Roles for the Troponin Tail Domain in Thin Filament Assembly and Regulation

A DELETIONAL STUDY OF CARDIAC TROPONIN T*

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Striated muscle contraction is regulated by Ca\(^{2+}\) binding to troponin, which has a globular domain and an elongated tail attributable to the NH\(_2\)-terminal portion of the bovine cardiac troponin T (TnT) subunit. Truncation of the bovine cardiac troponin tail was investigated using recombinant TnT fragments and subunits TnI and TnC. Progressive truncation of the troponin tail caused progressively weaker binding of troponin-tropomyosin to actin and of troponin to actin-tropomyosin. A sharp drop-off in affinity occurred with NH\(_2\)-terminal deletion of 119 rather than 94 residues. Deletion of 94 residues had no effect on Ca\(^{2+}\)-activation of the myosin subfragment 1-thin filament MgATPase rate and did not eliminate cooperative effects of Ca\(^{2+}\)-binding. Troponin tail peptide TnT1–153 strongly promoted tropomyosin binding to actin in the absence of TnI or TnC. The results show that the anchoring function of the troponin tail involves interactions with actin as well as with tropomyosin and has comparable importance in the presence or absence of Ca\(^{2+}\). Residues 95–153 are particularly important for anchoring, and residues 95–119 are crucial for function or local folding. Because striated muscle regulation involves switching among the conformational states of the thin filament, regulatory significance for the troponin tail may arise from its prominent contribution to the protein-protein interactions within these conformations.

Tropominin is a striated muscle regulatory protein (see reviews in Refs. 1–4) that is located at periodic, 38-nm spacing along muscle thin filaments. This spacing is due to the 1:1 complex formation of troponin with tropomyosin, an elongated coiled-coil protein that stretches along seven actin monomers. Ca\(^{2+}\) binding to troponin triggers conformational changes in the thin filament, thereby allowing actin and myosin to interact to produce force and movement. Troponin contains two domains: a globular region, which is composed of subunits TnC, TnI, and the COOH-terminal portion of TnH, and a highly extended region, or tail, containing the remainder of TnT (5, 6). The globular region has a central role in regulation, because it is the site of calcium binding. In contrast, the tail region of troponin, which is the subject of this report, has an uncertain role in conformational changes of the thin filament. One possibility is that it has little direct effect on regulation, acting instead as a calcium-insensitive anchor that holds troponin onto tropomyosin (7, 8). However, the details of the interactions of troponin with actin and tropomyosin are unknown, in any of the conformations of the thin filament. Moreover, there is increasing evidence that the structure of the troponin tail can alter thin filament function in a complex manner (9–16). To better understand the troponin tail region, the present study reports the properties of a series of troponin complexes containing progressively less of this region. Cardiac TnC plus TnI was reconstituted with either cardiac TnT or a series of recombinant NH\(_2\)-terminal truncation mutants of TnT. The NH\(_2\)-terminal region of TnT was also examined in isolation, in the absence of TnC or TnI. The results suggest that the anchoring function of this region involves interactions with actin as well as with tropomyosin and that this function is not confined to a small region of the tail, and they suggest that a critical peptide in the tail may be particularly important for proper function.

MATERIALS AND METHODS

Design of Recombinant TnT cDNAs for Bacterial Expression—Bovine cardiac TnT cDNA was cloned from a bovine heart cDNA library. The encoded amino acid sequence was almost completely consistent with the predominant adult bovine cardiac TnT isoform, as previously determined by protein sequencing (17). However, the cDNA contained no codon for Glu-42 of the protein sequence, possibly due to splice site variability within the alternative splicing region of TnT (18–20).

