Folding and Function of the Troponin Tail Domain

EFFECTS OF CARDIOMYOPATHIC TROPONIN T MUTATIONS*

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Troponin contains a globular Ca$^{2+}$-binding domain and an elongated tail domain composed of the N terminus of subunit troponin T (TnT). The tail domain anchors troponin to tropomyosin and actin, modulates myosin function, and is a site of cardiomyopathy-inducing mutations. Critical interactions between tropomyosin and troponin are proposed to depend on tail domain residues 112–136, which are highly conserved across phyla. Most cardiomyopathy mutations in TnT flank this region. Three such mutations were examined and had contrasting effects on peptide TnT-(1–156), promoting folding and thermal stability assessed by circular dichroism (F110I) or weakening folding and stability (T104V and to a small extent R92Q). Folding of both TnT-(1–156) and whole troponin was promoted by replacing bovine TnT Thr-104 with human TnT Ala-104, further indicating the importance of this cardiomyopathy site residue for protein folding. Mutation F110I markedly stabilized the troponin tail but weakened binding of holo-troponin to actin-tropomyosin 8-fold, suggesting that loss of flexibility impairs troponin tail function. The effect of the F110I mutation on troponin-tropomyosin binding to actin was much less, indicating this flexibility is particularly important for the interactions of troponin with tropomyosin. We suggest that most cardiomyopathic mutations in the troponin tail alter muscle function indirectly, by perturbing interactions between troponin and tropomyosin requisite for the complex effects of these proteins on myosin.

In striated muscles, including the heart and skeletal muscle, contraction is tightly regulated by the reversible binding of Ca$^{2+}$ to the thin filament protein troponin. Tight and specific attachment of troponin to the thin filament is mediated by the troponin tail domain, which is composed of the N-terminal portion of TnT and interacts with the tropomyosin C terminus. Hydrodynamic studies (1), rotary-shadowed electron micrographs of troponin (2), and intermediate resolution studies of both troponin-tropomyosin (3) and TnT-tropomyosin co-crystals (4) indicate that the tail domain is highly asymmetric and ~160 Å in length. Electron microscopy of tropomyosin-TnT co-crystals suggests that a long region of the tropomyosin C terminus may interact with the troponin tail (4). However, most of this extended interaction may be very weak, because a variety of other evidence suggests that only the C terminus of tropomyosin binds strongly to troponin (reviewed in Ref. 5). Recently, an x-ray crystallographic study of the tropomyosin C terminus identified and determined the structure of an 18-residue tropomyosin region that comprises a critical TnT-binding site (6). The TnT element that binds to this tropomyosin region is unknown.

A new approach to these important interactions has been provided by the discovery that any of several mutations in the troponin tail region can cause the autosomal dominant disorder, familial hypertrophic cardiomyopathy (FHC). Regardless whether because of mutations in thick filament or thin filament components of the cardiac sarcomere, the characteristic finding in FHC patients is missense or mild truncation mutations (7–13) that generally alter rather than abolish protein function. As many as 15% of FHC kindreds have cardiac TnT mutations, and the largest portion of these are in the troponin tail (see below). The disease phenotype has high penetrance, including myocardial disarray and risk of sudden death by arrhythmia. Therefore, troponin tail function must be altered and protein function studies, if sufficiently sensitive, are likely to identify abnormalities. Indeed, beginning in 1996 (14), numerous studies have described effects on unloaded sliding speed, force, Ca$^{2+}$ affinity, Ca$^{2+}$ sensitivity, and cooperativity (15–24). These effects support the view that the troponin tail has subtle effects on myosin, in addition to its critical function as an anchoring domain.

The present work concerns three FHC-inducing TnT mutations that are of interest because of their location in the important but poorly understood troponin tail region, and because their unexpected effects on troponin solubility suggested alterations in protein folding. In an earlier publication (18), we found that mutations R92Q and F110I (11) greatly impaired the solubility of bovine cardiac troponin. (Bovine TnT amino acids are designated in the present report, unlike some of our previous work (18, 25, 26), by the sequence positions of homologous human cardiac TnT residues (27).) Although it was possible to reconstitute troponin complexes containing these mutant TnTs by gradually dialyzing away denaturant under high salt conditions, R92Q troponin precipitated when the ionic strength was decreased below 0.5 M, and F110I troponin precipitated unless 10% glycerol was added in addition to a high ionic strength buffer. Insoluble proteins can cause human disease (28, 29), but there was no precedent for protein insolubility in FHC. Therefore, it seemed plausible that the observations were due to the use of bovine rather than human troponin. In this regard, it is notable that between residues 70 and 141, human and bovine TnTs are identical except at one position: bovine Thr-104 is Ala in the human sequence. Because this residue is located near both positions 92 and 110, the sites of mutations causing poor solubility, we created bovine

