Cooperative Interaction between Developmentally Regulated Troponin T and Tropomyosin Isoforms in the Absence of F-actin*

Troponin T (TnT) is the tropomyosin (Tm) binding subunit of the troponin complex that mediates the Ca$^{2+}$ regulation of actomyosin interaction in striated muscles. Troponin T isoform diversity is marked by a developmentally regulated acidic to basic switch that may modulate muscle contractility. We previously reported that transgenic expression of fast skeletal muscle TnT altered the cooperativity of cardiac muscle. In the present study, we have demonstrated that the binding of acidic TnT to tropinin I is weaker than that of basic TnT. However, affinity chromatography experiments showed that Tm bound to acidic TnT with a greater affinity than to basic TnT, consistent with the significantly higher maximal binding of acidic TnT to Tm in solid phase binding assays. Competition and co-immunoprecipitation experiments demonstrated that the binding of TnT to Tm was cooperative in the absence of F-actin. The cooperativity between TnT molecules for Tm binding can be initiated by the conserved COOH-terminal T2 fragment of TnT. This indicates that the interaction of TnT with Tm induces a conformational change in Tm, promoting interaction of TnT with adjacent Tm dimers. This finding suggests a role for TnT and its acidic and basic isoforms in the cooperative release of the inhibition of striated muscle actomyosin interaction.

Striated muscle contraction is regulated by Ca$^{2+}$ through the troponin-tropomyosin signaling pathway (1). The troponin complex, identified by Ebashi (2), contains three constituents (3): troponin T (TnT) (the tropomyosin (Tm) binding subunit), the inhibitory subunit troponin I (TnI), and the Ca$^{2+}$-binding subunit troponin C (TnC). The binding of Ca$^{2+}$ to TnC triggers a series of interactions which relieves troponin-tropomyosin inhibition of the actomyosin ATPase and allows for cross-bridge attachment and cycling (for review, see Ref. 4). Troponin T is at a central position in the thin filament regulatory system where it interacts with TnI (5), TnC (6), Tm (7), and actin (8). Three genes are responsible for the expression of TnT specific to fast skeletal, slow skeletal, and cardiac muscle fibers of vertebrates (9–11). For each TnT gene, RNA splicing generates isoform diversity primarily through alternative exons encoding the extended NH$_2$-terminal domain of the protein. The isoform diversity is best exemplified by fast skeletal muscle TnT wherein at least six (mammalian) and as many as thirteen (avian) exons are alternatively spliced to generate a large number of isoforms (10, 12–17). As a result of tissue-specific regulation by alternative RNA splicing, cardiac and fast skeletal muscle TnT isoform switches are observed during mammalian and avian development (9, 13, 15, 18). This developmental expression pattern can be classified by the predicted physical properties of the TnT isoforms expressed (15). Specifically, fetal and neonatal TnT isoforms are higher $M_r$ isoforms when compared with those expressed in adult striated muscles. Protein and cDNA sequencing have shown that the NH$_2$-terminal variable region of TnT is abundant with acidic amino acid residues. Nonetheless, the NH$_2$-terminal domains of high $M_r$ fetal and neonatal TnT isoforms have relatively acidic isoelectric points (pI) in comparison with adult TnT isoforms. The high to low $M_r$, acidic to basic developmental TnT isoform switch is conserved among species and may reflect the role of TnT isoforms in the functional adaptation of developing striated muscles. Cardiac (18) and slow skeletal muscle (19) TnT have more acidic NH$_2$-terminal domains compared with those in the fast skeletal muscle TnT (15), consistent with a role for TnT isoforms in the functional diversity of muscle fiber types. The regulation of muscle regulatory protein isoforms is not restricted to TnT because TnI and Tm show muscle specific and developmental control of gene expression (20–22). This suggests that expression of the muscle thin filament protein genes is coordinated. The importance of the TnT structure-function relationship to muscle contractility is further demonstrated by changes in cardiac TnT isoform expression during diabetes (23) and heart failure (24). In addition, differences in TnT isoform expression are able to alter the sensitivity and the cooperativity of the force response to Ca$^{2+}$ (25, 26). In this respect, TnT isoform expression may be important in the etiologic or compensatory mechanism involved in compromised contractility of cardiac muscle. Therefore, understanding the interaction of acidic and basic TnT isoforms with other thin filament proteins is important in deciphering the role of TnT in Ca$^{2+}$ regulation of striated muscle contraction. In the present study, we have investigated the functional properties of high $M_r$ acidic versus low $M_r$ cardiac TnT isoforms. Interestingly, acidic and basic TnT show differential interactions with Tm isoforms and TnI. In addition, the binding of TnT to Tm is cooperative in the absence of F-actin, an effect that is induced by the binding of the COOH-terminal domain of TnT to Tm. Taken together, the data imply that TnT isoforms and their interactions with Tm play important roles in the physiology of muscle contraction.

