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RESEARCH ARTICLE

Implications of Tropomyosin Phosphorylation in Normal and Cardiomyopathic Hearts

David F. Wieczorek[*1]

[1]Department of Molecular Genetics, Biochemistry and Microbiology
University of Cincinnati College of Medicine, 231 Albert Sabin Way
Cincinnati, OH 45267-0524

* David.wieczorek@uc.edu

Abstract
Numerous molecular and biochemical processes regulate protein production in the cell. One of these processes, phosphorylation, allows the cell to rapidly adapt to changing physiological situations. In terminally differentiated cells, such as cardiomyocytes, phosphorylation of sarcomeric proteins controls contraction and relaxation under both normal and stressful conditions. The focus of this review is how phosphorylation of sarcomeric proteins alters physiological performance in cardiac muscle with a particular emphasis on the thin filament protein tropomyosin. This topic is addressed by the examination of tropomyosin isoform expression and its phosphorylation state from embryonic to adult murine development. Next, studies are examined which utilize *in vivo* model systems to express phosphorylation mimetics and de-phosphorylation genetically-altered tropomyosin transgene constructs. Results show that tropomyosin isoform expression is highly regulated, along with its phosphorylation state. Transgenic mouse hearts which express high levels of a constitutively phosphorylated tropomyosin develop a severe dilated cardiomyopathy and die within a month. A more moderate expression of this phosphorylation mimetic leads to normal systolic performance, but impaired diastolic function. When tropomyosin is dephosphorylated, the transgenic mice develop a compensated cardiac hypertrophy without systolic or diastolic alterations. Interestingly, when dephosphorylated tropomyosin is co-expressed with a hypertrophic cardiomyopathy tropomyosin mutation, the pathological phenotype is rescued with improved cardiac function and no indices of systolic or diastolic dysfunction. These studies demonstrate the functional significance of tropomyosin phosphorylation in determining cardiac performance during both normal and pathological conditions.

Introduction

The regulation of gene expression plays a critical role in determining mRNA and protein levels in the cell. Multiple molecular processes, which include transcriptional, post-transcriptional, and translational mechanisms, determine these levels of gene expression. Transcriptional regulation has been studied extensively for decades and plays a dominant role in determining mRNA and protein levels; chromatin accessibility, histone protein modification, acetylation and methylation of both DNA and protein regulate the accessibility of the transcription apparatus to DNA. Also, co-transcriptional and post-transcriptional processes play crucial roles, with some of these mechanisms being recently discovered. For example, methylation of pre-mRNA and mRNA during transcription and the synthesis of circular RNAs regulate splicing, alternative splicing, and other essential key regulatory events. RNA and protein expression is also determined by mRNA and protein stability. Transcribed nucleotide sequences in the 3' untranslated region can confer message instability through AUUUA-rich binding RNA proteins and attraction of the exosome-mediated degradation complex. Coupled with these nucleotide-regulatory mechanisms, translational regulation plays a central role in controlling protein synthesis and function. Translational frameshifting allows multiple proteins to be expressed from the same processed RNA transcript, and RNA editing allows multiple proteins to be generated from a single mRNA, often in a tissue- or developmental stage-specific manner. The focus of this article will be on the phosphorylation post-translational regulatory mechanism and its role in controlling cardiac physiological function, with a particular emphasis on tropomyosin, a thin filament sarcomeric protein.