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* The abbreviations used are: TnT, troponin T; FHC, familial hypertrophic cardiomyopathy.
myosin was stoichiometrically labeled under denaturing conditions on with a TLA100 rotor in a Beckman TL100 centrifuge (34). The tropo-
state (three transitions) (data not shown).

demonstrated that the heat of unfolding was in fact gradual and could 156) – 156), F110I TnT-(1-156), 300 mM KCl, 15 mM NaH2PO4 (pH 6.5). Similar 156) was monitored as a function of temperature using an Aviv DS65 136 of the troponin tail domain. These residues comprise a tropomyosin-binding element. Also, note that this region is flanked by many hypertrophic cardiomyopathy alleles, including (# in Fig. 1) sites Arg-92, Ala-104, and Phe-110.

Although not the focus of the present study, the sequence alignment in Fig. 1 should also be considered in relationship to a preliminary, high resolution structure of a troponin complex containing TnC, most of TnI, and TnT fragment 188–288 (36). Of the 101 TnT residues crystallized, 70 (residues 202–271) are ordered and identifiable in the x-ray structure. As can be seen in the figure, these boundaries correspond to those residues of the C-terminal half of TnT that are evolutionarily conserved. Within this region there is particular conservation of amino acids 226–271, which form a coiled coil with subunit TnI and also interact with TnC (36).

Effects of Cardiomyopathy Causing TnT Mutations on Folding of the Troponin Tail—In an earlier study, we reported that TnT R92Q and F110I mutations interfered with the ability of bovine cardiac troponin subunits to reconstitute into a soluble troponin complex (18). To investigate this folding abnormality quantitatively, we isolated troponin tail fragments TnT-(1–156), finding them to be soluble regardless of mutation. No precipitation was observed in low ionic strength buffer at protein concentrations studied (≤1.5 mg/ml). Troponin tail fragment TnT-(1–156) was predominantly α-helical as assessed by circular dichroism (data not shown), as expected (37, 38). When ellipticity was examined as a function of temperature, the mutations caused large but disparate effects (Fig. 2A and Table 1). TnT-(1–156) altered the ellipticity in the manner expected for a destabilizing mutation, decreasing the temperature of the unfolding curve midpoint (Tm) by 4.2 °C and also decreasing the absolute magnitude of the maximal ellipticity observed at the lowest temperature examined (−5 °C). Cardiomyopathy causing mutation R92Q similarly decreased both the ellipticity

MATERIALS AND METHODS

Protein Purification and Construct Design—Bovine cardiac tropomyosin, native troponin, TnI, and TnC were purified as described previ-
ously (31, 32) from a heart muscle ether powder. Actin was obtained from an acetone powder of rabbit fast skeletal muscle (33). Whole troponin was reconstituted from denatured subunits mixed in a 1:1:1 ratio, followed by stepwise dialysis and size exclusion column chroma-
tography (32). Recombinant control and mutant bovine whole TnT and TnT-(1–156) were expressed using pET3d in DE3 cells and purified to homogeneity as described (25). TnT(1–156) or TnT(1–140) mutations were introduced into bovine cardiac TnT cDNA (18) by the same PCR-based approach used previously to create the R92Q and F110I mutations (18). The same method was also used to introduce the T104A mutation into TnT R92Q and F110I. To create the various TnT-(1–156) constructs, the correspond-
ing DNA fragment was amplified by PCR from the various full-
length plasmids and inserted into the NcoI/BamHI sites of pET3d. All coding sequences in expression plasmids were confirmed by automated DNA sequencing at the University of Iowa DNA Facility.

