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Tropomyosin Ser-283 pseudo-phosphorylation slows myofibril relaxation

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Abstract

Tropomyosin (Tm) is a central protein in the Ca²⁺ regulation of striated muscle. The Tm isoform undergoes phosphorylation at serine residue 283. While the biochemical and steady-state muscle function of muscle purified Tm phosphorylation have been explored, the effects of Tm phosphorylation on the dynamic properties of muscle contraction and relaxation are unknown. To investigate the kinetic regulatory role of Tm phosphorylation we expressed and purified native N-terminal acetylated Ser-283 wild-type, S283A phosphorylation null and S283D pseudo-phosphorylation Tm mutants from insect cells. Purified Tm's regulate thin filaments similar to that reported for muscle purified Tm. Steady-state Ca²⁺ binding to troponin C (TnC) in reconstituted thin filaments did not differ between the 3 Tm's, however disassociation of Ca²⁺ from filaments containing pseudo-phosphorylated Tm was slowed compared to WT Tm. Replacement of pseudo-phosphorylated Tm into myofibrils similarly prolonged the slow phase of relaxation and decreased the rate of the fast phase without altering activation kinetics. These data demonstrate that Tm pseudo-phosphorylation slows deactivation of the thin filament and muscle force relaxation dynamics in the absence of dynamic and steady-state effects on muscle activation. This supports a role for Tm as a key protein in the regulation of muscle relaxation dynamics.

Keywords

Tropomyosin; phosphorylation; myofibril; relaxation; human; calcium

Introduction

Tropomyosin (Tm) represents a critical myofilament protein in the Ca^{2+} regulation of actomyosin interaction and therefore of striated muscle contraction and relaxation [1, 2]. In striated muscle, Tm forms a coiled-coil dimer spanning 7 actins. The Tm dimer polymerizes in a head-to-tail fashion through interaction of 9-11 N- and C-terminal amino acids to form a continuous filament on actin [3]. In the absence of activating Ca^{2+} , the Tm strand occupies a position on actin directly blocking myosin binding sites [4–8]. In the presence of elevated intracellular Ca^{2+} , Ca^{2+} binding to troponin C (TnC) is transmitted through the troponin (Tn) complex to allow movement of the Tm filament on actin and exposure of myosin binding sites. Once bound, myosin further moves the Tm filament on actin promoting a fully active myofilament state and contraction. Relaxation requires the disassociation of Ca^{2+} from Tn, the detachment of myosin from actin and the return of Tm to its myosin blocking position. The strength of the Tm dimer interaction at the Tm head-to-tail overlap is an important factor in the position of Tm along the actin filament and therefore the regulation of muscle contractile activation and relaxation [3, 9–12].

The post-translational modification of myofilament proteins represents a critical mechanism to modulate the Ca^{2+} regulated contraction/relaxation of striated muscle [13]. Muscle Tm is primarily composed of or gene products that encode largely conserved 284 amino acid isoforms [2]. Both skeletal and cardiac muscle express varied amounts of the and Tm isoforms. In the mammalian heart and fast skeletal muscles the Tm isoform predominates. In addition to varied isoform expression, Tm is also modified by phosphorylation at a single Ser-283 residue [14]. The location of this phosphorylated residue is significant as it is located directly within the C-terminal portion of the head-to-tail overlap critical to Tm transmission of the Ca^{2+} activation signal. The significance of Tm phosphorylation to cardiac muscle contraction is demonstrated by its tight developmental regulation such that prior to birth the majority of Tm is phosphorylated with Tm phosphorylation decreasing to ~30% in the adult mammalian heart [15, 16]. Tm Ser-283 phosphorylation levels can also become altered in response to cardiac stress concurrent with altered contractile function [17–19]. At the molecular level, phosphorylation enhances head-to-tail interaction of neighboring Tm dimers and increases binding to troponin T [20–23]. These phosphorylation-dependent alterations in protein-protein interactions are affiliated with increased cooperative activation [24], maximal Ca^{2+} activated ATPase activity [22, 23] and variable reported effects on Ca^{2+} sensitivity of force development [22, 25]. While the functional effects of Tm phosphorylation have been investigated at steady-state equilibrium, the speed of striated muscle contraction and relaxation are dynamic non-equilibrium events. The current lack of knowledge regarding the dynamic effects of Tm phosphorylation on myofilament regulation limits our understanding of the functional role of Tm phosphorylation.

To determine the specific functional effects of Tm Ser-283 phosphorylation in the absence of other confounding post-translational modifications we investigated the role of recombinant Tm's. Native N-terminal acetylated Tm (Ser-283), pseudo-phosphorylated Tm (Ser-283 mutated to Asp) or phosphorylation null Tm (Ser-283 mutated to Ala) were expressed and purified from insect cells. Results employing these recombinant Tm's demonstrate Tm pseudo-phosphorylation does not alter calcium binding to regulated thin filaments or the equilibrium of myosin binding to Tm decorated actin. However, pseudo-phosphorylated Tm does decrease the dynamic dissociation of calcium from regulated thin filaments. Myofibrils exchanged with pseudo-phosphorylated Tm showed slowed relaxation of Ca^{2+} activated force following Ca^{2+} removal, in the absence of alterations of the kinetics of force activation. These results demonstrate for the first time a role of Tm Ser-283 phosphorylation in the modulation of striated muscle dynamic function.