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The abbreviations used are: TnT, tropinin T; TnC, tropinin C; TnI, troponin I; Pipes, 1,4-piperazinediethanesulfonic acid; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbant assay; TCEP, tris(carboxyethyl)phosphine; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; Tm, tropomyosin; TnT, chicken acidic fast skeletal muscle TnT; cTnT, bovine cardiac TnT.

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**Experimental Procedures**

**Specific Antibodies Against Acidic and Basic Fast Skeletal Muscle TnT Isoforms**—The anti-TnT monoclonal antibodies (mAbs) 3E4 and 6B8 (27) were used to specifically detect acidic and basic fast skeletal muscle TnT isoforms. The relative affinities of the mAbs to acidic and basic TnT were demonstrated by indirect enzyme-linked immunosorbent assay (ELISA). Chicken TnT was diluted to 5 μg/ml in Buffer A (1 mM Pipes, pH 7.0, 100 mM KCl, 3 mM MgCl₂, 0.1 mM TCEP, 0.1% coated overnight (100 μM, 4 °C) onto triplicate wells of microtiter plates (Falcon 3915). Control wells were coated with bovine serum albumin (BSA) at a concentration of 5 μg/ml. The wells were then washed once in Buffer A with 0.05% Tween 20 (Buffer B) and blocked at room temperature for 90 min using Buffer B containing 1% BSA. Following blocking, the wells were washed three times in Buffer B and incubated in dilutions of mAb 3E4 or 6B8 in Buffer B with 0.1% BSA at 90 min at room temperature. For antibody incubations, TCEP was omitted. The bound mAbs were detected by horseradish peroxidase-conjugated anti-mouse immunoglobulin second antibody and 2,2'-azino-bis-(3-ethylbenzthiazoline-sulfonic acid) substrate reaction as described previously (28).

**Purification of Skeletal Muscle Proteins**—Troponin T and TnI were prepared from fresh chicken skeletal muscles using a protocol developed by Dr. L. B. Smillie (University of Alberta, Edmonton, Canada). As demonstrated previously (29), adult chicken breast muscle exclusively expresses high Mᵣ acidic fast skeletal muscle TnT whereas the gastrocnemius muscle exclusively expresses low Mᵣ basic fast skeletal muscle TnT. Therefore, the two muscle types were used to purify acidic and basic TnT isoforms, respectively. Following homogenization and extraction of muscle with 50 mM KCl, a resolution protocol of TnI, TnT, or Tm antibodies to assure no cross-contamination or proteolytic degradation products in the samples. Protein concentrations were determined by absorption measurements and SDS-PAGE analyses against controls of known concentrations. The following extinction coefficients and molecular weights were used: acidic fast skeletal muscle TnT, E₂₈₀ = 0.44 (1 mg/ml), Mᵣ = 33,000; basic fast skeletal muscle TnT, E₂₈₀ = 0.52 (1 mg/ml), Mᵣ = 21,000; chicken α/α- or α/β-Tm, E₂₈₀ = 0.44 (1 mg/ml), Mᵣ = 66,000 (32, 33).

