systolic left ventricular internal dimensions (LVIDd, LVIDs), respectively. D, fractional shortening (% FS) is shown. E, heart weight (HW) to body weight (BW) ratio of NTG and TG sham-operated hearts and NTG and TG TAC-operated hearts is shown. F, cardiomyocyte cross-sectional area measurements are shown. n = 6 for all groups. * indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001. G, shown are tissue sections from NTG sham, TG sham, and NTG TAC– and TG TAC-operated hearts stained with hematoxylin and eosin (i), Masson’s Trichrome (ii), and wheat germ agglutinin (iii). All images were taken at 40×. The scale bar indicates 50 μm.
hypertrophy in the TG TAC operated animals, that this group would exhibit increases in hypertrophic markers as well as increased lethality.

This is the first study indicating that dephosphorylating a sarcomeric protein can result in maintenance of a compensated or physiological hypertrophic phenotype. Additionally, to our knowledge, this is the first study in which a TG animal with alterations in a sarcomeric protein results in increases in SERCA2a protein expression and PLN Ser-16 phosphorylation. Tm phosphorylation appears to be involved in the development of compensated or physiological hypertrophy, possibly through proteins involved in signaling at the z-disc. Novel PKCδ and PKCe are two molecules shown to promote physiological hypertrophy. Both molecules translocate to the z-disc upon cardiomyocyte stimulation (60, 61). PKCe, specifically, has been shown to associate with the myofilament and bind strongly to actin, resulting in constitutively active PKC (62).

Previous studies indicate that dephosphorylated Tm binds actin differentially from phosphorylated Tm, and it is possible that the replacement of Ser-283 with an Ala residue affects nearest neighbor interactions in the sarcomere, allowing PKCe greater access to the binding site on actin (4, 10). Mice expressing a PKCe-specific activator exhibit normal cardiac function and a compensated hypertrophic phenotype indicating that PKCe can be a positive modulator of compensatory cardiac hypertrophy (63). Additionally, activated PKCe can activate MEK1 through Raf1, which has been shown to also result in compensated hypertrophy, indicating that the PKCe-Raf1-MEK1-ERK1/2 pathway may be playing a role in the S283A TG mouse phenotype (64–66).

Studies examining the potential signaling pathways activated by Tm dephosphorylation are currently in progress. In summary, the results presented here firmly establish that the status of Tm phosphorylation can influence expression of Ca2+ regulatory proteins and the response of the heart to acute cardiac stress.

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