Materials and Methods

Baculoviral *Spodoptera frugiperda* (Sf9) insect cell expression and purification of recombinant Tm

cDNA encoding wild-type mouse Tm Ser-283 was mutated to Ala generating the phosphorylation null S283A encoding mutant or to Asp generating the pseudo-phosphorylation encoding mutant by site directed mutagenesis (Stratagene)[26]. Authentic N-terminal acetylated mouse WT, S283A and S283D Tm were expressed by identical means in *Spodoptera frugiperda* insect cells (Sf9) employing the Bac-to-Bac Baculovirus Expression System with slight adaptation of the manufactures direction (Invitrogen). Baculovirus was generated from Tm cDNA containing an L21 nucleotide leader sequence [27] cloned into the pFastBac1 vector (Invitrogen) at the BamHI and EcoRI restriction sites. The sequence verified construct was transformed into DH10Bac *E. coli* (Invitrogen), resultant colonies streaked and recombinant bacmid purified from liquid culture by HiPure Plasmid DNA Miniprep (Invitrogen). Resultant PCR positive recombinant bacmid was transfected into healthy Sf9 cells by Cellfectin II Reagent (Invitrogen) and 72 hours post transfection media collected as a viral stock. This initial viral stock underwent 2 subsequent amplifications and the resultant amplified stock used to optimize Tm expression conditions. Mouse Tm's were expressed individually by inoculating 2×10^6 Sf9 cells/mL in 1 L of Sf-900 II SFM complete media (Gibco) supplemented with GlutaMAX (Gibco) and 10 g/mL gentamycin with Tm baculovirus. Sf9 insect cells do not express detectable amounts of Tm. Following incubation in shaking flasks at 27°C for 72 hours, cells containing Tm were collected by centrifugation at $4,000 \times g$. Resultant mouse Tm's were purified similar to that previously described [28] with the following adaptations. The Sf9 cell pellet was lysed by freeze-thaw at -80°C following suspension in (200mM NaCl and 50 mM Tris-HCl, pH 8.0 containing 2.5 g/mL Leupeptin, 1 g/mL pepstatin and 0.1 mM PMSF). Following centrifugation as above, freeze/thaw was repeated and the solution passed 10 times through a 25 g needle before clarification at $20,000 \times g$ for 10 min. Resultant supernatant was brought to 1 M NaCl and Tm precipitated by decreasing pH to 4.58 for 30 min on ice. Tm precipitant was recovered by centrifugation at 6,000 g for 20 min and the pellet suspended in (1 M KCl, 0.5 mM DTT and 10 mM MOPS, pH 7.0 containing 2.5 g/mL Leupeptin, 1 g/mL pepstatin and 0.1 mM PMSF). Following clarification at $6,000 \times g$ for 10 min, the Tm precipitation was repeated. Resultant pellet from the second precipitation was suspended in 0.5 mM DTT and 10 mM MOPS, pH 7.0 and clarified. Tm containing supernatant was subjected to ammonium sulfate precipitation at 50% saturation and contaminant precipitated proteins removed by centrifugation at $11,000 \times g$ for 30 min. The Tm containing supernatant was then precipitated by the addition of ammonium sulfate to 65% saturation, Tm recovered by centrifugation, the pellet dissolved in Buffer A (0.1 M NaCl, 0.5 mM DTT and 10 mM MOPS, pH 7.0) and dialyzed overnight at 4°C against Buffer A. Resultant proteins were then fractionated on a Source 15Q media column (General Electric) equilibrated in Buffer A and fractionated by elution with a 0.1 M – 0.5 mM NaCl gradient. Fractions containing pure Tm were identified by SDS-PAGE, pooled, dialyzed against 1 mM ammonium bicarbonate and lyophilized. The N-terminal acetylation of select Tm protein fractions was verified by Edman degradation N-terminal sequencing performed by the Biotechnology Center Protein Science Facility, University of Illinois at Urbana-Champaign (Urbana, IL).

Protein purification

Actin was purified from rabbit fast skeletal muscle acetone powder as previously described [26, 29]. Myosin utilized for ATPase experiments was purified from fresh rabbit fast skeletal muscle and the myosin S1 subfragment generated as previously described [28, 29]. Myosin S1 subfragment utilized for myosin binding to actin was porcine cardiac and was prepared

as previously described [29]. Rabbit fast skeletal Tn utilized in myofibril reconstitution was prepared as previously described [30]. Recombinant human cardiac troponin T, troponin I, TnC and TnC containing C35/84S and T53C mutations were expressed, purified and Tn complexes formed as previously described [26, 28].

Gel electrophoresis and Western Blot

Proteins (*E. coli*, purified or myofibrils) were solubilized in denaturing sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue and 10% glycerol) and resultant protein separated by SDS-PAGE on cooled 8 × 10 cm (Hoefer) 12% (29:1) polyacrylamide as previously described [26, 31]. Resultant gels were either coomassie stained or transferred to PVDF membrane for Western blot with the Tm specific monoclonal antibody CH1 (Iowa hybridoma bank) detected on film by ECLplus chemiluminescence (GE Healthcare) as previously described [26, 31].

Myosin S1 ATPase in reconstituted thin filaments

Thin filaments were reconstituted with rabbit skeletal actin, human cardiac troponin and mouse WT, S283A or S283D Tm and Ca^{2+} activated myosin S1 ATPase rate was conducted at sub-activating (pCa 10.0) and maximally activating (pCa 4.0). Conditions were 6 M Actin, 0.5 M Myosin S1, 1 M Tm in 1.2 M Tn at 35mM NaCl, 5mM MgCl_2 , 14mM MOPS, pH 7.0. The reaction was initiated upon the addition of ATP to 1mM and conducted at 20°C [32].

