BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

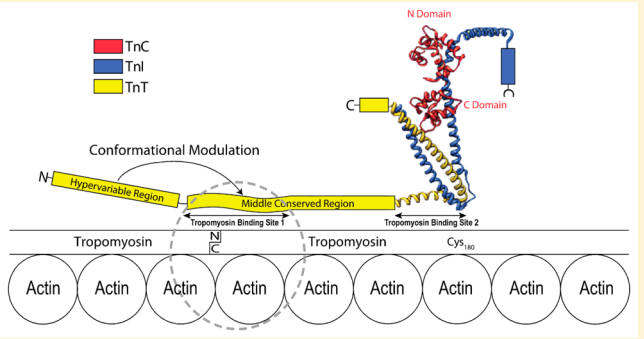
N-Terminal Hypervariable Region of Muscle Type Isoforms of Troponin T Differentially Modulates the Affinity of Tropomyosin-Binding Site 1

Chinthaka Amarasinghe and J.-P. Jin*

Department of Physiology, Wayne State University School of Medicine, Detroit, Michigan 48201, United States

Supporting Information

ABSTRACT: The troponin complex plays a central role in the contraction and relaxation of striated muscle by enacting Ca^{2+}-regulated allosteric changes in the sarcomeric thin filaments. The troponin T subunit (TnT) contains two binding sites for tropomyosin (Tm) and is responsible for anchoring the troponin complex to the thin filament. While the amino acid sequences of the regions containing the Tm-binding sites are highly conserved among the three muscle type isoforms of TnT, previous studies have observed significant discrepancies in the affinity of Tm-binding site 1 in the chymotryptic fragment T1 of different TnT isoforms. Here we cross-examined the Tm-binding affinity of TnT isoforms and molecular engineered fragments using affinity chromatography and microplate protein binding assays to investigate the effects of the evolutionarily diverged N-terminal region that is significantly variable among muscle type isoforms. The results demonstrated that the C-terminal T2 fragment of TnT containing the Tm-binding site 2 had similarly high affinity across isoforms. In the absence of the N-terminal variable region, Tm-binding site 1 in the conserved middle region of TnT also exhibited high intrinsic affinity. The presence of isoform specific N-terminal variable region differentially reduced the binding affinity of TnT for Tm, primarily at binding site 1 in the middle region. These findings indicate that the N-terminal variable region of TnT plays a pivotal role in the functional difference of muscle type-specific isoforms and the developmental and pathogenic splice variants by modulating the interaction with Tm during Ca^{2+} regulation of cardiac and skeletal muscle contraction and relaxation.



The troponin complex plays a central role in the Ca^{2+}-regulation of the sarcomeric thin filament during the contraction and relaxation of vertebrate striated muscle, i.e., skeletal and cardiac muscles.[1−5] The troponin complex consists of three protein subunits: Troponin C (TnC), the calcium sensor; troponin I (TnI), the inhibitory subunit; and troponin T (TnT), the tropomyosin-binding subunit.[2,3] Encoded by homologous genes, three TnT isoforms have evolved in vertebrates. The cardiac, fast, and slow skeletal muscle TnT isoforms are expressed in a muscle type specific manner, indicating that their diverged function corresponds to the diverged contractility of cardiac, fast, and slow skeletal muscle.[3,6]

The interaction between TnT and Tm is essential in muscle contraction and relaxation. TnT is known to have two binding sites for tropomyosin (Tm) and is responsible for anchoring the troponin complex to the thin filament. The structure of TnT was classically divided into two fragments, T1 and T2. These two fragments were produced by limited chymotrypsin digestion, with each fragment containing a Tm binding site.[7−11] Although the high resolution partial crystal structure of troponin revealed locations of TnT's binding sites for TnI and TnC,[4,5] a precise localization of the Tm-binding sites requires further crystallographic studies at the whole thin filament level.

Nonetheless, recent protein binding studies have further mapped the two Tm-binding sites using genetically engineered TnT fragments. In the T1 region, the Tm-binding site 1 corresponds to a 39-amino-acid segment in the N-terminal portion of the conserved middle region of TnT whereas the Tm-binding site 2 is located within a 25-amino-acid segment near the beginning of the C-terminal T2 region.[12]

After four decades of studies on the interaction between TnT and Tm,[7−13] an unresolved major discrepancy in the literature is the difference in Tm-binding affinity when comparing the T1 and T2 fragments of different TnT isoforms. The T1 fragment of rabbit fast skeletal muscle TnT has been shown to have a greater or equally high affinity for Tm compared to that of the rabbit fast TnT T2 fragment,[8,13] while the T1 fragment of mouse slow skeletal muscle TnT has been observed to have a significantly lower binding affinity for Tm than that of the mouse slow TnT T2 fragment.[12]

Received: April 2, 2015
Revised: May 13, 2015
Published: May 29, 2015

ACS Publications © 2015 American Chemical Society

DOI: 10.1021/acs.biochem.5b00348
Biochemistry 2015, 54, 3822−3830

Since the amino acid sequences of the Tm-binding sites are highly conserved among TnT isoforms and in all vertebrate species examined,[12] the significant discrepancy in Tm-binding affinity may be attributed to the modulatory function of the N-terminal region that is hypervariable among muscle type-specific TnT isoforms.[3,6,14,15] The hypothesis that the N-terminal variable region of TnT may affect the affinity of Tm-binding sites is supported by the role of the N-terminal region in modulating the overall molecular conformation of TnT and the binding affinities for TnI, TnC, and Tm.[16−19]

In the present study, we carried out cross-examination experiments using intact as well as representative fragments of fast, slow, and cardiac isoforms of TnT to investigate the modulatory effects of the N-terminal variable region on the Tm-binding affinity of TnT. To compare with previous studies primarily done using rabbit fast TnT fragments,[7−9,11,13] we also included the T1 and T2 fragments of rabbit fast TnT in the present study. The results demonstrated that the C-terminal T2 region of TnT containing the Tm-binding site 2 showed comparably high binding affinities across the three TnT isoforms. In the absence of the N-terminal variable region, Tm-binding sites 1 in the conserved middle region of TnT also exhibited high intrinsic Tm-binding affinity. The presence of the isoform specific N-terminal variable region differentially reduced the binding affinity of TnT for Tm, primarily at the middle region binding site 1. These novel findings indicate that the N-terminal variable region plays a pivotal role in the functional difference of muscle fiber type-specific TnT isoforms and the developmental and pathogenic splice variants by modulating the interactions with Tm during cardiac and skeletal muscle contraction and relaxation.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids and Preparation of Intact TnT and TnT Fragments. The representative TnT isoforms and fragments used in our study are outlined in Figure 1.

cDNAs encoding rabbit fast TnT were cloned using RT-PCR as described previously.[20] cDNAs encoding T1 and T2 fragments of rabbit fast TnT were amplified by PCR from its intact cDNA. cDNAs encoding intact mouse fast, slow, and cardiac TnT were amplified by PCR from the respective intact cDNAs and inserted into the pAED4 expression vector.[21] cDNAs encoding the T1, middle, and T2 fragments of mouse cardiac TnT (McTnT), mouse slow TnT (MsTnT), and mouse fast TnT (MfTnT) were amplified by PCR from their respective intact cDNA.[19,22,23] The cDNA inserts were subcloned into a pAED4-based expression vector employing an N-terminal fusion tag of 10−15 amino acids ($[HEEAH]_{2/3}$) derived from the N-terminal variable region of chicken breast muscle TnT[24] to facilitate the purification via metal affinity chromatography.[17,18,20,24]

The PCR primers (Table 1) were designed to add a translational initiation codon or termination codon at the 5′ and 3′ ends of the cDNA inserts with unique restriction enzyme sites for the subsequent cloning into the expression plasmids. All recombinant plasmids were sequenced to confirm the correct insertion of the specific cDNA inserts.