Many of the TnTs used in the present study are NH\(_2\)-terminal truncations, which therefore do not include the lone Cys (Cys-39) of native TnT. (Numbering follows the published protein sequence.) Therefore, to facilitate labeling of all the planned TnTs by carboxymethylation with radioactive iodoacetate, a single Cys mutant was designed by performing the double mutation C39S/D266C. Asp-266 was chosen because it would be present in all the truncation mutants, was in a charged region unlikely to be buried on folding, was not directly implicated in interactions with the other troponin subunits (2), and would have its negative charge restored by the planned carboxymethylation of the new Cys-266. The C39S mutation was created using polymerase chain reaction, with a primer producing both the desired codon change (G170C) and a protein-silent change (A177G) that created a SacII site to facilitate identification of recombinants. The D266C mutation was produced similarly by polymerase chain reaction, using a primer with TnT cDNA alterations G847T/A848G to encode the amino acid change, as well as A834T/C831A to introduce a silent BSoA1 site. Double mutant C39S/D266C was obtained by subcloning. To remove 94 NH\(_2\)-terminal residues, D266C TnT cDNA was inserted in pSP72 and digested with EcoRI and HindIII, and the TnT-encoding 3'-fragment was isolated. A similar digestion using BsoAI and HindIII yielded a 550-base pair fragment encoding the COOH-terminal portion of TnT starting at Leu-120. cDNAs encoding TnT COOH-fragments starting with Glu-133, Ala-154, or Ala-174 were created by polymerase chain reaction, using

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The abbreviations used are: Tn, bovine cardiac troponin; myosin S1, myosin subfragment 1; Tn95, ternary troponin complex composed of bovine heart TnC, TnI, and a recombinant TnT fragment from position 95 to the COOH terminus (Lys-284); Tn120, Tn133, Tn154, Tn174, similar to Tn95; CBMII, D65A/E66A cardiac TnC.

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D266C TnT as the template. Plasmids encoding NH2-terminal TnT fragments 1–94, 1–132, and 1–153, and 1–173 were created by polymerase chain reaction and contained Cys-39 rather than the C39S mutation. All cDNAs were inserted into the expression plasmid pET3d (21) and confirmed by automated chain termination sequencing.

Expression of TnT and TnT Expression and Purification—Recombinant TnT was obtained by modification of published protocols (10). Bacterially expressed and extracted TnTs were dialyzed against a denaturing buffer consisting of 10 mM Tris-HCl (pH 7.5), 5 mM urea, 1 mM dithiothreitol, 5 μM/ml 1-1-tosylamido-2-phenylethyl chloromethyl ketone, and 5 μM/ml 1-chloro-3-tosylamido-7-amino-2-heptanone. The full-length TnT molecules were applied on a Flow Q Sephadex G100 which was then washed with an 80 mM NaCl buffer, and then purified with a 0–0.6 M NaCl gradient. The truncated forms were similarly purified, but using an SP Sepharose column. An additional column purification step was performed using fast protein liquid chromatography columns, either Resource Q (full-length TnTs) or Resource S (truncated TnTs missing the negatively charged NH2-terminal region). The fast protein liquid chromatography step was sometimes omitted, and residual impurities after the Sepharose step removed instead by gel filtration of the troponin complex after reconstitution with TnI and TnC.

Reconstitution of Troponin Complexes Containing Truncated Forms of TnT—Recombinant TnI or TnT fragments were mixed under denaturing conditions with bovine cardiac myosin S1 and TnC in an 1:1:1 ratio. Protein molarities were calculated by combining measured absorbance with sequence-determined aromatic amino acid composition (21). However, NH2-terminal TnT fragments have weak molar absorbance (one Tyr and no Trp), so they were measured by protein assay ESL (Boehringer Mannheim), using a modification (22) of the biuret reaction. The mixtures were then dialyzed in several steps to remove urea and KCl (13). This procedure was modified for the truncated troponins, which precipitated when the protocol was tried without modification: the pH of all dialysis buffers was raised from 7.5 to 8.8 (Tris-HCl in both cases), and the final dialysis step retained 0.1M KCl, instead of removing all KCl as in the unmodified procedure. This succeeded in producing ternary troponin complexes containing bovine cardiac TnI, TnC and truncated TnTs (21). All samples contained 0.2 mM dithiothreitol, 0.3 mg/ml bovine serum albumin, and either 0.5 mM EGTA or 0.1 mM CaCl2. Displacement of the 3H-labeled troponin from the thin filament was produced by varying the concentration of competitive labeled troponin to the affinity of control labeled troponin was fit using the following expression,

\[ 0 = F_S + F_R + \frac{F_S (S + T - K_T)}{K_T + (S - C - T)} \]

where C (the independent variable) is the concentration of added competitor troponin, S is the concentration of troponin binding sites on F-actin, and T is the total concentration of [3H]troponin.