Ca^{2+} binding to TnC in reconstituted thin filaments

Thin filaments were reconstituted with rabbit skeletal actin, human cardiac troponin containing 2-(4'-iodoacetamido)anilino)naphthalene-6-sulfonic acid (IAANS)-labeled T53C, C34/85S TnC and mouse WT, S283A or S283D Tm and steady state Ca^{2+} binding to TnC measured by monitoring the change in IAANS fluorescence. Measurements were performed using a Perkin Elmer Life Sciences LS 55 fluorescence spectrometer at 15 °C. IAANS fluorescence was excited at 330 nm and monitored at 450 nm as microliter amounts of CaCl_2 were added to 2mL of each filament. Conditions were 4 M Actin, 0.5 M Tm and 0.3 M Tn in 150mM KCl, 3mM MgCl_2 , 0.5 mM DTT, 200 mM MOPS, pH 7.0 conducted at 15°C [33, 34].

Myosin S1 binding to Tm decorated actin

Steady-state binding of myosin to Tm-coated actin filaments was monitored through quenching of pyrene-actin fluorescence. Actin was labeled by pyrene through established methods [35]. Actin filaments were polymerized in the presence of the individual Tm proteins by dialyzing in a polymerization buffer that included 100 mM KCl, 1 mM MgCl_2 , 20 mM PIPES, pH 7.0. To follow the myosin S1 binding to the decorated actin filaments, the change in pyrene fluorescence upon addition of increasing concentrations of myosin S1 was measured on a Shimadzu RF5301PC spectrofluorometer using excitation and emission at 365 nm and 386 nm, respectively. The change in fluorescence was plotted as a function of myosin S1 concentration, and the concentration of myosin S1 required for half-maximal binding was reported.

Ca^{2+} disassociation from TnC in reconstituted thin filaments

Thin filaments were reconstituted with rabbit skeletal actin, human cardiac Tn containing IAANS-labeled T53C, C34/85S TnC and mouse WT, S283A or S283D Tm and the rate of Ca^{2+} disassociation from TnC measured as the change in IAANS fluorescence upon rapid mixing of 200 M Ca^{2+} saturated filaments with 10 mM EGTA. Measurements were conducted on an Applied Photophysics model SX.20 stopped-flow instrument with a dead

time of 1.4 ms at 15°C. IAANS fluorescence was excited at 330 nm and emission was monitored through a 510 nm broad band-pass interference filter [36, 37].

Myofibril experiments: Preparation, Tm-Tn replacement, force and kinetic measurements

Myofibrils were prepared by homogenization of glycerinated rabbit psoas muscles, as described previously [38]. All solutions were kept around 0 °C and contained a cocktail of protease inhibitors including 10 µM leupeptin, 5 µM pepstatin, 200 µM phenylmethylsulfonylfluoride, 10 µM E64, 500 µM NaN₃ and 0.5 mM DTT.

Endogenous Tm and Tn were extracted and replaced into myofibrils with recombinant Tm (mouse WT, S283A or S283D Tm) and rabbit fast skeletal Tn as previously described [30, 39]. Briefly, myofibrils were washed (5 – 7 times) in a low ionic strength solution (2 mM Tris-HCl, pH 8.0) to remove native Tm and Tn. Extracted myofibrils were then washed in a 200 mM ionic strength rigor solution (100 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 50 mM TrisHCl, pH 7.0) and reconstituted with exogenous Tm (5 µM) and Tn (2 µM) in a two steps protocol (0 °C, 2 hr incubation per step). Reconstituted myofibrils were washed and stored in 200 mM ionic strength rigor solution at 4 °C, and used within 3 days. At each stage of the protocol, samples were retained from both supernatant and pellet fractions and then used to determine the extent of the Tm-Tn extraction and replacement by SDS-PAGE analysis. The extent of the Tm-Tn extraction and replacement was determined by densitometry of the Tm band normalized to the Actin band. Average Tm replacement was 96% complete as assessed by 12% SDS-PAGE analysis [30].

In the present experiments we used previously published techniques to measure and control the force and length of isolated myofibrils activated and relaxed by fast solution switching [38, 40]. Briefly, a small volume of the myofibril suspension was transferred to a temperature controlled chamber (15 °C) filled with relaxing solution (pCa 8.0) and mounted on an inverted microscope. Selected preparations (single myofibrils or bundles of few myofibrils, 30–70 µm long, 1–3 µm wide) were mounted horizontally between two glass microtools. One tool was connected to a length-control motor that could produce rapid (<1 ms) length changes. The second tool was a calibrated cantilevered force probe (1–3 nm nN⁻¹; frequency response 2–5 kHz). Force was measured from the deflection of the image of the force probe projected on a split photodiode. Average sarcomere length and myofibril diameter were measured from video images (ca. 1800 X). The initial sarcomere length of the preparations was set around 2.4 µm. Myofibrils were activated and relaxed by rapidly translating the interface between two flowing streams of solution of different pCa (pCa 8.0, pCa 4.5) across the length of the preparation. The solution change took place with a time constant of 2–3 ms and was complete in < 10 ms [40]. Release–restretch protocols were applied to the myofibril to measure the rate of tension redevelopment under steady-state conditions of maximal activation. The rate constant of tension development following maximal Ca²⁺-activation (k_{ACT}), the rate constant of tension redevelopment following release–restretch (k_{TR}), and the rate constant of the final fast phase of relaxation following Ca²⁺ removal (fast k_{REL}) were estimated from mono-exponential fits of the tension records. The rate constant of the early slow force decline (slow k_{REL}) was estimated from the slope of the regression line fitted to the tension trace normalized to the entire amplitude of the tension relaxation transient; the duration of the slow relaxation phase was measured from tension traces from the onset of solution change at the myofibril to the intercept of the regression line with the fitted exponential [41, 42]. Activating and relaxing solutions, calculated as previously described (Tesi et al 2000), were at pH 7.0 and contained 10 mM total EGTA (CaEGTA/EGTA ratio set to obtain pCa 8.0 -fully relaxing solution- and 4.5 – maximally activating solution), 5 mM MgATP, 1 mM free Mg²⁺, 10 mM MOPS, propionate and sulphate to adjust the final solution to an ionic strength of 200 mM and monovalent cation concentration of 155 mM. Creatine phosphate (10 mM) and creatine kinase (200 unit

ml⁻¹) were added to all solutions. Contaminant inorganic phosphate (P_i) from spontaneous breakdown of MgATP and CP was reduced to < 5 M by a P_i scavenging system (purine-nucleoside-phosphorylase with substrate 7-methyl-guanosine)[38]. All solutions contained the cocktail of protease inhibitors mentioned above.