Competent BL21(DE3)pLysS *E. coli* cells were transformed with the expression plasmid encoding intact TnT and TnT fragments as previously described.[25] Freshly transformed BL21(DE3)pLysS colonies were used to inoculate LB media containing 100 μg/mL ampicillin and 12.5 μg/mL chloramphenicol. The cultures were incubated at 37 °C with vigorous shaking and induced at $O.D._{600\ nm}$ of ∼0.3 by adding IPTG to a final concentration of 0.4 mM. The cultures were continued for 3 h and harvested as previously described.[12,25]

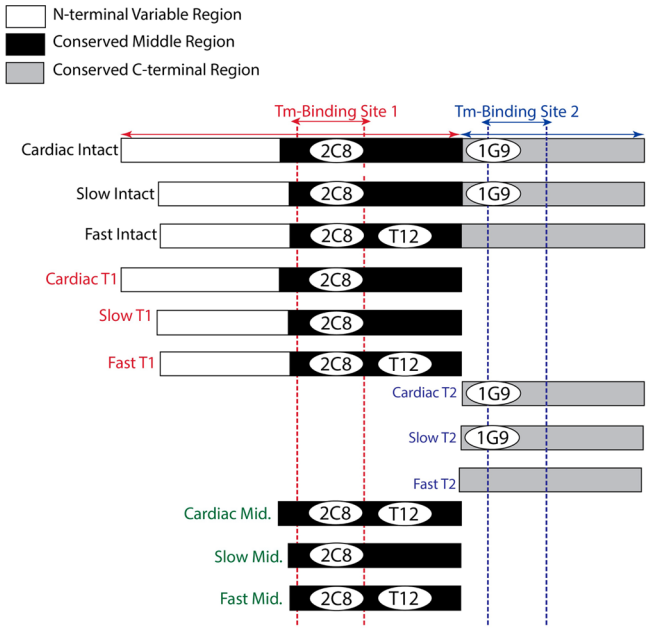


Figure 1. TnT constructs used in the present study. Fragments of cardiac, slow, and fast skeletal muscle TnT isoforms are constructed to represent the three functional regions of TnT: The N-terminal hypervariable region, the conserved middle region and the conserved C-terminal region. The T2 fragment contains the C-terminal region; the T1 fragment contains the conserved middle region and the N-terminal hypervariable region; while the middle fragment contains only the conserved middle region. Locations of the epitopes recognized by specific mAbs 2C8,[12] T12,[48] CT3,[19] and 1G9[14] are indicated on the TnT construct maps. The T12 epitope in cardiac TnT becomes more clearly recognizable by mAb T12 when the N-terminal variable region is removed.

The intact nonfusion TnT and TnT fragments were purified via ammonium sulfate precipitation. Nonfusion TnT and fragments were further purified using ion exchange chromatography and size exclusion chromatography as previously described.[12,25] The tagged TnT fragments were further purified via Zn(II) affinity chromatography.[17,24] The purified fractions of TnT fragments were dialyzed against 0.1% formic acid and lyophilized for use in functional studies.

SDS−Polyacrylamide Gel Electrophoresis (SDS−PAGE) and Western Blotting. Protein samples were homogenized in SDS−PAGE sample buffer containing 2% SDS, 0.3% bromophenol blue, 10% glycerol, 50 mM Tris−HCl, pH 8.8, and analyzed on gels in Laemmli buffer as described previously.[12,25] The resulting gels were stained with Coomassie Blue R250 to reveal the protein bands.

Duplicate gels were transferred to nitrocellulose membrane and Western blotting was performed as previously described.[25] Monoclonal antibodies (mAb) recognizing specific TnT epitopes[14] were used to verify the intact TnT and TnT fragments used in our study.

Tropomyosin Affinity Chromatography. Purified rabbit skeletal muscle α/β-Tm was prepared as previously described[26] and covalently conjugated to CNBr-activated Sepharose 4B (GE Healthcare) according to the manufacturer's protocol. A Tm affinity column of 0.5 mL bed volume was prepared and

DOI: 10.1021/acs.biochem.5b00348
Biochemistry 2015, 54, 3822−3830

Table 1. PCR Primers Used for the Construction of TnT Fragments[a]

TnT fragment	forward primer	reverse primer
Rabbit Fast T1	5′-AGCCCATATGTCTGACGAGGAAGT-3′ (NdeI)	5′-CCGAATTCTAGTAGCTGCTGTAGTTG-3′ (EcoRI)
Rabbit Fast T2	5′-CAACTACAGCCATATGCTGCTGGCCAA-3′ (NdeI)	5′-TCTCTCGAGTTAGTGCTTCTGG-3′ (XhoI)
Mouse Cardiac Middle	5′-AGCCCCATATGCTCTTCATGCCCA-3′ (NdeI)	5′-CTTGAATTCTCACCCTCCAAA-3′ (EcoRI)
Mouse Slow T1	5′-CTCTCCCATATGTCAGACACCGAAGAA-3′ (NdeI)	5′-CAGAACTTTCCATGGTTACTTCTTCTTGGC-3′ (NcoI)
Mouse Slow T2	5′-CTGTCCCATATGGGAGCTCAT-3′ (NdeI)	5′-CTGGCAGTCGAATTCTCACTTCCAGCG-3′ (EcoRI)
Mouse Slow Middle	5′-TCCTCATATGCCCCCGAAGAT-3′ (NdeI)	5′-CAGAACTTTCCATGGTTACTTCTTCTTGGC-3′ (NcoI)
Mouse Fast T1	5′-AGCCCATATGTCTGACGAGGAAGT-3′ (NdeI)	5′-CCGAATTCTAGTAGCTGCTGTAGTTG-3′ (EcoRI)
Mouse Fast T2	5′-CAACTACAGCCATATGCTGCTGGCCAA-3′ (NdeI)	5′-TCTCTCGAGTTAGTGCTTCTGG-3′ (XhoI)
Mouse Fast Middle	5′-AGACCCAAACATATGGCTCCTAAGA-3′ (NdeI)	5′-CAGCCTTGAATTCTCAGCTGCTGTA-3′ (EcoRI)

[a]In the primers used for the construction of representative fragments of cardiac, slow, and fast skeletal muscle TnT isoforms, restriction enzyme cutting sites introduced for cloning are underlined and the complementary sequences of stop codons engineered in the reverse primers are in bold letters.

equilibrated in a buffer containing 100 mM KCl, 3 mM $MgCl_2$, 20 mM PIPES, pH 7.0, 1 mM dithiothreitol, and 0.1 mM PMSF. After each use, the column was re-equilibrated with the column buffer for reuse.

Engineered T1 and T2 fragments of rabbit fast TnT were loaded as a mixture to the Tm affinity column. The column was washed with 10 bed volumes of the column buffer and eluted with a step gradient of 0.1−1.2 M KCl in 0.1 M increments. The eluted fractions were analyzed using SDS-PAGE.

Chicken breast muscle fast TnT was purified from adult chicken breast muscle as described previously[27] and treated with limited chymotryptic digestion in 4 M urea at pH 7.5 under conditions modified from that described previously.[9,16] The digestion was stopped at a series of time points by adding PMSF to 3 mM to aliquots. The digestion samples were examined by SDS-PAGE and Western blotting to verify the production of T1 and T2 fragments. After 4-fold dilution with the column buffer to reduce the concentration of urea carried over from the digestion to 1 M, the mixture of T1 and T2 fragments of chicken breast muscle TnT, containing some intact TnT and partial digestion products, was analyzed on Tm affinity column as above.