MgATPase Rate Assays—The actin-activated MgATPase rate of rabbit skeletal muscle myosin S1 (28) was measured by release of 32P from γ-labeled ATP (29). Skeletal muscle myosin S1 was used instead of bovine cardiac myosin S1 because of its greater stability, higher ATPase rates, and many functional similarities: (i) the effects of Ca2+ on Vmax, ATPase actin Km, and true myosin S1-ATP affinity for the thin filament (26, 30, 31); (ii) ATPase rate linearity with myosin S1 concentration, cooperative ATPase activation by the free Ca2+ concentration (30, 32, 33), and cooperative ATPase activation by the Ca2+ + troponin complex (31); (iii) the inactivation of the Ca2+ + troponin complex by MgATP (29). Rates were linear over 10 min (four or five aliquots removed and quenched with sulfuryl/silicotungstic acid) and were initiated by the addition of 10 μM of 20 mM ATP to 190–200 μM protein samples.

RESULTS

Formation of Troponin Complexes with Variously Long Tail Regions—Although the structure of troponin is unknown, available information aids interpretation of progressive NH2-terminal truncations of TnT. Troponin contains a globular domain and an elongated tail region (6). The globular domain contains TnI, TnC, and the COOH-terminal portion of TnT. Specifically, rabbit fast skeletal muscle TnT COOH-terminal residues 159–259 (35) form a stable ternary complex with TnI and TnC (36–39). The troponin tail is formed by an imprecisely defined part of TnT, which is highly elongated (6, 7). Mass attributable to TnT residues 1–71 is located far from the other subunits, at the end of the tail (5). The corresponding NH2-terminal region of bovine cardiac TnT, used in the present study, contains an additional 27 amino acids (17), and this produces a 20-Å increase in TnT length for the cardiac isoform (40). These observations suggest that serial deletions of the cardiac TnT NH2 terminus will qualitatively shorten the tail region, without preventing troponin complex formation. The structure of such complexes will depend on the original tertiary structure of the tail region and upon any folding alterations resulting from the deletion.

A series of troponin complexes was created lacking a variable amount of the TnT sequence at the NH2 terminus, from 0 to 173 residues. The least (94 residues) and the most extensive (173 residues) truncations correspond to skeletal muscle TnT deletions of 67 or 146 residues, which can be removed with relatively minor or major effects on troponin function, respectively (24, 36, 41). As predicted from results with skeletal muscle TnT, SDS-polyacrylamide gel electrophoresis analysis of gel filtered samples (Fig. 1A) indicated that all of these cardiac TnT fragments could be combined with TnC and TnI to...
form ternary complexes. In other words, none of the deletions caused sufficient unfolding of the remaining TnT fragment to preclude formation of a truncated troponin complex, nor did the deletions remove sequences critical for complex formation.

The hydrodynamic properties of the various troponin complexes were compared by gel permeation chromatography. There was a monotonic pattern of decreasing Stokes radius as the amino terminus was progressively shortened (Fig. 1B). There was a linear decrease in \( R_s \), the amino terminus of TnT was progressively removed. Errors are \( \pm 1 \AA \). Note that there is no discontinuity when 119 residues were removed instead of 94 residues. However, removal of 119 residues had a much greater effect on function (see below).

The preceding experiments were conducted in the presence of high ionic strength conditions \((0.3 \text{ m KCl})\), a condition that we found essential for quantitative assessment of the full-length control molecules \((24)\), which tend to polymerize with tropomyosin under lower ionic strength conditions. To better study the truncated troponins, they were also examined in the presence of 60 mM KCl. Under these conditions, the truncated troponins lacking 119 \((\bullet)\) or 132 \((\Theta)\) TnT residues promoted tropomyosin binding to actin (Fig. 2B), implying direct and/or indirect interactions between tropomyosin and these troponins under these lower ionic strength conditions. However, the effect on tropomyosin sedimentation was much less than the effects of full-length troponin \((\bigcirc)\) or troponin lacking 94 TnT residues \((\bigtriangleup)\).