Statistical analysis

Ca²⁺ dissociation and myosin binding to actin were fit with a single exponential to determine K_d and k_{off}. Steady-state Ca²⁺ binding was fit to the Hill equation to determine a dissociation constant (K_d) and Hill coefficient. Myofibril rate constants k_{ACT}, k_{TR} and fast k_{REL} were determined by fit to mono-exponential of tension records. The rate constant of the myofibril slow k_{REL} was determined from the slope of the regression line fit to the normalized tension trace. All data is presented as mean ± SEM. Statistical significance is defined as p < 0.05 as determined by ANOVA.

Results

Expression and purification of N-terminal acetylated Tm

Full functionality of Tm's ability to regulate the thin filament requires its N-terminal acetylation within the head-to-tail overlap region [43, 44]. Inasmuch as proteins expressed in *E. coli* are not modified by N-terminal acetylation, studies investigating the role of Tm phosphorylation to date have largely utilized Tm purified from tissue. Tissue purified Tm may contain unknown and/or potentially confounding post-translational modifications that complicate the interpretation of phosphorylation results. To obtain functionally active Tm protein of identical molecular composition, we expressed and purified recombinant authentic N-terminal acetylated mouse -Tm by baculoviral infection of eukaryotic Sf9 insect cells [43, 45]. The time course of protein expression following infection with baculovirus encoding mouse -Tm demonstrates the appearance of a band at approximately 32 kDa (Fig. 1A). Transfer of a similarly loaded gel for Western blot with the Tm specific CH1 antibody verifies the identity of this band as Tm. The Western blot further demonstrates a lack of Tm expression in uninfected Sf9 cells (Fig. 1A, 0 hours), suggesting that Sf9 serve as a good host to express and purify recombinant Tm. Pseudo-phosphorylated Tm has previously been used to investigate the biochemical effects of Tm phosphorylation and was demonstrated to mimic properties of native phosphorylated Tm [21]. Baculovirus encoding native mouse Tm (Tm WT), phosphorylation-null Tm containing Ser-283 mutated to Ala (Tm S283A) and pseudo-phosphorylated Tm with Ser-283 mutated to Asp (Tm S283D) were generated and employed to express variant Tm in Sf9 cells. Resultant recombinant Tm were purified to homogeneity as demonstrated by coomassie stained gel and Western blot of the resultant purified proteins (Fig. 1B). Purified Tm's subjected to N-terminal sequencing by Edman degradation demonstrated a fully blocked N-terminus indicative of N-terminal acetylation (Fig. 2) and consisted of a single protein spot by two dimensional gel electrophoresis indicating a single Tm species (Data not shown). These data demonstrate the expression of highly pure and molecularly identical N-terminal acetylated mouse Tm WT, S283A and S283D variants from Sf9 insect cells.

Pseudo-phosphorylated Tm increases maximal ATPase Activity

Phosphorylated Tm purified from tissue demonstrates increased maximal calcium activated myosin S1 ATPase activity in reconstituted thin filaments [22, 23]. To validate the regulatory functionality of Sf9 purified Tm we measured the myosin S1 ATPase rate of filaments reconstituted with Tm WT, Tm S283A or Tm S283D in the absence and presence of Ca²⁺. At sub-activating Ca²⁺ (pCa 10.0) the ATPase rates of filaments reconstituted with Tm S283A or S283D were not different from that of Tm WT, while at maximally activating Ca²⁺ (pCa 4.0) the filaments reconstituted with Tm S283D were increased by 1.45 fold

compared to Tm WT. Filaments reconstituted with either Tm WT or Tm S283A were not different from each other at either Ca^{2+} (Fig. 3; ATPase rate at sub-activating Ca^{2+} (s^{-1}), Tm WT = 0.32 ± 0.03 , Tm S283A = 0.31 ± 0.04 , Tm S283D = 0.35 ± 0.03 ; ATPase rate at maximal activating Ca^{2+} (s^{-1}), Tm WT = 0.65 ± 0.05 , Tm S283A = 0.67 ± 0.05 , Tm S283D = 0.93 ± 0.07). These results demonstrate Sf9 purified Tm is functionally active and that Tm S283D pseudo-phosphorylation regulates thin filaments similar to that of phosphorylated Tm purified from muscle. Sf9 expressed pseudo-phosphorylated Tm therefore provides a unique model to study the effects of a single phosphorylation change on Tm's regulatory function.

Tm pseudo-phosphorylation does not alter steady-state Ca^{2+} sensitivity of the thin filament

Calcium binding to TnC is a necessary upstream step in the regulation of Tm's position on actin and initiation of muscle contraction [4, 5]. To investigate the mechanism of Tm phosphorylation to alter ATPase activity we measured Ca^{2+} sensitive activation as the change in fluorescence upon steady-state Ca^{2+} binding to IAANS labeled TnC in Tm and Tn reconstituted actin filaments [33]. The results in figure 4 demonstrate filaments reconstituted with WT, phosphorylation null S283A or pseudo-phosphorylated S283D Tm all exhibit similar Ca^{2+} sensitivity of IAANS fluorescence (Fig. 4 and Table 1). Interestingly, while the k_d of TnC Ca^{2+} binding was unaltered, the Hill coefficient of Tm S283D filaments trended to be increased (ANOVA $p = 0.058$), suggesting Tm pseudo-phosphorylation increased cooperative activation of the thin filament (Table 1). These results demonstrate Tm phosphorylation does not directly affect steady-state Ca^{2+} sensitivity of thin filament activation, but may alter the spread of this Ca^{2+} activation.