Solid-Phase Microplate Protein-Binding Assay. ELISA-based solid-phase microplate protein-binding assay[28] was used to compare the Tm-binding affinities of different TnT constructs. Purified TnT or TnT fragments were dissolved in Buffer A (100 mM KCl, 1 mM EGTA, 3 mM $MgCl_2$, 20 mM PIPES, pH 7.0) at 5 μg/mL. The TnT solution was added to 96-well polystyrene microtiter plates at 100 μL/well and incubated at 4 °C overnight to noncovalently immobilize the protein on the solid phase. The subsequent steps were all performed at room temperature. Excess TnT proteins were removed by washing with Buffer T (Buffer A plus 0.05% Tween 20) for three times over a 10 min period. The plate was then blocked with Buffer T plus 1% bovine serum albumin (BSA) for 1 h. Serial dilutions of rabbit skeletal muscle α/β-Tm in Buffer T containing 0.1% BSA were added to the plate at 100 μL/well and incubated for 2 h. The plates were washed three times with Buffer T and an anti-Tm mAb CH1[29] was added to the plate at 100 μL/well and incubated for 1 h. After three Buffer T washes, horseradish peroxidase-labeled goat anti-mouse secondary antibody (Santa Cruz) was added to the plate at 100 μL/well and incubated for 45 min. After three Buffer T washes, the amount of Tm bound to the immobilized TnT in each well was quantified via the colorimetric H_2O_2−ABTS (2,20-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) substrate reaction. The $A_{415\ nm}$ value in the linear course of the color development was monitored for each assay well against a reference wavelength of 655 nm using a Bio-Rad Benchmark automated microplate reader and recorded to construct Tm-binding curves for each TnT construct. The experiments were done in triplicate wells and repeated.

Troponin and tropomyosin are present in muscle cells in high concentrations based on their compacted presence in sarcomeric thin filaments. In the solid-phase protein binding assay, the TnT constructs studied are immobilized on the surface of microtitering plate at saturated density. Tropomyosin was then incubated to reach equilibrium binding. Therefore, the assay system provides a physiologically relevant condition for evaluating the binding affinity between myofilament proteins.

Statistical Analysis. The raw absorbance values of each Tm-binding curve were compared with two-way ANOVA using GraphPad Prism software. The means were compared across raw data binding curves and a Tukey's multiple comparisons test was conducted with a 0.05 significance level. Tm concentration for 50% maximum binding to each TnT construct was obtained by fitting the sigmoidal curves with the least-squares method.

RESULTS

Purified TnT and TnT Fragments. The SDS-PAGE gel and Western blots in Figure S1 summarize the intact and representative fragments of fast, slow, and cardiac TnT isoforms engineered and purified for our study. The anticipated recognition by specific anti-TnT mAbs verified the authenticity of the recombinant TnT isoforms and fragments.

T1, but Not T2, Fragments of Rabbit and Chicken Breast Muscle Fast TnT Differing in the N-Terminal Variable Region Exhibit Different Binding Affinity for Tm. Tm-affinity column chromatography showed that the T1 and T2 fragments of rabbit fast skeletal muscle TnT eluted at similarly high KCl concentrations, demonstrating similarly high binding affinities for Tm (Figure 2A).