**Troponynosin Affinity Chromatography**—The relative binding strengths of acidic and basic TnT to α/β-Tm were determined using affinity chromatography. Troponynosin affinity chromatography was performed to CNBr-activated Sepharose 4B gel (Amersham Pharmacia Biotech) according to the manufacturer's instructions using 11 mg of protein per 0.5 ml of gel during coupling. A 0.5-mL column was packed and equilibrated with 100 mM NaCl, 20 mM Pipes, pH 7.0, 1 mM EDTA, 0.1 mM TCEP. Acidic and basic chicken fast TnT (1.5 nmol each) were dissolved in 10 μl of the equilibration buffer (TnT concentration of 0.3 μM) and loaded onto the column at a flow rate of ~0.2 ml/min. The column was washed with three volumes of the equilibration buffer and eluted by a gradient of 100–600 mM NaCl in the equilibration buffer (20-μl total volume, 0.5-ml fractions). All steps were carried out at room temperature. The fractions were resolved by 180:1 acrylamide/bisacrylamide 14% SDS-PAGE gel and silver stained to determine the high Mᵣ acidic and low Mᵣ basic TnT peaks. For silver staining, the resolved SDS-PAGE gel was shaken for 20 min in 20 volumes of 50% methanol, 12% trichloroacetic acid, 2% CuCl₂, followed by 10 min in 10% ethanol, 5% acetic acid and then 10 min in 0.01% K₂MnO₄. The gel was then shaken in 10% ethanol, 5% acetic acid for 10 min, 10% ethanol for 10 min, and in double-distilled H₂O for 10 min and then incubated in 0.1% AgNO₃ for 10 min. Following incubation in 0.1% AgNO₃, the gel was rinsed in double-distilled H₂O for 30 s, shaken in 10% K₂CO₃ for 60 s, and then incubated in 0.01% formaldehyde, 2% K₂CO₃ until the protein bands were clearly visible. The gel was then briefly incubated in 10% methanol, 5% acetic acid and photographed.

**Solid Phase Protein Binding Assay**—To study the high affinity interactions of TnT isoforms with TnI or Tm, we used a solid phase protein binding assay (29). Chicken TnT, α/α- or α/β-Tm, or BSA were separately dissolved to 5 μg/ml in Buffer A and coated overnight (100 μM, 4 °C) onto triplicate wells of microtiter plates (Falcon 3915). The plates were then washed with Buffer B and blocked for 90 min at room temperature with Buffer B containing 1% BSA. Following three washes with Buffer B, the plates were incubated with serial dilutions of acidic or basic fast TnT in Buffer B with 0.1% BSA for 90 min at room temperature. The bound TnT was detected by mAb 3E4 (1:2000 dilution) and color development was done as detailed above. The intensity of the bound TnT was well fit by single exponentials. The fits were used to determine the concentration of TnT required for 50% maximal binding. Differences in the concentration of TnT required to achieve 50% maximal binding were determined as a measure of the extent of TnT isoform interactions.

**Solid Phase Competition Assay of TnT-Tm Interaction**—The competition and cooperativity between acidic and basic TnT for Tm binding was monitored by TnT isoform-specific antibodies. We developed a solid phase ELISA method where the binding of acidic fast TnT to immobilized Tm was selectively detected by mAb 6B8 (see Fig. 2B) in the presence of basic fast TnT or bovine cardiac TnT. Briefly, following incubation of TnT isoforms or Tm with Buffer B (0.1% BSA) at 4 °C, the plates were blocked with Buffer B with 1% BSA. The immobilized Tm was incubated with TnT mixtures (in Buffer B, 0.1% BSA) wherein the concentration of acidic TnT remained constant (25 nm), but the concentration of basic or bovine cardiac TnT was titrated from 0 to 2.5 μM. Following incubation of Tm with TnT for 1.5 h, the binding of acidic TnT to Tm was determined by mAb 6B8 (1:2000 dilution). The detection of the bound antibody and color development was done as detailed above. The interaction of TnT isoforms with immobilized Tm was also analyzed using the same ELISA protocol.