In addition to Ca^{2+} , full activation of the myofilament and muscle force requires the downstream strong-binding of myosin to actin [4, 5]. To investigate the effects of Tm phosphorylation on this downstream activation we measured the change in fluorescence upon steady-state myosin binding to Tm decorated pyrene-actin. Myosin induced quenching of pyrene-labeled actin fluorescence demonstrates the affinity of myosin S1 binding to actin was not altered by the presence of WT, phosphorylation null S283A or pseudo-phosphorylated S283D Tm (Fig. 5; myosin concentration required to reach 50% maximal binding (nM); Tm WT = 18.9 ± 2.3 , Tm S283A = 17.8 ± 1.8 , Tm S283D = 17.1 ± 3.4). These results demonstrate Tm phosphorylation does not alter steady-state binding of myosin to actin differentially from that of WT, non-phosphorylated Tm.

Tm pseudo-phosphorylation decreases the rate of Ca^{2+} dissociation from regulated thin filaments

The kinetics of Ca^{2+} release from TnC are critical to regulate the position of Tm, cross-bridge cycling and thus the speed of muscle relaxation [7, 36]. To determine the effects of Tm phosphorylation on dynamic myofilament Ca^{2+} deactivation we measured the rate of Ca^{2+} disassociation from Tn and Tm reconstituted actin filaments as the change in Ca^{2+} bound IAANS-labeled TnC fluorescence following rapid mixing with EGTA [36]. Figure 6 demonstrates representative data for the change in IAANS fluorescence over time of various Tm containing filaments that has been normalized and staggered for clarity. These results demonstrate the rate of Ca^{2+} disassociation from filaments containing Tm S283D was slowed by 13% compared to those containing Tm WT. Ca^{2+} disassociation of Tm WT did not differ from that of Tm S283A filaments (Fig. 6 and Table 1). Utilizing this rate of disassociation and the previously measured K_d , we calculated the apparent rate of Ca^{2+} association. This calculated Ca^{2+} association rate was likewise decreased by 10% in Tm S283D compared to Tm WT filaments (Table 1). Together these data demonstrate the pseudo-phosphorylation of Tm slows dynamic Ca^{2+} regulation of the myofilament.

Tm pseudo-phosphorylation prolongs myofibril force relaxation

To determine whether Tm S283D induced alteration of Ca^{2+} regulated thin filament dynamics would translate into altered dynamic contractile function we measured force changes upon rapid alteration of Ca^{2+} in Tm replaced skeletal myofibrils [30, 39, 41]. Endogenous Tm and Tn in myofibrils were batch extracted and exogenous recombinant Tm and skeletal Tn were reconstituted by a 2-step procedure [30]. SDS-PAGE comparison of sham, extracted and reconstituted myofibrils demonstrating endogenous Tm and Tn removal upon extraction with the replacement of exogenous Tm and Tn upon reconstitution with Tm WT, S283A or S283D (Fig. 7A). Densitometry analysis revealed an average of $19 \pm 4\%$ endogenous Tm remained after extraction with subsequent reconstitution to 96% Tm (Tm WT $92 \pm 9\%$, Tm S283A $94 \pm 9\%$, $101 \pm 14\%$, $n=2-4$). Reconstituted myofibrils mounted in the force recording apparatus were then maximally Ca^{2+} -activated and fully relaxed by fast solution switching. In each activation-relaxation cycle the following measurements were made: i) the rate of force activation (k_{ACT}) upon maximal Ca^{2+} -activation (from pCa 8.0 to pCa 4.5); ii) maximal steady-state tension (P_0); iii) the rate of tension redevelopment upon a rapid release-restretch length maneuver at maximal activation (k_{TR}); iv) the duration and rate of the slow isometric phase of force relaxation and the rate of the fast relaxation phase upon rapid switch back to relaxing Ca^{2+} (k_{REL}). Representative activation-relaxation traces demonstrate that myofibrils reconstituted with the varied Tm's did not differ in maximal tension, in the rate of activation nor in the rate of tension redevelopment (Fig. 7C and Table 2). Interestingly, the analysis of normalized relaxation traces on expanded time scale shows force relaxation of Tm S283D myofibrils was prolonged (Fig. 7C). Following rapid switch from maximally activating Ca^{2+} to relaxing Ca^{2+} , Tm S283D myofibrils showed prolonged duration of the slow phase of relaxation and a decreased rate of the fast phase compared to WT (Fig. 7C-E and Table 2). In S283A or Tm WT reconstituted myofibrils relaxation was not different. These results demonstrate that Tm pseudo-phosphorylation with Tm S283D prolongs muscle relaxation and suggests Tm phosphorylation as an important mechanism for the modulation of striated muscle dynamic function.

Discussion

The phosphorylation of tropomyosin at Ser-283 was first identified as a striated muscle post-translational modification more than 30 years ago [14], yet its role in modulating muscle force remains unclear. To investigate the effects of Tm phosphorylation in the absence of other confounding post-translational modifications we employ molecularly identical recombinant, N-terminal acetylated wild-type (S283), Ser-283 phosphorylation null (S283A) or Ser-283 pseudo-phosphorylated (S283D) Tm. Our main findings demonstrate pseudo-phosphorylated Tm increases maximal myosin ATPase activity (Fig. 3) without altering the steady-state Ca^{2+} sensitive regulation of the myofilament (Fig. 4) similar to those previously reported for muscle purified Tm [22, 23]. Additionally we demonstrate for the first time that Tm pseudo-phosphorylation alters the time course of striated muscle force relaxation through altered thin filament dynamics (Figs. 6 and 7) without altering the rate of activation, maximal tension development (Table 2) or myosin binding to actin (Fig. 5). Together these findings expand our understanding of Tm as a modulator of dynamic striated muscle relaxation rather than steady-state, equilibrium contractile muscle function.