Phosphorylation of Sarcomeric Proteins and Their Influence on Cardiac Function

Cardiac muscle cells replicate during embryogenesis and fetal development, becoming terminally differentiated soon after birth. Because cardiomyocytes must respond to a wide variety of changing physiological conditions, these cells must adapt molecular and biochemical mechanisms that allow them to respond to these environmental changes. One means of adaptation is the implementation of various contractile protein isoforms which change their expression dependent upon the age (embryonic, newborn, or adult) or region within the heart (atria vs ventricle). For example, in the thick myofilament of the sarcomere, there are atrial vs ventricular specific myosin light chains; also in the atria, α-myosin heavy chain (MHC) is predominantly expressed, whereas the ventricular chambers transcribe α-or β-MHC dependent upon the age of the organism. In the thin filament, although α-tropomyosin (Tpm) is the predominant isoform in the heart, β-Tpm does have higher expression during fetal development.[1,2] With striated muscle actin, the cardiac actin isoform is restricted to the heart, whereas skeletal actin is expressed in both the heart and skeletal muscle (cardiac muscle expresses 80% cardiac actin and 20% skeletal actin).[3] These changes in isoform expression are critical during development, and serve as a long-term response to environmental stress. Since the half-life of many of the sarcomeric muscle mRNAs is very long (~ 80 hrs), the turnover in mRNA and protein isoform expression takes substantial time. An alternative to implementing changes in protein function in a rapid manner is through post-translational modifications of existing proteins. Phosphorylation is one such process that is utilized to accomplish this rapid adaptation to elicit necessitated changes in physiological function.

Phosphorylation of muscle contractile proteins is an essential process for the regulated control of the sarcomere. Cardiac function must respond to increased demand during exercise and stress by increasing contractility, oftentimes stimulating β-adrenergic receptors to increase activity by protein kinase A (PKA). Protein kinase A phosphorylates calcium (Ca^{2+}) handling proteins (i.e. L-type Ca^{2+} channel, ryanodine receptor, phospholamban) and several myofibrillar proteins (i.e. cardiac troponin I, cardiac myosin binding protein C) to increase muscle cell contractility. Another important phosphorylation signal is elicited by PKC through α- and β-adrenergic signaling. Protein kinase C epsilon phosphorylates a number of proteins, including troponin I, troponin T and myosin binding protein C (MyBP-C).[4-6] The consequence of these phosphorylation events on contractile proteins is to affect contractility, cross-bridge kinetics, and Ca^{2+} sensitivity of the myofiber.

Titin, a giant filamentous protein, spans the entire length of the sarcomere and contributes to cardiomyocyte stiffness. This stiffness is associated with site-specific phosphorylation of titin.[7] During exercise, PKA-mediated titin phosphorylation reduces this stiffness.[8] Interestingly, multiple sites within titin are targets for phosphorylation by different kinases (i.e. PKA, protein kinase G, and Ca^{2+}-dependent calmodulin kinase II (CaMKII).

In the heart, the primary cardiac sarcomeric thick filament proteins that are phosphorylated are

myosin light chains (MLCs) and MYBP-C. Myosin light chain 2, a protein associated with the myosin heavy chain (MHC) head region, is phosphorylated by MLC kinase, and MLC2 phosphorylation increases Ca^{2+} sensitivity and cross-bridge kinetics.[9,10] Cardiac MyBP-C (cMyBP-C) is a 130 kDa thick filament protein that interacts with both myosin and titin. It is ~ 40 nm in length and is composed of 11 globular immunoglobulin and fibronectin domains (C0 – C10) and an extensible M-domain that can be phosphorylated. Results show that cMyBP-C binds to F-actin and causes a shift in Tpm, promoting the ON state of the thin filament. In this way, cMyBP-C is considered to slow cross-bridge kinetics.[11,12] Phosphorylation of cMyBP-C relieves this brake by releasing its binding to the S2 region of myosin to actin, thus extending the cross-bridges further from the myosin backbone.[13,14] Seventeen phosphorylation sites of cMyBP-C have been found, located in the C0 – C2 regions, and within the M-domain. These sites are substrates for PKA, PKC, or CaMK II. Data shows there is increased cooperativity in cross-bridge recruitment, related to Ca^{2+} sensitivity of the thin filaments, with recent evidence suggesting that the precise impact of cMyBP-C phosphorylation on contractile function is phosphorylation site-specific.[15]