DOI: 10.1021/acs.biochem.5b00348
Biochemistry 2015, 54, 3822−3830

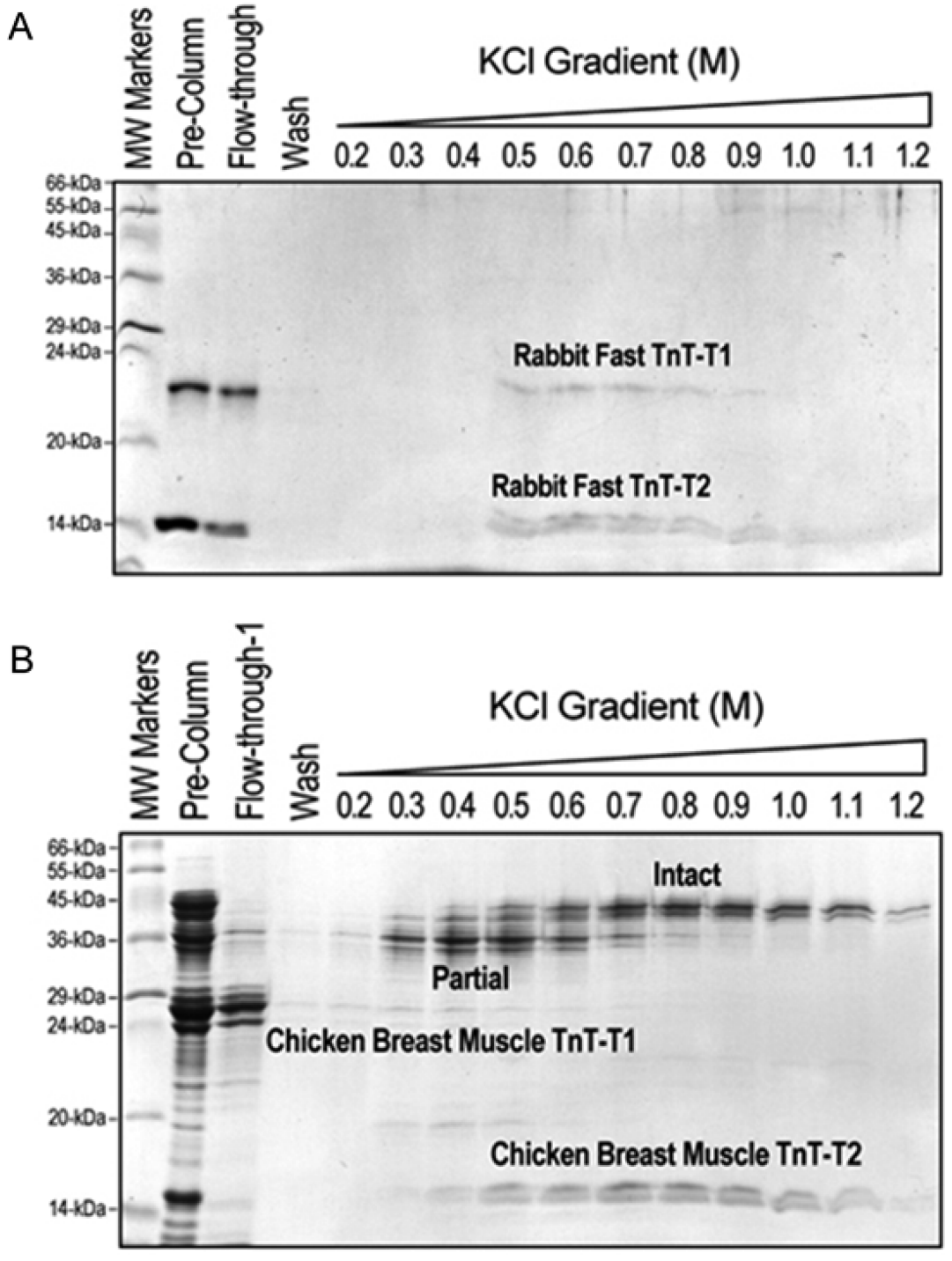


Figure 2. Tm-affinity chromatographic comparison of T1 and T2 fragments of rabbit fast skeletal muscle TnT and chicken breast muscle fast TnT. Rabbit fast skeletal muscle TnT and chicken breast muscle fast TnT that have significantly different N-terminal segments were compared. A. The SDS-PAGE gel shows the elution profiles of T1 and T2 fragments of rabbit fast skeletal muscle TnT analyzed on Tm-affinity column. The result demonstrated that the loading of mixed T1 and T2 fragments resulted in saturated binding to the Tm column as excess proteins were detectable in the flow-though. Both T1 and T2 fragments bound to the Tm column with high affinity, of which the T1 fragment was eluted at slightly higher KCl concentration. B. The SDS-PAGE gel shows the Tm-affinity column analysis of chymotryptic fragments of chicken breast muscle fast TnT. Limited chymotrypsin digestion produced a mixture of T1 and T2 fragments as well as some partial digestion products together with remaining intact TnT, which was loaded to the Tm affinity column. The elution profile showed that majority of the T1 fragment eluted in the flowthrough and only a small fraction bound to the Tm column and eluted very early. In contrast, almost all T2 fragment bound to the Tm column and eluted later at much higher KCl concentrations, only slightly earlier than the elution peak of intact protein. The multiple bands in the intact chicken breast muscle fast TnT preparation and the T1 fraction reflect the multiple N-terminal alternatively spliced isoforms naturally existing in adult chicken breast muscle.[20,24,27]

In contrast, the T1 fragment of chicken breast muscle fast TnT, which has an N-terminal region significantly different from that of rabbit fast TnT (Figure 3A), exhibited significantly lower Tm-binding affinity (eluted from the Tm-affinity column

DOI: 10.1021/acs.biochem.5b00348
Biochemistry 2015, 54, 3822−3830

A

```
Rabbit          1 MSD-EEVEHVEEQYEEEE--EAQ-EEAP----------SPAEVH--EPAPEVH-VPEEV-----HEEEKPRPKLT   6.17 kDa, pI:  4.02
Chicken Breast  1 MSDTEEVEHGEAHEAEEVHEEAHHEEAHHEEAHHEEAHHAEAHHAEAHHEEAHAHAEEVHEPAPPPEEKPRPKLT   8.59 kDa, pI:  5.00
```

B

```
Cardiac    1 MSDAEEVVEEYEEEQEEAVEEQEEAVEEEEAGGA----EPEPEGEAETEEANVEEVGPDEEAKDAEEGPVEDTKPKPSRLFMPN-L 8.98 kDa, pI: 3.44
Slow       1 MSDTEEQ--EYEEEQA----EDEEAVEEEEA-PE----EPEPVAER----------------------EEERPKPSRPVVPPLI 5.94 kDa, pI: 3.70
Fast       1 MSDEETE--QVEEE-A----QEEEVQEEEEV-QEDAVAEEEREEDE----------------------EEEKPRPKL------T 5.75 kDa, pI: 3.59
```

Figure 3. N-terminal variations of TnT isoforms. A. The amino acid sequence alignments demonstrate the significant differences between rabbit fast skeletal muscle TnT and chicken breast muscle fast TnT (GenBank accession numbers: ADY80034.1 and NP_990253.1, respectively). B. The amino acid sequence alignments demonstrate the significant differences among the N-terminal variable regions of mouse cardiac, slow and fast TnT (GenBank accession numbers: NP_001123650.1, NP_001264832.1, and NP_001157136.1, respectively).

largely in the flow-through) than that of the T2 fragment of chicken breast muscle fast TnT, which was eluted at high KCl concentrations (Figure 2B) similar to that of rabbit fast TnT T2 fragment (Figure 2A).

T2 Fragments of the Three Muscle Type TnT Isoforms Have Similar Binding Affinities for Tm. Microplate solid-phase protein binding assay showed that there was no significant difference in Tm binding affinity among the T2 fragments of mouse cardiac, slow, and fast skeletal muscle TnT (Figure 4). Consistent with the highly conserved amino acid

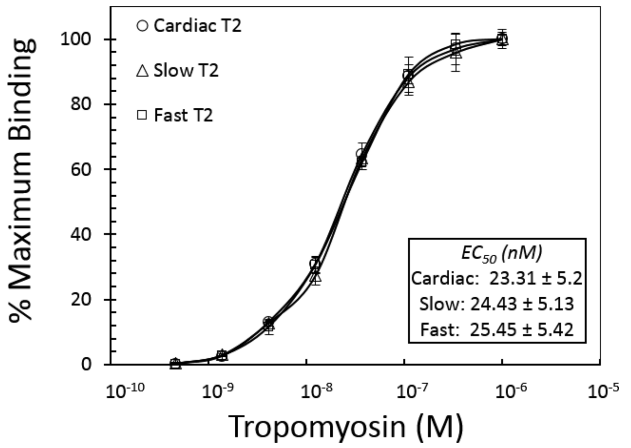


Figure 4. T2 fragments of the three muscle type TnT isoforms have similar binding affinity for Tm. Microplate protein binding experiments demonstrated nearly identical curves for the high affinity binding of T2 fragments of mouse cardiac, slow, and fast skeletal muscle TnT to Tm (n = 3).

sequences of the T2 fragment across TnT isoforms and species,[12] the results demonstrate nearly identical high affinities of Tm-binding site 2 residing in the T2 region of cardiac, fast and slow isoforms of TnT.

T1 Fragments of the Three Muscle Type TnT Isoforms Exhibit Different Binding Affinities for Tm. Microplate solid-phase protein binding assay showed significantly different Tm-binding affinities of the T1 fragments of mouse cardiac, fast, and slow skeletal muscle isoforms of TnT (Figure 5). Fast skeletal muscle T1 had the highest and slow skeletal muscle T1 had the lowest Tm-binding affinities, whereas the cardiac T1 was in between.

Middle Fragments of the Three Muscle Type TnT Isoforms Have Similar Binding Affinity for Tm. The T1 fragment is comprising the N-terminal variable region and the conserved middle region containing the Tm-binding site 1. To

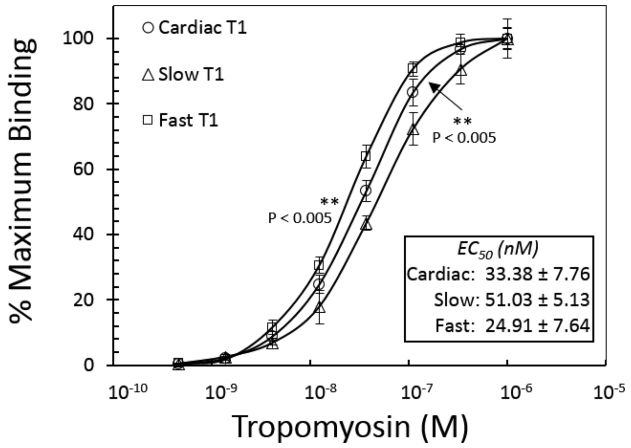


Figure 5. T1 fragments of three muscle type TnT isoforms have significantly different Tm-binding affinity. Microplate protein binding experiments demonstrated significantly different Tm binding affinities for T1 fragments of the three muscle type TnT isoforms in the order of fast > cardiac (** <0.005) > slow (**P < 0.005) by Fisher test in Two Way ANOVA, n = 3).

dissect the intrinsic property of Tm-binding site 1, we further studied the affinity of isolated middle fragment of the three muscle type TnT isoforms for Tm. Microplate solid-phase protein binding assay showed that there was no significant difference in Tm-binding affinities among the middle fragments of mouse cardiac, slow, and fast isoforms of TnT (Figure 6). Consistent with the highly conserved amino acid sequences of the middle fragment across TnT isoforms and species,[12] the results demonstrate nearly identical high affinity binding of Tm-binding site 1 residing in the middle region of cardiac, slow, and fast isoforms of TnT.