Pseudo-phosphorylated Tm is a model of Ser-283 phosphorylated muscle Tm

Experiments to date investigating the effects of Tm phosphorylation have employed Tm purified from muscle. Muscle purified Tm is inherently prone to complicating factors including the loss of phosphorylation over time, the presence of other muscle induced non-identified post-translational modifications, the selective co-purification of different modifications, the potential of multiple amino acid residue phosphorylations and the non-

specific effects of kinase/phosphatase treatment. These factors may contribute to a confounding interpretation of specific Tm Ser-283 phosphorylation functional effects. To investigate the sole role of Tm Ser-283 phosphorylation in the absence of these confounding factors we employed molecularly identical recombinant wild-type, phosphorylation null and pseudo-phosphorylated Ser-283 Tm. Tm requires N-terminal acetylation to be fully active [43, 44], however proteins expressed in *E. coli* are not acetylated.

Native N-terminal acetylation is significant to investigations of Tm Ser-283 phosphorylation effects since both modifications are located in the functionally important Tm head-to-tail overlap region [3, 9, 10, 12, 43]. We therefore chose to express and purify native N-terminal acetylated recombinant Tm in Sf9 insect cells to retain native N-terminal acetylated head-to-tail function. Similar to that previously demonstrated for Sf9 expressed Tm, our purified Tm's are fully N-terminal acetylated (Fig. 2)[45]. Purified Tm's consisted of a single protein spot by two dimensional gel electrophoresis supporting the expression and purification of a single molecular species (data not shown). Purified Tm's retain regulatory inhibition in the absence of Ca^{2+} (Fig. 3A), while Ser-283 pseudo-phosphorylated Tm increases maximal myosin ATPase (Fig. 3B) in the absence of altered Ca^{2+} sensitive activation (Fig. 4). These findings are similar to those demonstrated for muscle purified phosphorylated Tm [22, 23]. Together our results demonstrate the Ser-283 to Asp pseudo-phosphorylation of Sf9 expressed Tm serves as model to investigate the role of Tm phosphorylation on myofilament regulation in the absence of other confounding post-translational modifications.

Tm Ser-283 pseudo-phosphorylation does not alter steady-state myofilament regulatory function

To date the role of Tm phosphorylation to affect Ca^{2+} activation of the myofilament is unclear. Initial biochemical studies demonstrated Tm phosphorylation did not alter skeletal thin filament Ca^{2+} regulation of myosin activity [22, 23]. More recently gelsolin extracted cardiac fiber preparations that were reconstituted with skeletal actin, cardiac Tn and phosphorylated Tm were shown to exhibit increased Ca^{2+} sensitivity of force production [25]. Our data demonstrates steady-state Ca^{2+} activation of skeletal actin and cardiac Tn thin filaments reconstituted with Tm S283D were not different from that of non-phosphorylated WT or S283A Tm (Fig. 4 and Table 1). This finding is further supported by the lack of altered steady-state myosin binding to WT versus S283D Tm decorated actin (Fig. 5). Correspondingly, we demonstrate that the exchange of skeletal myofibrils with Tm S283D did not alter steady-state tension production at maximal activating Ca^{2+} (Table 2). While it remains possible that the ability of Tm phosphorylation to alter Ca^{2+} sensitivity requires coordinated components of the cardiac muscle myofilament, our data supports a lack of Tm S283D impact on steady-state, equilibrium Ca^{2+} regulation of the myofilament.

Tm Ser-283 pseudo-phosphorylation slows dynamic muscle relaxation

While steady-state, equilibrium measurements of myofilament activation are informative, measurements of the dynamic rate of myofilament activation and deactivation are important to determining the speed of contraction/relaxation. To date there are no investigations into the effects of Tm phosphorylation on the dynamic properties of muscle activation/deactivation. In addition to the 13% slowed Ca^{2+} disassociation in the Tm S283D thin filament we also calculated a concurrent 10% slowing of the calculated Ca^{2+} association rate (Table 1). Although we did not measure a statistical difference in Tm S283D contractile activation or the rate of force activation at the muscle level, both trended to be slowed (Table 2). The similar slowing of both disassociation and association supports why Tm S283D does not alter steady-state Ca^{2+} sensitive activation as we (Fig. 4 and Table 1) and others [22] have observed.

Our data demonstrates that Tm phosphorylation slows Ca^{2+} deactivation of the thin filament (Fig. 6) resulting in prolongation of the slow phase duration and decreased rate of fast phase force relaxation (Fig. 7C–E). We propose 2 potential mechanisms for this Tm regulation of muscle relaxation. 1) Tm phosphorylation slows the intrinsic disassociation rate of Ca^{2+} from the thin filament. This mechanism is consistent with our finding that Tm phosphorylation is sufficient to decrease Ca^{2+} disassociation in the isolated thin filament (Fig. 6 and Table 1). Tm altered Ca^{2+} disassociation is also in agreement with our previous demonstration that a TnC mutant with decreased Ca^{2+} disassociation similarly prolonged the duration of the slow phase of force relaxation [41]. We therefore propose Tm phosphorylation directly slows Ca^{2+} disassociation to prolong thin filament deactivation and slow muscle force relaxation. 2) Tm phosphorylation induces increased cooperativity. Our data demonstrates Tm S283D trends towards increased cooperative Ca^{2+} activation of the thin filament (ANOVA $p = 0.058$; Table 1), presumably as a result of a phosphorylation induced increase in head-to-tail interaction strength [21, 23]. This finding is in agreement with data from Rao *et al.* who demonstrated phosphorylated Tm increased the myosin induced cooperative activation of the thin filament [24] and increased myosin cross-bridge lifetime [46]. We therefore propose the Tm induced strengthening of the head-to-tail overlap increases thin filament cooperativity resulting in a slowed thin filament deactivation, an extended cross-bridge lifetime and maintenance of isometric force to prolong muscle force relaxation. Such a mechanism is consistent with our observation that Tm S283D lengthened the slow phase of relaxation (Fig. 7 and Table 2). These 2 mechanisms are by no means exclusive and it is more likely that both mechanisms contribute to the Tm altered relaxation phenomenon.