The principle proteins of the cardiac sarcomeric thin filament are filamentous actin, Tpm, and the troponin complex: the Ca^{2+}-binding subunit (cTnC), and inhibitory subunit (cTnI) and a Tpm-binding subunit (cTnT). There are no identified phosphorylation sites in striated-muscle actin, and Tpm phosphorylation will be discussed below. Cardiac troponin C also does not appear to be phosphorylated. However, extensive phosphorylation occurs in both TnT and TnI. Mouse cTnT is phosphorylated at 4 PKC targeted amino acid residues: Thr 197, Ser 201, Thr 206, and Thr 287, whereas human cTnT is phosphorylated at Ser179, Ser 198, Thr 203, and Thr 284. Phosphorylation of mouse Thr 206 (human 203) appears to reduce tension and actomyosin Mg-ATPase activity, myofilament Ca^{2+} sensitivity, and cooperative activation of the thin filament.[16,17]

Cardiac TnI contains several phosphorylation sites targeted by different kinases and phosphatases. Phosphorylation by PKA at Ser 23/24 results in increased crossbridge cycling and decreased myofilament Ca^{2+} sensitivity, whereas phosphorylation by PKC at Ser 43/45 decreases maximum Ca^{2+} activated force and crossbridge cycling rate. In addition, phosphorylation at Thr 144 increases myofilament sensitivity to Ca^{2+} and decreases myofilament sliding velocity.[18] Thus, phosphorylation of both TnT and TnI by PKC and PKA contribute to precisely regulate sarcomeric contractility and relaxation through thick and thin myofilament interactions.

Tropomyosin Isoform Expression in the Heart

Prior to addressing Tpm phosphorylation in the heart, it is necessary to examine Tpm isoform expression with respect to development and in response to cardiomyopathic conditions. To investigate Tpm expression in the mammalian heart, we conducted a molecular analysis during murine embryogenesis and in developing embryonic stem cells. Our results show that Tpm genes ((α, β, γ, and δ) are all expressed in differentiated embryoid bodies and during murine embryogenesis.[1] Interestingly, the striated muscle β-Tm isoform and the α-TM smooth muscle isoform are constitutively expressed during development. In contrast, the striated muscle α-Tm isoform is not expressed until the day 5 embryoid body and the day 7.5 post coitus embryo. Further analyses show the α- and β-Tpm striated muscle isoforms are expressed during cardiogenesis (embryonic day 11 – 19), with the striated muscle β-Tpm isoform expression changing from 20% to 2% during the embryonic to adult transition in the mouse heart.[1] Also, our results show the adult human heart predominantly expresses striated muscle α-Tpm, with lower levels of β-Tpm and α-Tpm(k), an isoform not expressed in mice.[19] Tropomyosin kappa is a novel striated muscle isoform, generated via alternative splicing from the α-Tpm gene that is expressed in the heart but not skeletal muscle. Expression of this isoform decreases fractional shortening, and causes a decrease in myofilament Ca^{2+} sensitivity, with no change in maximum developed tension.[19]

To determine whether the striated muscle β-Tpm isoform could substitute for α-Tpm, we generated transgenic mice that express the striated muscle β-Tpm isoform specifically in the heart. There is 86% amino acid identity between α- and β-Tpm with 39 amino acid differences distributed throughout the entire protein. We generated multiple β-Tpm transgenic lines which showed a 150-fold increase in β-Tpm mRNA, along with a 34-fold increase in the associated protein.[20] There is a concomitant decrease in α-Tpm mRNA and protein expression in these β-Tpm transgenic mouse hearts, with no net change in total Tpm production which illustrates the regulatory translational feedback mechanism that controls Tpm protein production in the heart.