To determine the effect of the NH₂- and COOH-terminal domains of TnT on the cooperative binding of full-length TnT to Tm, the NH₂-terminal N165 fragment of chicken fast skeletal muscle TnT (Ref. 28, equivalent to the chymotryptic fragment T1 (7)) and the COOH-terminal T2 fragment of rabbit skeletal muscle TnT were used in serial dilutions as the competing TnT species. For these experiments, mAb 3E4 (1:2000) was used to selectively detect bound full-length acidic TnT. This mAb does not cross-react to fragment N165 or the T2 fragment (27). The ELISA titration results are presented as the average ± S.D. The binding curves of TnT to Tm and TnI were well fit by single exponentials. The fits were used to determine the concentration of TnT required for 50% maximal binding. Differences in the concentration of TnT required to achieve 50% maximal binding were determined as a measure of the extent of TnT isoform interactions.

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**Co-immunoprecipitation**—We measured the competition of TnT isoforms for Tm binding by co-immunoprecipitation. In these assays, the concentration of chicken acidic fast skeletal muscle TnT and chicken breast muscle α/α-Tm dimers were fixed at 0.1 μM and 0.05 μM, respectively. An increasing concentration of bovine cardiac TnT (0–0.1 μM) was used as a competitor of the acidic fast skeletal muscle TnT for α/α-Tm binding. The TnT/Tm mixture was incubated for 2 h at room
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RESULTS

Purified Thin Filament Regulatory Proteins Used in This Study—The purified thin filament regulatory proteins used in this study were resolved by 29:1 acrylamide/bisacrylamide 12% SDS-PAGE and were transferred to nitrocellulose membrane and immunoblotted using antibodies specific for TnT (Anti-TnT), Tm (Anti-Tm), and TnI (Anti-TnI). SDS-PAGE and Western blots indicate that the protein preparations are homogenous without cross-contamination with other thin filament proteins or proteolytic fragments. Migration of standard molecular mass markers (arrowhead) is shown.

Temperature in 100 mM NaCl, 10 mM PIPES, pH 7.0, 3 mM MgCl₂, 0.05% Tween 20. Following the initial incubation, anti-Tm mAb CH1 was added (~3 μg) and was allowed to incubate for 1 h at room temperature. The antibody-bound TnT-Tm complexes were precipitated by incubation with Protein A-Sepharose beads (Amersham Pharmacia Biotech) for 35 min at room temperature. Following a brief centrifugation at 8000 × g to pellet the beads, excess buffer was removed with a 27-gauge needle. The beads were washed with 100 volumes of the incubation buffer, excess liquid was removed, and the beads were resuspended in 20 μl of SDS-PAGE sample buffer. The samples were heated at 80 °C for 3 min and resolved by 180:1 acrylamide/bisacrylamide 14% SDS-PAGE. Protein bands were visualized by silver staining and analyzed by densitometry as described above.

Differential Interactions between TnT and Tm Isoforms—Affinity chromatography was used to determine the relative affinity of mAbs 3E4 and 6B8 toward the two classes of purified chicken fast skeletal muscle TnT. Both acidic and basic TnT were identified by 3E4 without significant differences in the titration curves (Fig. 2A). Because 3E4 detects TnT isoforms with equal relative affinity, it was suitable for comparing the interaction of acidic and basic TnT with TnI and Tm. In contrast, indirect ELISA titration showed that mAb 6B8 was highly selective for acidic TnT over basic TnT (Fig. 2B). The two antibodies allow for either comparable (mAb 3E4) or selective (mAb 6B8) detection of chicken fast skeletal muscle TnT isoforms.

Specific Monoclonal Antibodies Distinguishing TnT Isoforms—Indirect ELISA was done to determine the relative affinity of mAbs 3E4 and 6B8 toward the two classes of purified chicken fast skeletal muscle TnT. Both acidic and basic TnT were identified by 3E4 without significant differences in the titration curves (Fig. 2A). Because 3E4 detects TnT isoforms with equal relative affinity, it was suitable for comparing the interaction of acidic and basic TnT with TnI and Tm. In contrast, indirect ELISA titration showed that mAb 6B8 was highly selective for acidic TnT over basic TnT (Fig. 2B). The two antibodies allow for either comparable (mAb 3E4) or selective (mAb 6B8) detection of chicken fast skeletal muscle TnT isoforms.