**Affinity of Troponin-Tropomyosin for Actin**—To explore the phenomenon illustrated in Fig. 2 in more detail, actin binding was assessed as the concentrations of the various troponin-tropomyosin complexes were varied. If the tail region of troponin merely holds the molecule onto tropomyosin, then deleting part of the tail would have little effect on the affinity of the troponin-tropomyosin complex for actin. However, Fig. 3A shows that progressive deletion of the tail had a large and progressive effect on this process in the absence of calcium. Removal of 94 residues \((\text{Tn95, } \bigtriangleup)\) caused a 2-fold shift from control results \((\bullet)\) in this representative experiment. Deletion of another 25 residues \((\text{Tn120, } \bigstar)\) had an additional 7-fold effect, for a 14-fold effect relative to whole troponin. Similar results were found for Tn120 and for Tn133 \((\bigstar)\). These last two complexes had only a small effect on tropomyosin binding to actin, as seen by comparison to data with tropomyosin alone \((\bigstar)\). The truncations produced a similar pattern in the presence of saturating calcium concentrations \((\text{Fig. 3B}),\) suggesting similar importance for the troponin tail in thin filament assembly, regardless whether calcium is bound to TnC.

**Actin Binding Properties of Tropomyosin Complexed to a Troponin Tail Peptide, TnT1153**—An alternative means to evaluate the function of the troponin tail is to examine it directly, rather than studying the effects of its deletion. This was investigated with several NH2-terminal TnT fragments, with mixed success. TnT1153 (Fig. 1A) was the easiest to examine in cosedimentation experiments because, like whole troponin \((24)\), it caused tropomyosin to sediment in the presence of 300 mM KCl only when actin was also present (data not shown). Fig. 3C shows the effects of this TnT NH2-terminal fragment alone \((\text{●})\), in the absence of TnI and TnC, on tropomyosin binding to actin. For comparison, binding of the tropo-
Fig. 2. Troponin tail length is critical for troponin-induced promotion of tropomyosin binding to actin. The binding of 0.5 μM [3H]tropomyosin binding to 10 μM actin was induced by adding varying concentrations of native or recombinant troponin and measured by cosedimentation in the presence of 300 mM KCl (A) or 60 mM KCl (B). For native (●) and recombinant (■) control troponins (TnT C39S/D266C), the tropomyosin binding patterns were indistinguishable. Under both high and low ionic strength conditions, deletion of the first 94 residues of TnT (▲) decreased tropomyosin binding to actin moderately, and this effect was much more profound when the TnT NH2-terminal truncations were larger: (●), 119 residues; (○), 132 residues; (□), 153 residues; (△), 173 residues. The truncations produced a similar pattern in the presence of lower ionic strength conditions (B), except that binding is tighter under these conditions. A second set of experiments gave similar results (not shown).

**FIG. 2.** Troponin tail length is critical for troponin-induced promotion of tropomyosin binding to actin.

The binding of 0.5 μM [3H]tropomyosin binding to 10 μM actin was induced by adding varying concentrations of native or recombinant troponin and measured by cosedimentation in the presence of 300 mM KCl (A) or 60 mM KCl (B). For native (●) and recombinant (■) control troponins (TnT C39S/D266C), the tropomyosin binding patterns were indistinguishable. Under both high and low ionic strength conditions, deletion of the first 94 residues of TnT (▲) decreased tropomyosin binding to actin moderately, and this effect was much more profound when the TnT NH2-terminal truncations were larger: (●), 119 residues; (○), 132 residues; (□), 153 residues; (△), 173 residues. The truncations produced a similar pattern in the presence of lower ionic strength conditions (B), except that binding is tighter under these conditions. A second set of experiments gave similar results (not shown).
Fig. 3. Effect of the troponin tail on the affinity of the troponin-tropomyosin complex for actin. Tropomyosin, troponin-tropomyosin complexes, or TnT-tropomyosin complexes were added to 5 μM actin in increasing concentrations, and binding was measured by monitoring cosedimentation of [3H]labeled tropomyosin. For Tn120 and Tn133, composite data from three experiments are shown. For TnT1–153 and for the other complexes, representative titrations are shown. A shows progressively weaker binding of troponin-tropomyosin complexes to actin as the troponin tail is shortened, determined in the presence of EGTA and 300 mM KCl. K_app values for native troponin (●), Tn95 (■), Tn120 (▲), Tn133 (●), and tropomyosin in the absence of troponin (△) were 6.9 ± 0.5, 3.4 ± 0.2, 0.48 ± 0.04, 0.36 ± 0.03 and 0.30 ± 0.01 x 10^6 M⁻¹, respectively. Best fit McGhee-von Hippel cooperativity parameters (72) were 13 ± 2 for native troponin, 40 ± 10 for Tn95, and 180 ± 98 for tropomyosin alone, and they were set at 30 for Tn120 and Tn133 because they could not be measured from these data. In the absence of any troponin, tropomyosin binding to actin is weak but very cooperative, as shown previously (24, 73).