We conducted morphological and physiological analyses on these β-Tpm transgenic mouse hearts.[20] When there was 60% β-Tpm protein expressed in the transgenic hearts, no morphological changes were observed in the transgenic hearts nor in their associated sarcomeres. Although there were no physiological changes associated with myocardial contractility, there were significant effects on diastolic function; there was a significant delay in the time of relaxation and a decrease in the maximum rate of relaxation. In addition, myofilaments isolated from these transgenic hearts exhibit an increase in the activation of the thin filament by strongly bound cross-bridges and an increase in the Ca^{2+} sensitivity.[21] Also, studies demonstrated reduced maximal rates of contraction and relaxation in isolated cardiomyocytes from these β-Tpm transgenic hearts.[22] Additional studies show that transgenic mice that express 80% β-Tpm in their hearts die between 10 – 14 days postnatally.[23] Severe pathological abnormalities in these high expression β-Tpm hearts include thrombus formation in both atria and ventricles, atrial enlargement and fibrosis and diffuse myocytolysis; physiological changes include severe systolic and diastolic dysfunction. These studies conclusively demonstrate there are distinct functional differences between Tpm isoforms in the regulation of cardiac performance.

Effects of Tropomyosin Phosphorylation on the Sarcomere

Tropomyosin, an α-helical coiled coil protein dimer, plays an essential role in the regulation of contraction and relaxation in the thin filament of the sarcomere through its interactions with actin and the troponin complex. As mentioned above, phosphorylation is a major regulator of cardiac function by affecting numerous membrane, cytoplasmic, and sarcomeric proteins. Although less is known regarding Tpm phosphorylation than some sarcomeric proteins, recent investigations have demonstrated its importance for sarcomeric performance and cardiac function.

All three striated muscle Tpm isoforms (α- , β-, and γ -Tpm) have a single phosphorylation site located at amino acid S283, the penultimate amino acid. The carboxyl terminus plays an essential role in its interaction with actin and TnT to facilitate polymerization with other Tpm molecules in a head-to-tail fashion.[24,25] Tropomyosin phosphorylation was first demonstrated using radioisotope labeling on leg muscles of frogs.[26] In the heart, studies indicate there is a progressive developmental decline in Tpm phosphorylation in the rat ventricle; during fetal development ~ 70% Tpm is phosphorylated, which subsequently decreases to to ~ 30% post-partum.[27,28] In addition, there is differential Tpm phosphorylation among the 4 cardiac chambers, with atria exhibiting the highest levels of phosphorylation[29] (Sheikh and Wieczorek, unpub. results). Our studies also found there are substantial levels of Tpm phosphorylation in human hearts, specifically the ventricles and the interventricular septum; the amount of Tpm phosphorylation in the human atria is currently unknown.[28]

Structural and Biochemical Effects of Tropomyosin Phosphorylation

As mentioned, phosphorylation in striated muscle Tpm occurs at the penultimate amino acid, serine 283, in the C-terminal region that interacts with the N-termini of adjacent overlapping Tpm molecules. In addition, there are also protein-protein interactions with both TnT and actin in this region. Previous investigations addressed the functional consequences of Tpm phosphorylation using *in vitro* biochemical systems. Biochemical assays, myofiber analyses, and molecular simulations indicate Tpm phosphorylation enhances the stiffness of the head-to-tail Tpm overlap region and its binding to TnT, while decreasing myofibril relaxation without significantly affecting cooperativity or altering activation kinetics.[30-32] These *in vitro* biochemical studies provide insight into the biophysical properties of phosphorylated Tpm.

Our approach to address the physiological importance of Tpm phosphorylation was to employ *in vivo* transgenic mouse model systems where striated muscle Tpm was modified using phosphorylation mimetics or altering the Ser283 amino acid to Ala, which cannot be phosphorylated. Expression of a genetically-modified Tpm cDNA was restricted to cardiomyocytes by employing the α-myosin heavy chain promoter (α-MHC). By using these transgenic models, we could ascertain the morphological, biochemical, and physiological significance of Tpm phosphorylation in an *in vivo* functioning heart.