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maximal binding. In addition, the absolute value of the binding curve was obtained after washing under non-equilibrium conditions, which appeared to be a more sensitive measurement of the binding affinity than that determined by the elution profile from an affinity column.

**Differential Interaction of Acidic and Basic TnT with TnI**—Using the solid phase binding assay, the relative affinity of acidic or basic TnT for fast skeletal muscle TnI was determined. The titration curves for TnI binding (Fig. 5) demonstrate that the maximal binding of basic TnT to TnI was significantly higher than the binding of acidic TnT to TnI (p < 0.05). Similar to the Tnt-Tm interactions (Fig. 4), no statistical difference in the relative affinities of the TnT isoforms for TnI was detected by the concentrations of TnT required to achieve 50% maximal binding (Table I).

**Cooperative Interaction of TnT with Tm**—Troponin T isoform competition assays were performed to further characterize the different binding strengths of TnT isoforms to Tm. Instead of a simple competition between the two TnT isoforms, the results showed cooperativity between TnT isoforms for Tm binding. The initial increase of the concentration of basic TnT did not produce a competitive effect on the binding of acidic TnT. In contrast, between 25.0 and 75.0 nM total TnT in the assay (with molar ratios of basic/acidic TnT between 0 and 2), the binding of acidic TnT to αβ-Tm was significantly increased (Fig. 6). These results indicate that the binding of basic TnT to Tm promoted the binding of acidic TnT to Tm by a cooperative effect in the absence of F-actin. As expected, the binding of acidic TnT to Tm rapidly decreased at higher molar ratios of basic to acidic TnT, reflecting effective competition by the excess basic TnT.

In similar competition experiments analyzing the binding of TnT isoforms to immobilized TnI, no such cooperative effect was observed. Increasing concentrations of basic TnT effectively competed with acidic TnT for TnI binding (Fig. 7). The competition experiments were done with two concentrations of TnI during coating (1 and 5 µg/ml) to ensure that the coating density of TnI was not responsible for the absence of cooperativity. Basic TnT competed more effectively with acidic TnT for TnI binding when 1 µg/ml TnI was used during coating versus 5 µg/ml. Unlike the cooperativity seen between TnT isoforms for Tm binding (Fig. 6), the interaction of TnT isoforms with TnI was strictly competitive.

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**TABLE I**

The concentration of TnT required for 50% maximal binding to TnI or Tm

| TnT Isoform | 50% Maximal Binding (nM) | 95% Maximal Binding (nM) |
|------------|--------------------------|--------------------------|
| αα-Tm      | 0.083 ± 0.047             | 0.109 ± 0.014             |
| αβ-Tm      | 0.125 ± 0.021             | 0.076 ± 0.030             |
| TnI        | 0.086 ± 0.019             | 0.095 ± 0.009             |

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**Fig. 3. αβ-Tm affinity chromatography of acidic and basic TnT.** αβ-Tropomyosin was coupled to CNBr-activated Sepharose 4B for affinity column chromatography to examine the binding affinity of acidic and basic TnT isoforms to Tm. A mixed solution of acidic and basic TnT (0.15 µM each) was allowed to interact with the immobilized αβ-Tm. After collecting the flow through, the column was eluted by an increasing [NaCl] gradient. The column fractions were examined by SDS-PAGE and silver staining. Although acidic and basic TnT elute closely together, the basic TnT elution precedes acidic TnT at lower [NaCl], reflecting a lower binding affinity.
We used a competition assay to monitor the binding of acidic TnT to α- or αβ-Tm in the presence of increasing molar ratios of basic TnT. Both experiments show that a significant molar excess of basic TnT was required to compete acidic TnT from Tm. The binding of acidic TnT to αβ-Tm was stronger than αα-Tm. However, at an optimal concentration of total TnT (~75 nM), the presence of basic TnT significantly enhanced the binding of acidic TnT to Tm showing a cooperative effect. Bars indicate mean ± S.D.