Fig. 4. Troponin binding to actin-tropomyosin is greatly weakened by progressive shortening of the tail region. A shows displacement of troponin from the thin filament, monitored by the increase in the free, non-actin-bound concentration of radiolabeled troponin as increasing concentrations of competing unlabeled troponin are added. Full-length troponin (●) causes more displacement than is caused by Tn95 (■), and Tn120 (▲) causes only minimal displacement of troponin. Solid lines are best fit curves using Equation 1, and they demonstrate the ratio of the affinities of the unlabeled troponins for actin-tropomyosin, relative to the affinity of the labeled control troponin. K_app values for Tn95 ± 0.11, 0.14 ± 0.02, and 0.002 ± 0.001 for native troponin, Tn95, and Tn120, respectively. B is a noncompetitive demonstration that radiolabeled Tn120 binds to actin nonspecifically. In the absence of actin (○), Tn120 does not sediment. In the presence of actin, it cosediments with 5 μM actin to an extent that is indistinguishable whether 3 μM tropomyosin is absent (□ and ▲) or present (● and ▲) or if Ca²⁺ is absent (○ and ▲) or present (● and ▲).
FIG. 5. Effects of troponin tail truncation on the energetics of thin filament assembly. A schematic representation of equilibria relevant to troponin and tropomyosin binding to actin. Cooperative aspects of these processes are not shown in the figure but are included implicitly, measured values for ΔG include cooperative components. Energetic linkage among the reactions is not obligatory unless cooperative aspects are excluded (not done in the present analysis). B shows the effects, in kJ/mol, of serial truncations of the troponin tail on ΔG2, the free energy of troponin-tropomyosin binding to actin (EGTA, °; Ca2+, +). The dashed line is the average of all the plotted ΔG2 determinations, with Ca2+ and EGTA combined because they do not differ. This panel also shows the effects of the truncations on ΔG1, which is the free energy of troponin binding to actin-tropomyosin. The solid line is the average of the plotted ΔG1 values, combining EGTA (□) and Ca2+ (○). The effect of deleting 119 residues on ΔG1 may be underestimated, because ΔG2 is so weak that it is hard to measure (Fig. 4A).

displace very little of the control [3H]troponin. This parallels the effects of the same deletion on binding of the troponin-tropomyosin complex to actin. Truncation by 119 residues has a severe effect on troponin function.

To further elucidate the properties of Tn120, it was radiolabeled on Cys-266, and its binding to actin or actin-tropomyosin assessed by cosedimentation, i.e., by direct measurement rather than competition assay. Under the high ionic strength conditions of these and the preceding experiments, removal of 119 residues from the TnT tail was sufficient to eliminate specific positioning of the troponin complex on actin-bound tropomyosin. This conclusion is supported by Fig. 4B, which shows that the binding of Tn120 to actin was unaffected by the presence of tropomyosin and was weakened to the μM range.

Effects of Truncation of the Troponin Tail on the Energetics of Thin Filament Assembly—The assembly of troponin and tropomyosin onto the actin filament can be approached as an equilibrium problem, in which alterations in troponin structure can affect processes shown in Fig. 5A. Fig. 5B summarizes effects of troponin tail truncation on troponin binding to actin-tropomyosin (ΔG2), calculated from Kapp measurements, such as those in Fig. 4A, and on troponin-tropomyosin binding to actin (ΔG3), taken from changes in Kapp, measured as in Fig. 3, A and B. Truncation of the troponin tail weakens both processes. Effects on ΔG2 (Fig. 5B, □ and ○) are larger than effects on ΔG3, (° and +), consistent with the known importance of the troponin tail for binding to tropomyosin (5, 7, 36, 45, 46). The results suggest that the troponin tail has a dual role in thin filament assembly, facilitating not only troponin binding to tropomyosin (from previous work), but also the association of these proteins to actin. The simplest explanation would be that the troponin tail interacts directly with actin as well as with tropomyosin. However, these data could also be explained by an indirect effect of the tail, increasing the strength of tropomyosin-actin interactions.