Intact Muscle Type TnT Isoforms Have Different Binding Affinities for Tm. Microplate solid phase protein binding assay further demonstrated significant differences in the Tm-binding affinity among the three intact mouse muscle type TnT isoforms. The results in Figure 7 showed that fast TnT has the highest Tm-binding affinity and slow TnT the lowest with the cardiac TnT in between, a pattern the same as that of the T1 fragments of the TnT isoforms (Figure 5). These data demonstrate that the differentially modulatory effect of the significantly diverged N-terminal variable regions of the three muscle type TnT isoforms (Figure 3B) on the affinity of the Tm-binding site 1 does occur in native TnT molecules.

Direct Comparison of Tm-Binding Affinity of Intact, T1, T2, and Middle Fragments of Mouse Cardiac TnT. Microplate solid-phase protein binding assay showed that intact

DOI: 10.1021/acs.biochem.5b00348
Biochemistry 2015, 54, 3822−3830

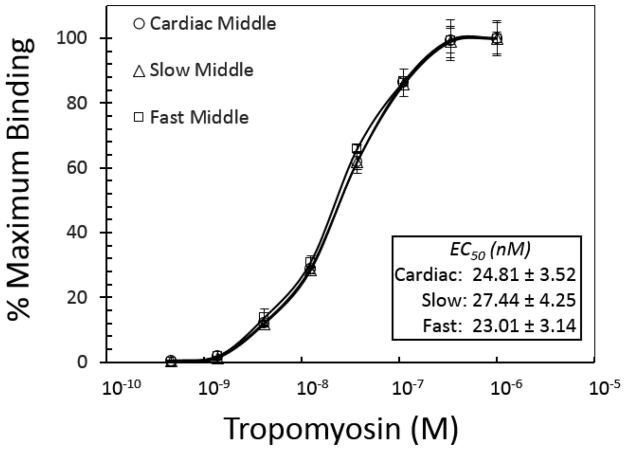


Figure 6. Middle fragments of three muscle type TnT isoforms have similar binding affinity for Tm. Microplate protein binding experiments demonstrated nearly identical curves for the high affinity binding of the middle fragments of cardiac, slow, and fast skeletal muscle TnT to Tm (n = 3).

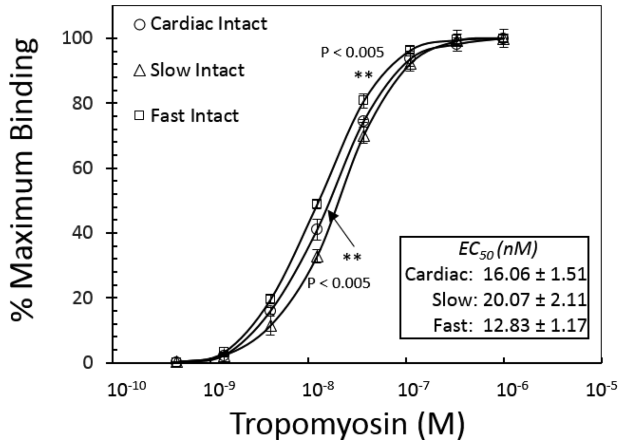


Figure 7. Intact muscle type TnT isoforms have significantly different Tm-binding affinity. Microplate protein binding experiments demonstrated significantly different Tm-binding affinities for intact muscle type TnT isoforms in the order of fast > cardiac (**P < 0.005) > slow (**P < 0.005) by Fisher test in Two Way ANOVA, n = 3).

mouse cardiac TnT had the highest Tm-binding affinity, consistent with the presence of both Tm-binding sties 1 and 2. The middle and T2 fragments containing Tm-binding sites 1 and 2, respectively, had detectably lower binding affinities for Tm with insignificant difference between each other. The T1 fragment combining the N-terminal variable region with the Tm-binding site 1 in the middle region had the lowest Tm binding affinity (Figure 8).

DISCUSSION

Revisiting the Two Tm-Binding Sites of TnT. The interactions between TnT and Tm are at the center of the Ca^{2+}-mediated thin filament regulation of striated muscle contraction.[1−3,6,15,30] After pioneer studies carried out nearly four decades ago to identify the functions of several classic enzymatic or chemically cleaved TnT fragments, i.e., the T1, T2, and a few CNBr fragments,[7−9,11,13,31,32] the regions of TnT molecule containing the two Tm-binding sites were not further mapped until recently,[12] while the Tm-binding affinity of site 1 remained controversial.

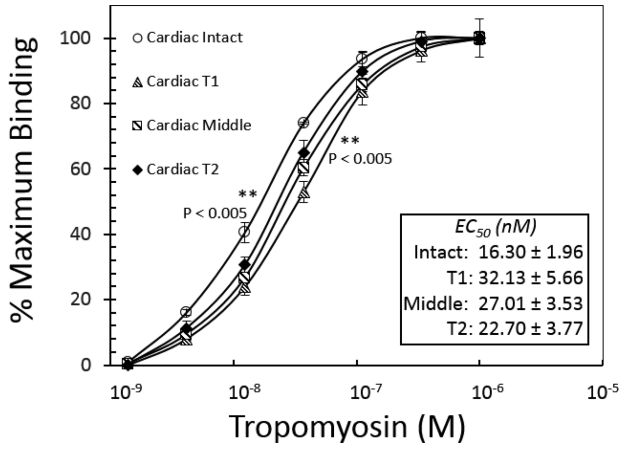


Figure 8. Comparison of the Tm-binding affinity of intact, T1, T2, and middle fragments of cardiac TnT. Microplate protein binding experiments demonstrated significant differences in Tm-binding affinity in the order of Intact > T2 (**P < 0.005) ≈ Middle > T1 (**P < 0.005) by Fisher test in Two Way ANOVA, n = 3).

In the present study, we employed the more quantitative microplate protein binding assay together with the classic affinity chromatography method used in the previous studies to comprehensively determine and compare for the first time the Tm-binding affinities of intact and molecular engineered representative fragments of the three muscle type isoforms of TnT. To elucidate the intrinsic binding affinity of Tm-binding sites 1 and 2 and to investigate the modulatory effect of the N-terminal variable region, we constructed and purified representative TnT fragments (Figure 1) based on current knowledge of the structure of troponin and TnT-Tm interaction, rather than limited to the availability of proteolytic fragments. In order to compare our data with previous studies primarily done using rabbit fast TnT fragments,[7−9,11,13,31,32] we also included rabbit fast TnT T1 and T2 fragments in the present study (Figure 2A).

While most vertebrate skeletal muscle express both α- and β-Tm, adult cardiac muscle[33] and chicken breast muscle[20] express only α-Tm. It is known that intact TnT, T1 and T2 fragments all have a slightly higher binding affinity for α-Tm than that for β-tropomyosin.[9] Therefore, we chose to use rabbit skeletal muscle α/β-Tm in the binding assays for the comparison of overall affinity of the two Tm-binding sites of TnT. The results of these new investigations provided quantitative data demonstrating the following insights into the structure−function relationship of TnT and the regulation of TnT-Tm interaction.

The N-Terminal Hypervariable Region Regulates the Tm-Binding Affinity of TnT. The level of saturated binding was similar for the different TnT isoforms, reflecting their full capacity of incorporating into the myofilaments. One of the main findings in this study is that TnT's N-terminal variable region is responsible for the significant differences in Tm-binding across the three muscle type isoforms. While intact cardiac, slow, and fast isoforms of TnT and their T1 fragments exhibit significant differences in Tm-binding affinity (Figures 3, 6, and 8), removal of the N-terminal variable region from the T1 fragment to isolate the highly conserved middle region containing Tm-binding site 1 of the three TnT isoforms produced nearly identical Tm-binding affinities (Figure 6).