Protein Folding and Circular Dichroism—The ellipticity of TnT-(1–156) was monitored as a function of temperature using an Aviv DS65 circular dichroism spectrometer, recording from −5 to 80 °C. Two sets of protein preparations gave similar results. Conditions are as follows: 0.1 mg/ml TnT-(1–156), 300 mM KCl, 15 mM NaH2PO4 (pH 6.5). Similar results were also obtained for one of the sets of protein preparations, examined in the presence of 150 mM KCl. Data were fit to a two-state, temperature (T)-dependent transition, with RT Inα − AG = AH(1 − T/Tm). The ellipticity of the unfolded state was taken as constant, but satisfactory fits required that the ellipticity of the folded state be assumed to vary linearly with temperature. This procedure should be considered as semi-empirical, because the unfolding process is not two-
state as assumed in the modeling. Differential scanning calorimetry of wt TnT-(1–156), F110I TnT-(1–156), and of F110I/T104A TnT-(1–156) demonstrated that the heat of unfolding was in fact gradual and could not be described accurately with models including as many as four states (three transitions) (data not shown).

Binding of Troponin or Tropomyosin to the Thin Filament—Binding of radiolabeled tropomyosin to actin was measured by cosedimentation with a TLA100 rotor in a Beckman TL100 centrifuge (34). The tropo-
myosin was stoichiometrically labeled under denaturing conditions on Cy3-labeled cysteic acid (34). Binding was calculated from the decrease in supernatant radioactivity following sedimentation. As de-
scribed in the figures, conditions were chosen in which the tropomyosin bound negligibly to actin unless troponin or a troponin fragment was added.

Tropomyosin binds very tightly to the thin filament, making its affinity problematic to measure by sedimentation. Therefore, the relative affinity of troponin for actin-tropomyosin was measured by its ability to displace a control, [3H]labeled troponin from the thin filament. Bound and free [3H]troponin were separated by ultracentrifugation as de-
scribed previously (25). By fitting the data to Equation 1 from Ref. 25, the affinity of troponin for actin-tropomyosin was measured, relative to the affinity of the [3H]labeled troponin. Conditions are as follows: 25 °C, 7 μm actin, 3 μm tropomyosin, 1 μm [3H]-labeled troponin, 10 mM Tris (pH 7.5), 300 mM KCl, 3 mM MgCl2, 0.2 mM dithiothreitol, 0.3 mg/ml bovine serum albumin, and 0.5 mM EGTA.

RESULTS

Sequence Alignment of Multiple Troponin Ts—The strongest interaction between troponin and tropomyosin involves the TnT N terminus and a tropomyosin C-terminal region found specifically in tropomyosins that bind to troponin (reviewed in Ref. 6). Moreover, a newly reported crystallographic study of tropomyosin shows that conserved C-terminal residues of mammalian striated muscle tropomyosin effect a distinctive structure, a troponin T recognition site. This structural and sequence conservation in tropomyosin suggests a similar con-
servation in the protein target, i.e. in the troponin tail. To help identify this region, a multiple sequence alignment of 15 TnTs was performed (Fig. 1). In the figure, chordate (mammals, birds, and tunicate) TnT conservation is indicated in yellow, and blue indicates conservation among three invertebrate phyla (nematode, mollusks, and insect). Regions conserved among both vertebrate and invertebrate TnTs are indicated in green and are readily apparent.

Fig. 1 shows that no TnT region is more conserved than residues 112–136 of the troponin tail domain. These residues are 70% homologous across the analyzed sequences. Signifi-
cantly, engineered cardiac troponin constructs bind tightly to the thin filament only if they contain this entire region (see “Discussion”), regardless whether the construct is a troponin tail fragment containing only the TnT N terminus or instead is a truncated ternary troponin complex with an N-terminal de-
letion (25, 34, 35). The present sequence alignment and these earlier deletional experiments together suggest that residues 112–136 comprise a tropomyosin-binding element. Also, note that this region is flanked by many hypertrophic cardiomyopathy alleles, including (# in Fig. 1) sites Arg-92, Ala-104, and Phe-110.

Within this region there is particular conservation of amino acids 226–271, which form a coiled coil with subunit TnI and also interact with TnC (36).