Regulatory Properties of Thin Filaments with Truncated TnT—To study meaningfully the effects of troponin tail truncation on regulatory function, it is necessary for the truncated troponin to bind specifically to tropomyosin on the thin filament. Otherwise, one cannot presume that the troponin is positioned once per seven actins. As indicated above (see especially Fig. 4B), this requirement is satisfied for Tn95 but not for the other truncations. Tn95 or control reconstituted troponin (1–1.5 μM) were combined with tropomyosin (1.5 μM) and actin (7 μM), and Ca2+-sensitive regulation of the myosin S1 MgATPase rate was examined in the presence of 20 mM imidazole (pH 7.1), 30 mM KCl, 3.5 mM MgCl2, and 0.3 μM myosin S1. Troponin tail truncation had no effect on the regulated MgATPase rates: 1.0 ± 0.1 s−1 (n = 5) for Tn95 and 0.9 ± 0.1 (n = 3) for control troponin in the presence of pCa 4, and <0.1 s−1 for both troponins in the presence of pCa>8. These identical results are consistent with the absence of Ca2+ sensitivity in the effects of this truncation on thin filament assembly (Fig. 5B).

Tn95 is missing a portion of TnT that is located at the tropomyosin-tropomyosin overlap joint (5), and aspects of cooperative thin filament activation by Ca2+ may depend upon this missing portion. As we have shown recently (34), Ca2+ binding to the thin filament has a cooperative effect on the ATPase rate of low myosin S1 concentrations, with activation paralleling the fraction of adjacent troponins that both have bound Ca2+, rather than simply matching the fraction of troponins with bound Ca2+. This observation was dependent upon a novel method for controlling fractional Ca2+ binding; manipulating the ratio of two forms of troponin, one that can bind Ca2+ and the other that cannot because of an inactivating mutation in TnC regulatory binding site II (TnC mutant CBMII) (27). If Ca2+ binding to adjacent troponins exerts no cooperative effect, the MgATPase rate will increase linearly with the fraction of troponins to which Ca2+ is bound. In Fig. 6, this experiment is presented using thin filaments that contain mixtures of Tn95 and Tn95 with CBMII. Despite the absence of TnT residues 1–94, nonlinearity is seen. ATPase activation (79) is poorly
from the dashed line, the cooperative effect of Ca\(^{2+}\) residues are deleted; this could explain the poor function of Tn153, but may not be able to fold or function once Tn119 terminal to these could also be critical, particularly residues 120–153, which poorly describes the data, shows the pattern expected if activation were proportional to Ca\(^{2+}\) binding. The data deviate from the dashed line, indicating cooperativity. The solid line is not a fitted curve. It is included to indicate the results previously observed when this experiment was performed with troponin containing full-length TnT (34). Conditions and protein concentrations are described under “Materials and Methods.” Normalized data from two separate preparations are shown. The non-normalized rates using Tn95 and CBMII-Tn95 were 4.4 and 0.2 s\(^{-1}\) for one preparation, respectively, and 4.7 and 0.3 s\(^{-1}\) for the other.

described by the dashed line, indicating that activation is not proportional to Ca\(^{2+}\) binding. The data are not adequate to measure the degree of cooperativity, but the deviation from the dashed line indicates that cooperativity was present. For comparison purposes, the pattern previously reported for nontruncated troponin is shown (Fig. 6, solid line). Removal of this portion of the troponin tail did not eliminate this aspect of cooperative regulation, further emphasizing that the most important functional regions of TnT lie COOH-terminal to residue 94.

DISCUSSION

TnT and tropomyosin are approximately co-linear, and they may interact for at least one-third of the length of tropomyosin (5, 7, 40). Protein fragment studies and analyses of conserved sequence regions suggest that the strongest interaction between tropomyosin and troponin involves the 27 most COOH-terminal residues of tropomyosin and some portion of TnT between residues 71 and 151 for the skeletal muscle isoform (5, 36, 45–51), which corresponds to positions 98–178 in bovine cardiac TnT analyzed in the present study. The present work examines a similar region of TnT, with a focus on its contributions to troponin and tropomyosin-tropomyosin binding to the thin filament, rather than on troponin binding to tropomyosin. We found that serial NH\(_2\)-terminal truncations of TnT resulted in troponin complexes that were progressively smaller as judged by Stokes radius, had a progressive loss of ability to interact with tropomyosin and actin, and had a particularly sharp fall-off in function when a specific 25-residue segment was deleted. The current deletion studies suggest that a cardiac TnT region including residues 95–119 (residues 68–92 for rabbit skeletal muscle TnT) is critical for function. TnT residues COOH-terminal to these could also be critical, particularly residues 120–153, but may not be able to fold or function once 119 TnT residues are deleted; this could explain the poor function of Tn120. However, the smooth Stokes radius results (Fig. 1) suggest there is not an extensive unfolding process.