The work produced by striated muscles is regulated by factors including the rate of force relaxation, however Tm phosphorylation slowed relaxation may be of differential significance to cardiac and skeletal muscle function. Increased cardiac muscle function (cardiac output) requires increased rates of relaxation to maintain adequate ventricular filling [47]. Tm phosphorylation induced slowing of cardiac relaxation may function to prolong the diastolic portion of the contraction cycle such that it could encroach on ventricular filling to decrease cardiac output. Tm phosphorylation prolongation of relaxation would function opposite to that of the well-described troponin I protein kinase A phosphorylation induced increase in cardiac relaxation [48–50]. Alternatively, skeletal muscle function may be improved upon Tm phosphorylation. The force produced by skeletal muscle is primarily regulated by fiber recruitment and Tm phosphorylation induced prolongation of relaxation may decrease the threshold for skeletal fiber summation and increase force production. To date the organ level functional effects of Tm phosphorylation on cardiac and skeletal muscle function are unknown.

Conclusions

Our data demonstrates Tm S283D pseudo-phosphorylation functions to slow deactivation of the thin filament and muscle force relaxation dynamics in the absence of dynamic and steady-state effects on muscle contraction. We propose the phosphorylation of Tm Ser-283 is therefore detrimental to cardiac output as the result of impeded ventricular filling. Future efforts are necessary to determine the ultimate contribution of this myofilament phosphorylation during adaptation to physiological and pathological cardiac stress and dysfunction.

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Abbreviations

Tm	tropomyosin
Tn	troponin
TnC	troponin C
Tm WT	Tm S283 wild-type
Tm S283A	Tm phosphorylation null Ser-283 to Ala mutant
Tm S283D	Tm pseudo-phosphorylation Ser-283 to Asp mutant
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
IAANS	2-(4'-iodoacetamidoanilo)naphthalene-6-sulfonic acid

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Highlights

Sf9 insect cell expressed and purified Tm is N-terminal Acetylated.

Pseudo-phosphorylated Tm S283D regulates filaments like phosphorylated muscle Tm.

Tm S283D does not alter steady-state Ca^{2+} binding to the thin filament.

Tm S283D slows filament Ca^{2+} dissociation and prolongs myofibril force relaxation.

Tm phosphorylation contributes to the modulation of muscle relaxation.

FIGURE 1.

Baculoviral Tm expression and purification in Sf9 cells. (A) Sf9 insect cells were infected with Tm WT encoding baculovirus and samples taken at various time point post infection. Coomassie stained SDS-PAGE gel and Western blot with the anti-Tm specific monoclonal antibody (CH1) demonstrate no expression of Tm in non-infected Sf9 cells and time course of Tm expression. (B) Coomassie stained SDS-PAGE gel and Western blot with the anti-Tm specific monoclonal antibody (CH1) of purified variant Tm's demonstrates their purity. Sf9+Tm, Sf9 Tm control; Mr Marker, molecular weight marker; Tm WT, Tm S283 wild-type; Tm S283A, Tm phosphorylation null Ser-283 to Ala mutant; Tm S283D, Tm pseudo-phosphorylation Ser-283 to Asp mutant.

FIGURE 2.

Sf9 expressed Tm is N-terminal Acetylated. Purified Tm fractions were subjected to N-terminal sequencing by Edman degradation to determine the extent of N-terminal acetylation. Representative chromatography traces demonstrating the signal from (A) the solution Blank and (B) a mixed amino acid Standard. Chromatography traces for 3 consecutive cycles of Edman degradation of Tm; (C) Cycle 1, (D) Cycle 2 and (E) Cycle 3. The lack of a detectable amino acid signal in each of the 3 rounds of Edman degradation demonstrates the inability of the Edman process to liberate individual amino acid residues from the Tm polypeptide and is indicative of full N-terminal blockage by acetylation.

FIGURE 3.

Myosin S1 ATPase of Tm variant reconstituted thin filaments. Thin filaments were reconstituted with actin, Tn and various Tms in the presence of sub-activating or maximally activating Ca^{2+} and the rate of myosin S1 ATPase measured. (A) Graph representing myosin ATPase activity at pCa 10.0. (B) Graph representing myosin ATPase activity at pCa 4.0. Tm WT, Tm S283 wild-type (black bar); Tm S283A, Tm phosphorylation null Ser-283 to Ala mutant (blue bar); Tm S283D, Tm pseudo-phosphorylation Ser-283 to Asp mutant (red bar); *, $p < 0.05$.

FIGURE 4.

Ca^{2+} binding to TnC in reconstituted thin filaments. Thin filaments were reconstituted with actin, Tn, variant Tm's and Ca^{2+} binding to TnC measured as the percent normalized change in IAANS-labeled TnC fluorescence at various Ca^{2+} concentrations. Tm WT, Tm S283 wild-type (black solid line); Tm S283A, Tm phosphorylation null Ser-283 to Ala mutant (blue dashed line); Tm S283D, Tm pseudo-phosphorylation Ser-283 to Asp mutant (red dashed line).