**Fig. 6. Cooperative binding of acidic and basic TnT isoforms to Tm.** We used a competition assay to monitor the binding of acidic TnT to αα- or αβ-Tm in the presence of increasing molar ratios of basic TnT. Both experiments show that a significant molar excess of basic TnT was required to compete acidic TnT from Tm. The binding of acidic TnT to αβ-Tm was stronger than αα-Tm. However, at an optimal concentration of total TnT (~75 nM), the presence of basic TnT significantly enhanced the binding of acidic TnT to Tm showing a cooperative effect. Bars indicate mean ± S.D.

**Fig. 7. Competitive binding of TnT isoforms to TnI.** A competition assay was carried out similar to that in Fig. 6 but used immobilized TnI as a TnT-binding partner. The increase in basic TnT concentration did not promote cooperative binding to TnI but instead demonstrated only competition. As expected, a lower amount of coated TnI (1 μg/ml versus 5 μg/ml) emphasized the competition between TnT isoforms for TnI binding. The results show that rather than cooperative, the binding of acidic and basic TnT isoforms to TnI is a competitive interaction. Bars indicate mean ± S.D.

Co-immunoprecipitation of TnT-Tm—The cooperativity between TnT isoforms for Tm binding was independently shown by co-immunoprecipitation experiments. Using a constant concentration of chicken fast skeletal muscle TnT (0.1 μM), Fig. 8A shows that increasing concentrations of up to 0.025 μM bovine cardiac TnT promoted cooperative binding of the fast skeletal muscle TnT to Tm. This resulted in an increase in the apparent affinity of fast skeletal muscle TnT for Tm. As the concentration of bovine cardiac TnT was further increased (0.025–0.1 μM), the fast skeletal muscle TnT was effectively competed from binding Tm. The cooperative TnT binding between cardiac and fast skeletal muscle TnT was also seen in the solid phase protein binding experiment (Fig. 8B). The relative binding of skeletal and cardiac TnT at equal concentrations (0.1 μM) showed that the affinity of cardiac TnT for Tm was higher (Fig. 8A), consistent with a more effective cooperative effect of cardiac TnT than that of basic fast TnT on the binding of acidic fast TnT. Denaturing SDS-PAGE shows that using a constant concentration of chicken acidic fast skeletal muscle TnT (fTnT), subsaturating concentrations (0–0.025 μM) of bovine cardiac TnT (cTnT) were able to increase the apparent affinity of TnT for Tm. At saturating concentrations of total TnT, cTnT was able to compete with fTnT for Tm binding. The plot of densitometric analysis data showed that total TnT binding to Tm (B) was saturable at ~1:1 molar ratio of TnT/Tm dimers. Increasing the concentration of cTnT in the reaction resulted in increased binding to Tm (C). However, increasing the cTnT concentration in the reaction up to 0.025 μM also promoted cooperative binding of cTnT to Tm (D). Once the TnT-Tm interaction was saturated (at 0.125 μM total TnT), increasing cTnT concentrations effectively competed with fTnT for Tm binding. The dashed line represents the molar ratio of cTnT/fTnT/Tm, B, solid phase binding assays. The cooperative binding of cardiac and fast skeletal TnT was analyzed by the competition assay as in Fig. 6. Similar to that observed for the acidic and basic fast skeletal muscle TnT isoforms, the results show that the increasing concentrations of bovine cardiac TnT promoted cooperative binding of chicken fast skeletal muscle TnT. Excess bovine cardiac TnT competed off the acidic fast skeletal muscle TnT. Bars indicate mean ± S.D.