Effects of Cardiomyopathy Causing TnT Mutations on Folding of the Troponin Tail—In an earlier study, we reported that TnT R92Q and F110I mutations interfered with the ability of bovine cardiac troponin subunits to reconstitute into a soluble troponin complex (18). To investigate this folding abnormality quantitatively, we isolated troponin tail fragments TnT-(1–156), finding them to be soluble regardless of mutation. No precipitation was observed in low ionic strength buffer at protein concentrations studied (≤1.5 mg/ml). Troponin tail fragment TnT-(1–156) was predominantly α-helical as assessed by circular dichroism (data not shown), as expected (37, 38). When ellipticity was examined as a function of temperature, the mutations caused large but disparate effects (Fig. 2A and Table 1). TnT-(1–156) altered the ellipticity in the manner expected for a destabilizing mutation, decreasing the temperature of the unfolding curve midpoint (Tm) by 4.2 °C and also decreasing the absolute magnitude of the maximal ellipticity observed at the lowest temperature examined (−5 °C). Cardiomyopathy causing mutation R92Q similarly decreased both the ellipticity
FIG. 1. Troponin T sequence alignment. ClustalW was used to align the amino acid sequences of human cardiac TnT (top line), five other vertebrate TnTs (mammalian, avian, and fish sequences from several muscle types), one tunicate TnT (Halocynthia roretzi, a chordate grouped here with vertebrate sequences), and eight TnTs from species in other invertebrate phyla: three insect, two molluscan, and three nematode TnTs. Yellow or blue identify residues homologous in a majority of either chordate or non-chordate sequences, respectively. Green indicates residues that are conserved in a majority of all examined TnTs, and in addition are found in two or more sequences from both chordate and non-chordate groups. Red rectangles indicate sites of hypertrophic cardiomyopathy mutations, with rectangle height indicating one, two, or three mutant alleles. Purple squares are sites of dilated cardiomyopathy mutations. According to a preliminarily reported troponin atomic structure, amino acids 227–271 (black line) form one strand of a coiled-coil with TnI and also bind to other portions of TnI and to TnC (36). Note that from Asn-112 through Glu-136 of...
Folding and Function of the Troponin Tail Domain

There is 70% homology across phyla. From top to bottom the TnTs and GenBank™ accession codes are as follows: Homo sapiens cardiac (AAD92231), Meleagris gallopavo cardiac (AY005139), Homo sapiens slow skeletal muscle (NP003074), Oryctolagus cuniculus heart (AAK92231), Halocynthia roretzi (BAA09463), Schistosoma mansoni (BAD28680), and Periplaneta americana muscle (AAD38600).

**FIG. 2.** Temperature-dependent folding of troponin tail fragment TnT-(1–156) is altered by cardiomyopathy-inducing mutations. The ellipticity at 222 nm of control and mutant bovine cardiac troponin tail fragments are shown as a function of temperature. A, effect of cardiomyopathic mutations on ellipticity of the TnT peptide. B, effect of the mutations on TnT peptides containing human TnT Ala-104 instead of bovine TnT Thr-104. WT, wild type.

**FIG. 3.** TnT mutations decrease the ability of the troponin tail to induce tropomyosin-actin binding. Tropomyosin (total 0.5 μM) binding to actin was negligible under the examined conditions (which notably included 300 mM KCl), unless either tropinin or the troponin tail domain was added. The cardiomyopathy mutations greatly diminished this effect of the tropinin tail. Lines are arbitrary curves. Conditions are as follows: 25 °C, 10 μM F-actin, 0.5 μM tropomyosin, 3 mM MgCl₂, 300 mM KCl, 10 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 0.2 mM dithiothreitol, 0.3 mg/ml bovine serum albumin, and between 0 and 1.6 μM troponin or TnT-(1–156). WT, wild type.

**Table 1.** Thermal denaturation midpoint of bovine cardiac troponin tail peptide TnT-(1–156) containing cardiomyopathy mutations and/or bovine versus human difference T104A

| Cardiomyopathy mutation | Bovine Thr-104 (control and single mutants) °C | Human Ala-104 (control and double mutants) °C |
|-------------------------|-----------------------------------------------|-----------------------------------------------|
| None (control)          | 30.5 ± 0.5                                    | 33.2 ± 0.2                                    |
| R92Q                    | 27.7 ± 0.5                                    | 33.1 ± 0.2                                    |
| F110I                   | 32.3 ± 0.3                                    | 38.8 ± 0.1                                    |
| T104V                   | 25.3 ± 0.5                                    | NA                                            |