DOI: 10.1021/acs.biochem.5b00348
Biochemistry 2015, 54, 3822−3830

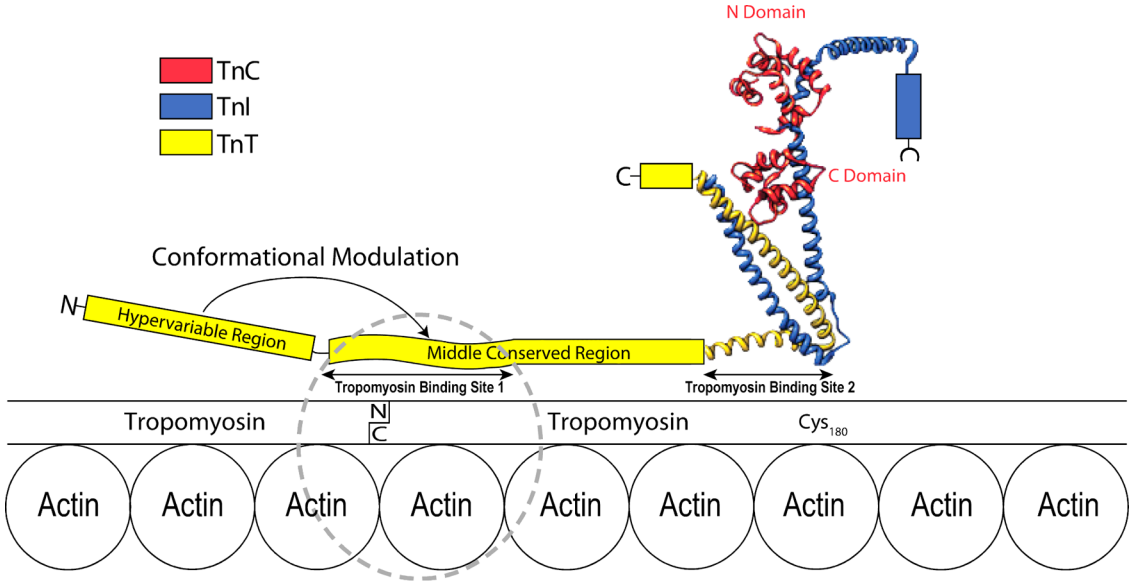


Figure 9. The N-terminal variable region of TnT modulates the affinity of Tm-binding site 1. A model is proposed for the mechanism by which the N-terminal variation in the muscle type isoforms or splice forms of TnT differentially modulates the conformation and function of other regions of the molecule with effects primarily on Tm-binding site 1 that interacts with the head−tail junction of Tm in muscle thin filament (highlighted by the dashed circle).

The literature discrepancy for the Tm-binding affinity of T1 vs T2 fragments of different TnT isoforms[9,12,13] is, therefore, explained by the data demonstrating that the Tm-binding affinity of site 1 is differentially modulated across isoforms by the N-terminal variable region. This modulatory effect of the N-terminal variable region was first demonstrated using the classic method of Tm-affinity chromatography, in which rabbit fast and chicken breast muscle TnT T1 fragments that have significantly different N-terminal structure (Figure 3A) but conserved sequences in the middle region[12] showed significantly different Tm-binding affinities (Figure 2). This modulation of the affinity of Tm-binding site 1 by the N-terminal variable region was then more quantitatively demonstrated in the microplate assays (Figures 6 and 7).

The current understanding is that the N-terminal variable region of TnT is an extended structure in the "tail" of the TnT molecule and does not bind to any known myofilament proteins.[15,33] On the other hand, the structure of the N-terminal region constitutes the major difference among the three evolutionarily diverged muscle type TnT isoforms[14] and is regulated via alternative splicing during cardiac and skeletal muscle development.[20,23,34] To investigate the function of the N-terminal variable region of TnT, previous studies have demonstrated its effects on altering TnT's overall molecular conformation and binding affinity for TnI, TnC, and Tm,[16,18,19,35] which modulates muscle contractility.[36] Further demonstrating this mechanism underlying the functional difference of TnT isoforms and splice forms, our new experimental data showed that the N-terminal variable region of TnT (Figure 3B) functions by differentially decreasing the high intrinsic binding affinity of Tm-binding site 1 (Figure 5 vs Figure 6).

Consistent with the above functional effects, the N-terminal variable region-generated difference in Tm-binding affinity is retained in intact TnT isoforms with the presence of both Tm-binding sites 1 and 2 despite the near identical high affinity of Tm-binding site 2 across TnT isoforms (Figure 4). Therefore, the results indicate a dominant conformational and functional effect of the N-terminal variable region of TnT on modulating the affinity of Tm binding site 1. Nonetheless, a potential effect on Tm binding site 2 could not be excluded. Previous studies have shown that the TnI- and TnC-binding sites of TnT involving structures downstream of Tm-binding site 2 (Figure 9) are modulated by the N-terminal variable region,[16,19,35] which indicates that the N-terminal variable region's conformational effect is transmitted involving the segment of Tm-binding site 2.

Differentiated Functions of Tm-Binding Sites 1 and 2 of TnT. Our study compared the Tm-binding affinities of the two Tm-binding sites across muscle type TnT isoforms as well as within one isoform of a given species. The thorough cross comparisons consistently showed that the T2 and middle fragments of TnT, containing Tm binding sites 1 and 2, respectively, have nearly identical high binding affinities for Tm (Figures 3 and 9).

When the T1 and T2 fragments were compared in the same system, our Tm affinity chromatography showed that in line with previous observations,[9] rabbit fast TnT T2 has similar Tm-binding affinity to that of rabbit fast T1 (Figure 2A). In contrast, chicken breast muscle TnT T2 has significantly higher Tm-binding affinity than that of chicken breast muscle TnT T1 (Figure 2B). When the N-terminal variable region is removed, the middle fragment of cardiac, fast, and slow isoforms of TnT exhibits similarly high Tm-binding affinity as that of T2. This indicates the modulatory effect of the N-terminal variable region plays a role on reducing the intrinsic affinity of Tm-binding site 1.

The results demonstrate that the Tm-binding site 2 of TnT exhibits high affinity in all isoforms, indicating a key role in the anchoring of troponin complex on the thin filament. This notion is consistent with the recessive phenotype of a nemaline myopathy mutation in human slow skeletal muscle TnT, in which a truncation of the TnT polypeptide chain deletes Tm-binding site 2, and results in the complete loss of myofilament incorporation.[37] On the other hand, Tm-binding site 1 may play a regulatory role in tuning thin filament function. Among

DOI: 10.1021/acs.biochem.5b00348
Biochemistry 2015, 54, 3822−3830

the T1 fragments across isoforms, fast TnT T1 was observed to have the highest binding affinity for Tm, followed by cardiac and then slow (Figure 5). This pattern was retained in intact TnT isoforms (Figure 8). This differential N-terminal regulation via long-range conformational modulation demonstrated a pivotal function of the Tm-binding site 1 of TnT involving interactions with the Tm head−tail junction in the thin filament (Figure 9). This modulation of TnT−Tm interaction subsequently modulates the calcium regulation of muscle contraction and relaxation[20,37,38] and would contribute to the differentiated contractility of cardiac, slow, and fast muscle fibers, during development and adaptation to pathogenic conditions. Previous studies demonstrated that fast TnT with less negative charge in the N-terminal variable region than that in cardiac TnT and slow TnT, corresponded to lower myofilament Ca^{2+} sensitivities but higher cooperativity.[37,44] With the results of the present study, the trend appears to be that a higher TnT−Tm binding affinity may produce lower Ca^{2+} sensitivity and higher cooperativity.