FIGURE 5.

Myosin S1 binding to Tm decorated actin. Pyrene-labeled actin was fully decorated with the varied Tms and the steady-state change in fluorescence upon the addition of varied myosin S-1 concentrations measured as myosin binding to actin. Tm WT, Tm S283 wild-type (black line); Tm S283A, Tm phosphorylation null Ser-283 to Ala mutant (blue dashed line); Tm S283D, Tm pseudo-phosphorylation Ser-283 to Asp mutant (red dashed line).

FIGURE 6.

Dynamic Ca^{2+} disassociation from TnC. Thin filaments were reconstituted with actin, Tn, variant Tm's and the rate of Ca^{2+} disassociation from TnC measured as the change in IAANS-labeled TnC fluorescence over time upon rapid mixing with EGTA. The time course of Ca^{2+} disassociation was fit with a single exponential equation to calculate the kinetic rates and represent an average of 3 to 5 individual traces. The data traces have been normalized and staggered for clarity. Tm WT, Tm S283 wild-type (black line); Tm S283A, Tm phosphorylation null Ser-283 to Ala mutant (blue line); Tm S283D, Tm pseudo-phosphorylation Ser-283 to Asp mutant (red line); V, volts; ms, milliseconds.

FIGURE 7.

Tm exchanged myofibril tension kinetics. Endogenous Tm and Tn was extracted from myofibrils and force kinetics measured upon rapid solution switching between sub-activating (pCa 8.0) and high (pCa 4.5) Ca^{2+} . (A) Representative Coomassie stained SDS-PAGE gel demonstrating the efficiency of endogenous Tm and Tn extraction from myofibrils and subsequent reconstitution with recombinant Tn and Tm variants. (B) Representative normalized myofibril activation-relaxation force traces demonstrating the rate of activation and maximal tension development following rapid solution switching from low to high Ca^{2+} and the rate of tension redevelopment following rapid change in length. (C) Representative normalized myofibril relaxation traces over an expanded time frame

demonstrate Tm S283D myofibrils exhibit slowed relaxation following rapid solution switching from high to low Ca^{2+} . (D) Graph demonstrating the significant prolongation of Tm S283D myofibril slow phase relaxation duration. (E) Graph demonstrating the significant slowing of the Tm S283D myofibril fast phase relaxation rate. k_{REL} , rate of relaxation; Rec, reconstituted; Tm WT, Tm S283 wild-type (black); Tm S283A, Tm phosphorylation null Ser-283 to Ala mutant (blue); Tm S283D, Tm pseudo-phosphorylation Ser-283 to Asp mutant (red); *, $p < 0.05$.

Table 1

Effects of Tm S283 WT, Tm S283A phosphorylation null or Tm S283D pseudo-phosphorylation reconstitution on thin filament calcium binding characteristics.

THIN FILAMENT	$\text{Ca}^{2+} k_d$ (μM)	n_H	$\text{Ca}^{2+} k_{\text{off}}$ (/s)	Apparent $\text{Ca}^{2+} k_{\text{on}}$ ($\times 10^6 \text{M}^{-1} \text{s}^{-1}$)
Tm WT	1.71 ± 0.08	2.00 ± 0.06	237 ± 2	13.9 ± 0.7
Tm S283A	1.75 ± 0.08	2.31 ± 0.31	235 ± 4	13.4 ± 0.7
Tm S283D	1.65 ± 0.08	$2.45 \pm 0.07^\#$	$206 \pm 2^*$	12.5 ± 0.6

Values are mean $\pm$ SEM. k_d , Ca^{2+} concentration at 50% maximal activation; k_{off} , rate of Ca^{2+} disassociation from TnC; k_{on} , rate of Ca^{2+} binding to TnC calculated from k_d and k_{off} . ANOVA;

*
p < 0.05,

p = 0.058.

Effects of Tn-Tm extraction and reconstitution with skeletal Tn and WT, phosphorylation null, or pseudo-phosphorylation Tm on rabbit psoas myofibrils tension kinetics.

Table 2

MYOFIBRIL TREATMENT	TENSION GENERATION			RELAXATION	
	P ₀ (mN mm ⁻²)	k _{ACT} (s ⁻¹)	k _{TR} (s ⁻¹)	Slow Phase	
				Duration (ms)	k _{REL} (s ⁻¹)
Sham (9)	443 ± 29 *	6.86 ± 0.44	7.14 ± 0.25	62 ± 4	1.63 ± 0.16
Tm WT (9)	364 ± 36	6.75 ± 0.40	7.15 ± 0.25	68 ± 8	1.70 ± 0.23
Tm S283A (12)	353 ± 18	6.82 ± 0.36	6.68 ± 0.39	67 ± 3	1.81 ± 0.22
Tm S283D (8)	324 ± 22	6.59 ± 0.27	6.61 ± 0.34	90 ± 5 *	1.64 ± 0.21
					29 ± 3 *

Each group of data are from different myofibril batches. All values are given as mean ± SEM with the number in parentheses representing the number of myofibrils. Sham, solution treated but not extracted or reconstituted. Tm WT, Ser-283 reconstituted; Tm S283A, phosphorylation null Tm reconstituted; Tm S283D, pseudo-phosphorylation Tm reconstituted; P₀, maximum isometric tension; k_{ACT}, rate constant of tension rise following step-wise pCa decrease (8.0-4.5) by fast solution switching; k_{TR}, rate constant of tension redevelopment following release-restretch of maximally activated myofibrils; k_{REL}, rate constant of tension relaxation for slow and fast phases of tension decrease following step-wise pCa increase (4.5-8.0).

* p<0.05 (ANOVA).