Co-immunoprecipitation of TnT-Tm—The cooperativity between TnT isoforms for Tm binding was independently shown by co-immunoprecipitation experiments. Using a constant concentration of chicken fast skeletal muscle TnT (0.1 μM), Fig. 8A shows that increasing concentrations of up to 0.025 μM bovine cardiac TnT promoted cooperative binding of the fast skeletal muscle TnT to Tm. This resulted in an increase in the apparent affinity of fast skeletal muscle TnT for Tm. As the concentration of bovine cardiac TnT was further increased (0.025–0.1 μM), the fast skeletal muscle TnT was effectively competed from binding Tm. The cooperative TnT binding between cardiac and fast skeletal muscle TnT was also seen in the solid phase protein binding experiment (Fig. 8B). Similar to the cooperativity observed between the acidic and basic fast skeletal muscle TnT (Fig. 6). The relative binding of skeletal and cardiac TnT at equal concentrations (0.1 μM) showed that the affinity of cardiac TnT for Tm was higher (Fig. 8A), consistent with a more effective cooperative effect of cardiac TnT than that of basic fast TnT on the binding of acidic fast TnT. Denaturing SDS-PAGE shows that using a constant concentration of chicken acidic fast skeletal muscle TnT (fTnT), subsaturating concentrations (0–0.025 μM) of bovine cardiac TnT (cTnT) were able to increase the apparent affinity of TnT for Tm. At saturating concentrations of total TnT, cTnT was able to compete with fTnT for Tm binding. The plot of densitometric analysis data showed that total TnT binding to Tm (B) was saturable at ~1:1 molar ratio of TnT/Tm dimers. Increasing the concentration of cTnT in the reaction resulted in increased binding to Tm (C). However, increasing the cTnT concentration in the reaction up to 0.025 μM also promoted cooperative binding of cTnT to Tm (D). Once the TnT-Tm interaction was saturated (at 0.125 μM total TnT), increasing cTnT concentrations effectively competed with fTnT for Tm binding. The dashed line represents the molar ratio of cTnT/fTnT/Tm, B, solid phase binding assays. The cooperative binding of cardiac and fast skeletal TnT was analyzed by the competition assay as in Fig. 6. Similar to that observed for the acidic and basic fast skeletal muscle TnT isoforms, the results show that the increasing concentrations of bovine cardiac TnT promoted cooperative binding of chicken fast skeletal muscle TnT. Excess bovine cardiac TnT competed off the acidic fast skeletal muscle TnT. Bars indicate mean ± S.D.
The NH$_2$-terminal region of TnT (T2 fragment) resulted in increased binding of acidic TnT to Tm. Therefore, the COOH-terminal Tm binding site of TnT alone may be the primary determinant to initiate in the cooperative binding of TnT to Tm.

**DISCUSSION**

The NH$_2$-terminal Variable Region of TnT Affects Binding to TnI and Tm—Isoform and sequence diversity of the NH$_2$-terminal extension of TnT indicate that this fragment is a non-essential element. A genetically expressed TnT with an NH$_2$-terminal truncation has been shown to conserve the core functions of full-length TnT (34). Therefore, the primary role of TnT in striated muscle thin filaments as an intermediary to tether TnI and TnC to Tm can be accomplished in the absence of the NH$_2$ terminus of TnT. Consistent with these observations, the highly conserved COOH-terminal region of TnT is responsible for TnI and TnC binding and has the higher affinity Tm binding site. The second Tm binding site resides in the central region of TnT (3, 35). However, an increasing amount of data demonstrates the functional importance of the variable NH$_2$-terminal region of TnT (36–38). This domain overlaps the head-to-tail junction of adjacent Tm dimers (39). Variations in the NH$_2$-terminal primary structure are able to modify the overall conformation of TnT (27, 28), affecting its interaction with Tm (29) and TnI (27) while changing the Ca$^{2+}$ sensitivity of force and stiffness in skinned skeletal muscle fibers (25). The effects of TnT isoforms on muscle contractility are likely to be mediated through a single binding site near the COOH terminus of TnT (40). The differences in TnT binding between the acidic and basic TnT isoforms supports the conclusion that changes in the primary structure of the NH$_2$-terminal variable region dictate changes in the overall tertiary structure of the TnT, including the functionally important COOH-terminal domain. Although the variable NH$_2$-terminal region of TnT does not directly interact with TnI or Tm, its effects on the binding of intact TnT to these thin filament proteins is evident.