Physiological and Pathophysiological Significance of the N-Terminal Modification-Based Regulation of TnT. The Tm-binding site 1 interacts with the Tm head−tail junction in striated muscle thin filament,[13,31,33,39,40] which is an important site determining the cooperative allosteric functions during Ca^{2+}-regulated contraction and relaxation.[13,41−44] The finding that the N-terminal variable region differentially modulates TnT−Tm interactions at Tm-binding site 1 indicates a submolecular mechanism for adaptive and potentially therapeutic alterations of muscle contractility.

The results of our present study also corroborated previous observations that removal of the entire N-terminal variable region of cardiac TnT results in an overall increase in TnT's binding affinity for Tm.[45,46] The mechanism of this phenomenon is supported by data shown in Figure 8 where the cardiac TnT middle fragment exhibited a higher Tm binding affinity than that of the T1 fragment.

Restrictive N-terminal truncation of cardiac TnT has been found as acute adaptation in ischemia reperfusion and pressure overload.[46,47] Transgenic overexpression of N-terminal truncated cardiac TnT in mouse heart increased stroke volume against high afterload by a moderate decrease in contractile velocity that beneficially prolongs left ventricular rapid ejection time.[47] Therefore, the N-terminal modification-regulated function of Tm-binding site 1 of TnT may represent a novel post-translational mechanism for the pathophysiological adaptation of cardiac muscle, and the development of targeted treatment for systolic heart failure.

In conclusion, the present study demonstrated that the N-terminal variable region of TnT is a regulatory structure for the differentiated function of muscle type isoforms and splice forms of TnT. While the Tm-binding sites of TnT are highly conserved across isoforms and species and have similarly high affinity for Tm, the presence of isoform-specific, alternatively spliced, or post-translationally modified N-terminal variable region, modulates the affinity of Tm-binding site 1 and fine-tunes muscle contractility in developmental, physiological, and pathophysiological adaptations.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Gel and Western blot images of the TnT proteins used in this study. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00348.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: jjin@med.wayne.edu. Tel: 313-577-1520. Fax: 313-577-5494.

Funding

This study was supported in part by the National Institutes of Health grants AR048816 and HL098945 and a Multidisciplinary Incubator Grant from the Office of Vice President for Research at Wayne State University to J.-P.J.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Ms. Hui Wang for technical assistance.

■ ABBREVIATIONS

BSA Bovine serum albumin; cDNA Complementary DNA; CNBr Cyanogen bromide; cTnT Cardiac troponin T; EGTA Ethylene glycol tetraacetic acid; ELISA Enzyme-linked immunosorbent assay; fTnT Fast TnT; IPTG Isopropyl β-D-1-thiogalactopyranoside; McTnT Mouse cardiac troponin T; MfTnT Mouse fast troponin T; PCR Polymerase chain reaction; PMSF Phenylmethylsulfonyl fluoride; SDS Sodium dodecyl sulfate; SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis; sTnT Slow troponin T; Tm Tropomyosin; TnC Troponin C; TnI Troponin I; TnT Troponin T

■ REFERENCES

(1) Ebashi, S., and Endo, M. (1968) Calcium ion and muscle contraction. *Prog. Biophys. Mol. Biol. 18*, 123−183.

(2) Greaser, M. L., and Gergely, J. (1971) Reconstitution of troponin activity from three protein components. *J. Biol. Chem. 246*, 4226−4233.

(3) Sheng, J. J., and Jin, J. P. (2014) Gene regulation, alternative splicing, and posttranslational modification of troponin subunits in cardiac development and adaptation: a focused review. *Front. Physiol. 5*, 165.

(4) Takeda, S., Yamashita, A., Maeda, K., and Maeda, Y. (2003) Structure of the core domain of human cardiac troponin in the Ca(2+)-saturated form. *Nature 424*, 35−41.

(5) Vinogradova, M. V., Stone, D. B., Malanina, G. G., Karatzaferi, C., Cooke, R., Mendelson, R. A., and Fletterick, R. J. (2005) Ca(2+)-regulated structural changes in troponin. *Proc. Natl. Acad. Sci. U.S.A. 102*, 5038−5043.

(6) Jin, J. P., Zhang, Z., and Bautista, J. A. (2008) Isoform diversity, regulation, and functional adaptation of troponin and calponin. *Crit. Rev. Eukaryotic Gene Expression 18*, 93−124.

(7) Nakamura, S., Yamamoto, K., Hashimoto, K., and Ohtsuki, I. (1981) Effect of chymotryptic troponin-T subfragments on the Ca-2+ sensitivity of super-precipitation. *J. Biochem. 89*, 1639−1641.

(8) Pearlstone, J. R., Carpenter, M. R., and Smillie, L. B. (1977) Primary structure of rabbit skeletal muscle troponin-T. Purification of cyanogen bromide fragments and the amino acid sequence of fragment CB2. *J. Biol. Chem. 252*, 971−977.

(9) Pearlstone, J. R., and Smillie, L. B. (1982) Binding of troponin-T fragments to several types of tropomyosin sensitivity to Ca-2+ in the presence of troponin-C. *J. Biol. Chem. 257*, 587−592.

(10) Tanokura, M., and Ohtsuki, I. (1984) Interactions among chymotryptic troponin-T subfragments, tropomyosin, troponin-I and troponin-C. *J. Biochem. 95*, 1417−1421.