Using the α-MHC promoter, we constructed an α-Tpm cDNA construct that incorporated an aspartic acid residue replacement of serine at amino acid 283 (S283D).[33] The usage of an aspartic acid residue, which is negatively charged, mimics the negative charges found with phosphorylated amino acids, thus representing a

pseudo-phosphorylation event. Results show that Tpm S283D mice that express high levels of the transgene exhibit severe dilated cardiomyopathy within 2 weeks postpartum. Similar with other Tpm transgenic mouse models, expression of the transgene leads to a reciprocal decrease in the endogenous Tpm protein, thereby demonstrating a translational feedback mechanism that regulates the total amount of Tpm protein in the cardiomyocytes.[20] These high expression Tpm S283D mice have a significant increase in their heart:body weight ratio, and usually die by 1 month of age.[33] Interestingly, transgenic mouse hearts that express moderate levels of the Tpm S283D transgene show a mild cardiomyocyte hypertrophy with limited fibrosis. This moderate phenotype does not progress to a more severe condition even after 1 year.

To explore further the functional significance of Tpm phosphorylation, we conducted various physiological measurements of cardiac and myofilament function.[33] In assessing *ex vivo* cardiac performance in different moderate expression transgenic lines, we found there are no differences in the systolic performance (rates of contraction and time to peak pressure); however, there was impaired diastolic function (Table 1). The rate of relaxation is significantly reduced, concomitant with an increase in the half-time to relaxation. We analyzed skinned fiber bundles from the papillary muscle of moderate and high expression mice and found no significant changes in absolute or normalized tension, Ca^{2+} sensitivity, cooperativity, or Ca^{2+}-ATPase activity in the myofibers. This work provided the first extensive *in vivo* assessment of Tpm phosphorylation in the vertebrate heart and its functional role in cardiac performance.

Table 1: Physiological Parameters of Cardiac and Myofiber Function in Select Tropomyosin Mouse Models

Mouse Model	Maximum Rate of Contraction	Maximum Rate of Relaxation	Myofiber Ca^{2+} Sensitivity	Sarcomere Tension Development
α-Tpm	100%	100%	100%	100%
β-Tpm	100%	↓	↑	↓
α-Tpm S283D	100%	↓	100%	100%
α-Tpm S283A	100%	100%	100%	100%
HCM α-Tpm E180G	100%	↓	↑	↑
HCM α-Tpm E180G/ S283A	↑	100%	100%	100%

Tropomyosin Dephosphorylation Leads to Compensated Cardiac Hypertrophy

As mentioned above, *in vitro* biochemical studies have investigated the significance of Tpm phosphorylation on protein-protein interactions, biophysical properties, and myofiber analyses. However, little is known regarding the *in vivo* importance of Tpm dephosphorylation to cardiac morphology and performance. To address this area using an *in vivo* approach, we generated transgenic mice which incorporate a striated muscle Tpm cDNA with an alanine substitution for serine at amino acid 283 (S283A).[34] In this work, we also employed the cardiac specific α-MHC promoter and generated mice that expressed 86 – 94% transgenic Tpm S283A protein; the endogenous wildtype Tpm protein was decreased reciprocally. Echocardiographic and histological analyses show a mild increase in cardiomyocyte disarray and a significant increase in cardiomyocyte area. There were no alterations in cardiac systolic or diastolic performance, myofilament Ca^{2+} sensitivity, cooperativity, or response to β-adrenergic stimulus (Table 1). When these Tpm S283A transgenic mice were subject to cardiac stress through transaortic constriction to induce pressure overload hypertrophy, there were greater functional defects in the transgenic mice than in controls; these differences were observed with diastolic and systolic left ventricular dimensions, fractional shortening, pressure gradients, and cardiomyocyte cross-sectional area. These results demonstrate that modification of Tpm phosphorylation modulates cardiac morphology which leads to a compensated hypertrophic phenotype.[34]

Tropomyosin Dephosphorylation Can Rescue the Cardiomyopathic Phenotype

Previously, our laboratory developed 2 transgenic mouse models of familial hypertrophic cardiomyopathy (FHC): (1) a substitution of asparagine for aspartic acid at codon 175 of α-Tpm (N175D);[35] and (2) substitution of glutamine for glutamic acid at codon 180 (E180G).[36,37] These

amino acid changes are identical to mutations that occur in human cardiovascular FHC disease.[38] Histological analyses show that the transgenic Tpm 175 hearts exhibit a mild hypertrophy, affecting only ~5% of the myocardium. In contrast, the Tpm 180 hearts develop a severe concentric hypertrophy with significant ventricular fibrosis and atrial enlargement that progressively increases from 2.5 months and results in death between 4 and 6 months. *In vivo* physiological analyses show severe deficits in both contractility and relaxation in both FHC models (Table 1). In addition, isolated myofilaments demonstrate an increased activation of the thin filament through enhanced Ca^{2+} sensitivity of steady-state force, which is a common feature of many of the FHC-associated mutations in thin filament proteins.