The present work emphasizes the importance of the troponin tail for interactions with actin, both in the presence and in the absence of calcium. An advantageous method for analyzing the binding of troponin and tropomyosin to actin is to separate contributions from the cooperativity of binding and from non-cooperative binding of a single troponin or tropomyosin to a bare actin filament (24, 52–55). This has not been emphasized in the present study, because we find the variability of such cooperativity measurements (up to 3-fold) to be larger than the variability in the measurements of overall affinity. It is possible that cooperativity contributes to the energetic differences in Fig. 5B, which then would not be attributable simply to actin binding. However, if the contributions of cooperativity are excluded, and the noncooperative actin binding of the various troponin-tropomyosin complexes are compared, the Fig. 3 data imply effects of the deletions that are even larger than the values used in Fig. 5B. Therefore the effects of the deletions must be attributed primarily to alterations in interactions with actin. Furthermore, because either Tn95 or TnT1–153 causes tropomyosin to bind actin almost as tightly as does whole troponin, this implicates a 59-amino acid region of TnT in anchoring of troponin-tropomyosin to actin. TnT residues 95–153, which correspond to skeletal muscle TnT residues 68–126.

The TnT residues that are absent from Tn95 include “hot spots” for dominant-negative TnT mutations causing familial hypertrophic cardiomyopathy (56–58) and also include the TnT hypervariable region that is developmentally regulated by alternative splicing (19). Deleting all or part of this region from cardiac or skeletal muscle TnT has produced only small effects in a variety of assays, at most 2-fold (12, 24, 41, 47, 59, 60). However, the present report is the first measurement of the effect of such a deletion on the affinity of troponin for the thin filament. Figs. 4A and 5B show this to be a significant effect, at least 7-fold, implying a greater importance for this region than established previously.

Because the COOH-terminal portion of TnT is associated with TnI and TnC, Ca\(^{2+}\)-sensitive changes in its interactions have obvious significance for understanding regulation, as recently emphasized by mutational studies of TnI and TnT (11, 15, 61–63). Perhaps less obvious is the regulatory (as opposed to structural) importance of the troponin tail. Nevertheless, the troponin tail may affect regulatory function: as an isolated peptide, the troponin tail increases the cooperativity of myosin S1 binding to the thin filament (16) and alters the actin-tropomyosin-myosin MgATPase rate (15); point mutations in the tail cause abnormally fast thin filament sliding and cardiomyopathy (10, 56); Ca\(^{2+}\)-sensitive regulation is altered either by isoform variation in this region (13, 14, 64) or by small NH\(_2\)-terminal truncations (12, 47), although these latter effects are not always seen (present study and Refs. 59 and 60); in rat cardiac myofibrils, TnT NH\(_2\)-terminal truncation reduces force and MgATPase rate (65). It has been suggested that TnT helps to activate the thin filament (9, 11, 15, 66), possibly due to the tail region specifically (15). However, thin filament activation is functionally (2, 34, 67–69) and structurally (70, 71) complex, and is incompletely understood.

We suggest that the regulatory significance of the troponin tail can be understood by considering the involvement of the tail region in the energetics of assembly of the different conformational states of the thin filament. (We have made a similar argument previously regarding the entire TnT subunit (9).) Recent structural results (70), supported by preliminary work from another group (71), imply there are at least three quaternary structures for the regulated thin filament, defined by

![Graph](image-url)
Ca$^{2+}$- and myosin-induced movements of tropomyosin. The free energies of these states, and the equilibrium constants for transitions among them, include contributions from the direct and indirect interactions of tropomyosin with actin. The present report quantitatively demonstrates the great importance of the tropomyosin tail for these interactions and for stabilizing the various conformational states of the thin filament. This is sufficient to explain, qualitatively, how the tropomyosin tail may also be important for regulation: its large influence on assembly implies effects on tropomyosin movement. Detailed evaluation of this suggestion will require further structural information concerning the conformation of tropomyosin and its position(s) on the thin filament. Studies of tropomyosin that distinguish critical and less critical regions of the molecule, such as the present work, help delineate what structures are required to achieve a compelling understanding of regulation.

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