DOI: 10.1021/acs.biochem.5b00348
Biochemistry 2015, 54, 3822−3830

(11) Tanokura, M., Tawada, Y., Ono, A., and Ohtsuki, I. (1983) Chymotryptic subfragments of troponin-t from rabbit skeletal-muscle - interaction with tropomyosin, troponin-I and troponin-C. *J. Biochem. 93*, 331−337.
(12) Jin, J. P., and Chong, S. M. (2010) Localization of the two tropomyosin-binding sites of troponin T. *Arch. Biochem. Biophys. 500*, 144−150.
(13) Heeley, D. H., Golosinska, K., and Smillie, L. B. (1987) The effects of troponin T fragments T1 and T2 on the binding of nonpolymerizable tropomyosin to F-actin in the presence and absence of troponin I and troponin C. *J. Biol. Chem. 262*, 9971−9978.
(14) Chong, S. M., and Jin, J. P. (2009) To investigate protein evolution by detecting suppressed epitope structures. *J. Mol. Evol. 68*, 448−460.
(15) Wei, B., and Jin, J. P. (2011) Troponin T isoforms and posttranscriptional modifications: Evolution, regulation and function. *Arch. Biochem. Biophys. 505*, 144−154.
(16) Wang, J., and Jin, J. P. (1998) Conformational modulation of troponin T by configuration of the NH2-terminal variable region and functional effects. *Biochemistry 37*, 14519−14528.
(17) Ogut, O., and Jin, J. P. (1996) Expression, zinc-affinity purification, and characterization of a novel metal-binding cluster in troponin T: metal-stabilized alpha-helical structure and effects of the NH2-terminal variable region on the conformation of intact troponin T and its association with tropomyosin. *Biochemistry 35*, 16581−16590.
(18) Jin, J. P., and Root, D. D. (2000) NH_2-terminal variable region-based modulation of conformation and flexibility of troponin T: A fluorescence spectral study. *Biophys. J. 78*, 365a−365a.
(19) Jin, J. P., Chen, A., Ogut, O., and Huang, Q. Q. (2000) Conformational modulation of slow skeletal muscle troponin T by an NH(2)-terminal metal-binding extension. *American journal of physiology. Cell physiology 279*, C1067−1077.
(20) Ogut, O., and Jin, J. P. (1998) Developmentally regulated, alternative RNA splicing-generated pectoral muscle-specific troponin T isoforms and role of the NH2-terminal hypervariable region in the tolerance to acidosis. *J. Biol. Chem. 273*, 27858−27866.
(21) Jin, J. P. (1995) Cloned rat cardiac titin class I and class II motifs. Expression, purification, characterization, and interaction with F-actin. *J. Biol. Chem. 270*, 6908−6916.
(22) Jin, J. P., Wang, J., and Zhang, J. Y. (1996) Expression of cDNAs encoding mouse cardiac troponin T isoforms: Characterization of a large sample of independent clones. *Gene 168*, 217−221.
(23) Wang, J., and Jin, J. P. (1997) Primary structure and developmental acidic to basic transition of 13 alternatively spliced mouse fast skeletal muscle troponin T isoforms. *Gene 193*, 105−114.
(24) Jin, J. P., and Smillie, L. B. (1994) An unusual metal-binding cluster found exclusively in the avian breast muscle troponin-T of galliformes and craciformes. *FEBS Lett. 341*, 135−140.
(25) Wang, X., Huang, Q. Q., Breckenridge, M. T., Chen, A., Crawford, T. O., Morton, D. H., and Jin, J. P. (2005) Cellular fate of truncated slow skeletal muscle troponin T produced by Glu180 nonsense mutation in amish nemaline myopathy. *J. Biol. Chem. 280*, 13241−13249.
(26) Smillie, L. B. (1982) Preparation and identification of alpha- and beta-tropomyosins. *Methods Enzymol. 85*, 234−241.
(27) Jin, J. P., and Samanez, R. A. (2001) Evolution of a metal-binding cluster in the NH2-terminal variable region of avian fast skeletal muscle troponin T: Functional divergence on the basis of tolerance to structural drifting. *J. Mol. Evol. 52*, 103−116.
(28) Biesiadecki, B. J., and Jin, J. P. (2011) A high-throughput solid-phase microplate protein-binding assay to investigate interactions between myofilament proteins. *J. Biomed. Biotechnol. 2011*, 421701.
(29) Lin, J. J., Chou, C. S., and Lin, J. L. (1985) Monoclonal antibodies against chicken tropomyosin isoforms: production, characterization, and application. *Hybridoma 4*, 223−242.
(30) Gordon, A. M., Homsher, E., and Regnier, M. (2000) Regulation of contraction in striated muscle. *Physiol. Rev. 80*, 853−924.
(31) Mak, A. S., and Smillie, L. B. (1981) Structural interpretation of the two-site binding of troponin on the muscle thin filament. *J. Mol. Biol. 149*, 541−550.
(32) Tanokura, M., Tawada, Y., and Ohtsuki, I. (1982) Chymotryptic subfragments of troponin-T from rabbit skeletal-muscle 0.1. Determination of the primary structure. *J. Biochem. 91*, 1257−1265.
(33) Perry, S. V. (2001) Vertebrate tropomyosin: distribution, properties and function. *Journal of Muscle Research and Cell Motility 22*, 5−49.
(34) Jin, J. P., and Lin, J. J. C. (1989) Isolation and characterization of cdna clones encoding embryonic and adult isoforms of rat cardiac troponin-T. *J. Biol. Chem. 264*, 14471−14477.
(35) Biesiadecki, B. J., Chong, S. M., Nosek, T. M., and Jin, J. P. (2007) Troponin T core structure and the regulatory NH_2-terminal variable region. *Biochemistry 46*, 1368−1379.
(36) Ogut, O., Granzier, H., and Jin, J. P. (1999) Acidic and basic troponin T isoforms in mature fast-twitch skeletal muscle and effect on contractility. *Am. J. Physiol.-Cell Physiol. 276*, C1162−C1170.
(37) Jin, J. P., Brotto, M. A., Hossain, M. M., Huang, Q. Q., Brotto, L. S., Nosek, T. M., Morton, D. H., and Crawford, T. O. (2003) Truncation by Glu180 nonsense mutation results in complete loss of slow skeletal muscle troponin T in a lethal nemaline myopathy. *J. Biol. Chem. 278*, 26159−26165.
(38) Gollapudi, S. K., Gallon, C. E., and Chandra, M. (2013) The tropomyosin binding region of cardiac troponin T modulates crossbridge recruitment dynamics in rat cardiac muscle fibers. *J. Mol. Biol. 425*, 1565−1581.
(39) Yang, S. X., Barbu-Tudoran, L., Orzechowsk, M., Craig, R., Trinick, J., White, H., and Lehman, W. (2014) Three-dimensional organization of troponin on cardiac muscle thin filaments in the relaxed state. *Biophys. J. 106*, 855−864.
(40) Murakami, K., Stewart, M., Nozawa, K., Tomii, K., Kudou, N., Igarashi, N., Shirakihara, Y., Wakatsuki, S., Yasunaga, T., and Wakabayashi, T. (2008) Structural basis for tropomyosin overlap in thin (actin) filaments and the generation of a molecular swivel by troponin-T. *Proc. Natl. Acad. Sci. U.S.A. 105*, 7200−7205.
(41) Ogut, O., and Jin, J. P. (2000) Cooperative interaction between developmentally regulated troponin T and tropomyosin isoforms in the absence of F-actin. *J. Biol. Chem. 275*, 26089−26095.
(42) Schaertl, S., Lehrer, S. S., and Geeves, M. A. (1995) Separation and characterization of the two functional regions of troponin involved in muscle thin filament regulation. *Biochemistry 34*, 15890−15894.
(43) Willadsen, K. A., Butters, C. A., Hill, L. E., and Tobacman, L. S. (1992) Effects of the amino-terminal regions of tropomyosin and troponin-T on thin filament assembly. *J. Biol. Chem. 267*, 23746−23752.
(44) Huang, Q. Q., Brozovich, F. V., and Jin, J. P. (1999) Fast skeletal muscle troponin T increases the cooperativity of transgenic mouse cardiac muscle contraction. *J. Physiol. (Oxford, U.K.) 520*, 231−242.
(45) Fisher, D., Wang, G., and Tobacman, L. S. (1995) NH_2-terminal truncation of skeletal-muscle troponin-T does not alter the Ca^{2+} sensitivity of thin filament assembly. *J. Biol. Chem. 270*, 25455−25460.
(46) Zhang, Z., Biesiadecki, B. J., and Jin, J. P. (2006) Selective deletion of the NH_2-terminal variable region of cardiac troponin T in ischemia reperfusion by myofibril-associated mu-calpain cleavage. *Biochemistry 45*, 11681−11694.
(47) Feng, H. Z., Biesiadecki, B. J., Yu, Z. B., Hossain, M. M., and Jin, J. P. (2008) Restricted N-terminal truncation of cardiac troponin T: a novel mechanism for functional adaptation to energetic crisis. *J. Physiol. 586*, 3537−3550.
(48) Lin, J. J., Matsumura, F., and Yamashiro-Matsumura, S. (1984) Tropomyosin-enriched and alpha-actinin-enriched microfilaments isolated from chicken embryo fibroblasts by monoclonal antibodies. *J. Cell Biol. 98*, 116−127.

DOI: 10.1021/acs.biochem.5b00348
Biochemistry 2015, 54, 3822−3830