Because transaortic coarctation is a severe stress inducing procedure, we explored whether there would be a differential response in the Tpm S283A mice when exposed to a prolonged congenital cardiac stress, such as a cardiomyopathic condition. For these studies, we needed to determine the baseline levels of Tpm phosphorylation in control and Tpm E180G mouse hearts. We examined these levels over a period of 1.5 – 5 months. In control mice, levels of Tpm phosphorylation decreased from ~ 36% to 23% over this time period; in the Tpm E180G hearts, the levels are relatively constant at 28%.[28] Interestingly, the levels of Tpm phosphorylation are higher in the transgenic hearts over controls in the 4-5 month time period; these results were confirmed with the FHC D175N model of FHC (Sheikh and Wieczorek, unpub).

To study the effects of reduced Tpm phosphorylation on hypertrophic cardiomyopathy, we generated transgenic mice encoding 2 mutations within the Tpm molecule: S283A – which prevents Tpm phosphorylation; and E180G – which causes FHC.[39] Surprisingly, we found that mice simultaneously expressing both of these mutations were rescued from the pathological hypertrophic phenotype. These mice exhibit no signs of cardiac hypertrophy and displayed improved cardiac function. These double transgenic mice show improved systolic function with increased ejection fraction and fractional shortening when compared with Tpm E180G mice and control littermates (Table 1). Diastolic function is significantly improved, and there is a decrease in myofilament Ca^{2+} sensitivity when compared with Tpm E180G values. In essence, the decreased phosphorylation of Tpm can morphologically and physiologically rescue the pathological phenotype associated with FHC. We speculate that the increased flexibility of the Tpm E180G mutation[40,41] may be offset by the loss of phosphorylation in the overlap region of adjacent Tpm molecules. This "normalization" of sarcomeric function may be mediated either by long-range structural effects on Tpm or through interactions with actin and/or TnT. This was the first study to demonstrate that decreasing Tpm phosphorylation could rescue a FHC phenotype.[28] A summary of various physiological parameters in the select forementioned Tpm transgenic mouse models is shown in Table 1. Interestingly, a recent paper by Nefedova et al. demonstrates that pseudo-phosphorylation in Tpm with S283D or S61D (a phosphorylation site in cytoskeletal non-muscle Tpm) can sometimes prevent/negate biochemical alterations induced by cardiomyopathic mutations.[42] Collectively, these studies suggest that alterations in Tpm phosphorylation may play a key role in future treatments of cardiomyopathic conditions.

Conclusions

As mentioned previously, Tpm phosphorylation is present in significant quantities in human hearts.[28] What is currently unknown is whether the levels of Tpm phosphorylation can be causative or are a consequence of human cardiomyopathies. This information is vital for developing therapeutic strategies for the treatment of these diseases. This information may be useful in identifying drug targets for various kinases or phosphatases and their downstream signaling factors that could potentially treat various cardiomyopathic conditions. Various kinases have been associated with Tpm phosphorylation (i.e. Tpm kinase, PKCz, casein kinase 2);[33] however, it is unknown whether all or only a subset of these kinases phosphorylates Tpm, dependent upon a specific developmental stage or stress-associated/pathological condition. Another important area of investigation is whether phosphorylation levels of the β-Tpm striated muscle isoform or α-Tpmk isoform changes in the human heart to either cause cardiac disease or is activated in response to the pathological phenotype. By answering questions such as these and the employment of therapeutic biochemical and molecular approaches, we can hope to improve the treatment of this often-fatal condition.

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