**The TnT-Tm Interaction Is Cooperative Independent of F-actin—**In the solid phase competition assays (Fig. 6), basic TnT required a molar excess to effectively compete with acidic TnT most likely because of its weaker binding to Tm (Fig. 4). However, with an optimal range of concentration of total TnT in the reaction, the addition of basic TnT was able to increase the binding of acidic TnT to Tm by a cooperative effect. This was also shown in the co-immunoprecipitation experiments (Fig. 8) wherein bovine cardiac TnT increased the apparent affinity of acidic TnT for Tm. The cooperativity between TnT molecules for Tm binding appears to be a general feature because chicken basic fast TnT, bovine cardiac TnT, and the conserved COOH-terminal T2 fragment all induced cooperative binding of the acidic fast TnT to Tm. However, we have not excluded the possibility that the presence of two or more TnT isoforms (e.g. in most vertebrate fast skeletal muscles) may enhance the cooperative interaction with Tm. Although it has been previously shown that the troponin interaction with Tm-F-actin filaments is cooperative (38), our experiments demonstrate that TnT-Tm cooperativity also exists in the absence of F-actin. Increased acidic TnT binding to Tm is well explained by cooperative recruitment of TnT molecules to adjacent Tm dimers following the initial interaction between TnT and Tm molecules. In effect, the binding of TnT to a Tm dimer would promote a conformational change not only in the Tm dimer bound to TnT but also in the adjacent naked Tm dimer. This conformational change would "prime" the adjacent Tm dimer for interaction with TnT, explaining the cooperativity seen in Figs. 6 and 8. Further, co-immunoprecipitation experiments demonstrated that the cooperative TnT-Tm interaction remained stoichiometric, mimicking the physiological ratio of one TnT molecule per Tm dimer. We found that the COOH-terminal Tm binding site of TnT was the initiator of the cooperative TnT-Tm interactions, as the COOH-terminal T2 fragment, not the NH$_2$-terminal N165 fragment, promoted cooperative binding (Fig. 9). Because TnT from different genes (cardiac versus fast skeletal muscle) and species (avian versus mammalian) promoted cooperative TnT binding to Tm, this conserved function of TnT is expected to be mediated primarily by a conserved domain of the molecule. The importance of the T2 region of TnT in this cooperativity is intuitive, because it is the site of interaction for TnI-TnC and would be the primary intermediary following activation when TnC binds Ca$^{2+}$. Because our detection of the cooperative TnT-Tm interaction was through the binding of intact TnT in the assay systems, we cannot exclude that the central region Tm binding site and the NH$_2$-terminal domain of TnT contributed to the responding effect. Note that cooperative TnT binding to Tm in the presence of the T2 fragment was not as effective as in the presence of intact TnT. This is predicted and in agreement with previous data that the NH$_2$-terminal variable region of TnT increases but is not critical to the cooperative binding of troponin to Tm-F-actin filaments (38). However, our results suggest that association with F-actin is not absolutely required for the cooperativity and/or cross-talk between adjacent Tm dimers. The results suggest that there is a determinant of cooperativity in muscle activation/relaxation intrinsic to troponin-Tm interaction.
**TnT Isoform Regulation and Thin Filament Signal Transduction**—Our experiments have demonstrated that acidic and basic TnT isoforms show distinct interactions within the thin filament. These changes in TnT function are predicted to be because of NH₂-terminal variation-originated changes in the tertiary structure of the COOH-terminal T2 region where both TnI and Tm show strong interactions. However, the mechanism governing how functional differences in TnT isoforms contribute to muscle contractility remains unclear. Previous studies have demonstrated that striated muscle fibers expressing more acidic TnT isoforms show increased sensitivity to Ca²⁺ for both force and stiffness development (25, 41, 42). In addition, transgenic expression of a relatively basic fast skeletal TnT-T2 fragment and helpful discussions and Dr. J. J.-C. Lin for the anti-Tm mAb CH1.

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