Circular dichroism results in Fig. 2 were analyzed to determine values for \( T_m \), the temperature corresponding to 50% thermal denaturation. Cardiomyopathic mutation T104V decreased thermal stability; F110I increased thermal stability, and R92Q had little effect. Thermal stability was increased when Human Ala-104 was inserted into the bovine sequence, in either the absence (“none”) or the presence of the R92Q and F110I mutations. NA indicates not applicable.
similarly promoted binding of tropomyosin (0.5 μM) or R92Q/T104A troponin (Δ), T104A troponin promoted tropomyosin binding to actin but to a lesser extent. Similar effects were observed in the presence of MgCl2/EGTA (A) or CaCl2 (B), except for the expected, slight weakening of binding in the latter condition (63, 64). Lines are arbitrary curves. Conditions are the same as in Fig. 3.

Effects of the Mutations on Binding of Troponin to Actin—

Effects of the Mutations on Binding of Troponin-Tropomyosin to Actin—The modest effect of the F110I/T104A mutation on troponin-tropomyosin binding to actin (Fig. 5) contrasts sharply with the large effect (see Fig. 3) of this mutation on the properties of TnT-(1–156), the troponin tail fragment. This suggests the possibility that the large effect of this mutation in Fig. 2, essentially a loss of all ability to promote tropomyosin-actin binding and producing less of a maximal effect. Each of these observations was unchanged by the addition of calcium (bottom versus top panel).

To delineate these effects better, and to determine whether the altered properties of F110I/T104A troponin primarily reflected changes in interactions with actin or instead with tropomyosin, saturating amounts of troponin were added to variable concentrations of tropomyosin. Fig. 5 presents the binding of tropomyosin-F110I/T104A troponin for actin was 56% the affinity found for control tropomyosin-tropomyosin complexes were routinely isolated at concentrations of 5 μM in low ionic strength buffer.

Fig. 4 shows the same experiment as in Fig. 3, except binding of tropomyosin to actin is promoted by whole troponin with or without cardiomyopathy mutations, instead of by troponin tail fragments. T104A troponin behaved similarly to control tropomyosin, as did R92Q/T104A troponin. Both the maximal tropomyosin-actin binding and the troponin concentration dependence of the effect were indistinguishable from data for control troponin. In contrast, F110I/T104A troponin was less effective, requiring higher concentrations to promote tropomyosin-actin binding and producing less of a maximal effect. Each of these observations was unchanged by the addition of calcium (bottom versus top panel).

Effects of the Mutations on Binding of Troponin-Tropomyosin—The modest effect of the F110I/T104A mutation on troponin-tropomyosin binding to actin (Fig. 5) contrasts sharply with the large effect (see Fig. 3) of this mutation on the properties of TnT-(1–156), the troponin tail peptide. This suggested the possibility that the large effect of this mutation in Fig. 2, essentially a loss of all ability to promote tropomyosin-actin binding, reflected only the properties of the troponin tail and not those of whole troponin. To evaluate this possibility,
Folding and Function of the Troponin Tail Domain

Fig. 6. Binding of troponin to actin-tropomyosin, measured by competition. Saturating amounts of radiolabeled control troponin were added to actin-tropomyosin, and the relative affinity of competing, unlabeled troponin was measured by displacement of the radiolabel from the thin filament. T104A (squares) and R92Q/T104A (diamonds) TnT mutations did not significantly alter the affinity of troponin for the thin filament ($K_R = 0.92 \pm 0.09$ and $1.06 \pm 0.12$, respectively, where $K_R = 1$ indicates no effect). In contrast, F110I/T104A troponin (triangles) had a considerably decreased affinity, $K_R = 0.113 \pm 0.015$. Lines are best-fit curves.

the effects of the TnT mutation on whole troponin binding to actin-tropomyosin were measured.

Troponin binds very tightly to actin-tropomyosin, complicating measurement of binding affinity. Therefore, the effect of the TnT mutations on this process was measured by competition (25). T104A and R92Q/T104A troponins displaced control, radiolabeled troponin from the thin filament (Fig. 6), with patterns consistent with normal (i.e. $K_R = 1$) binding of these complexes to the thin filament. ($K_R$ equals the fold change in affinity, so $K_R = 1$ indicates no effect of the mutation.) In contrast, F110I/T104A troponin was much less effective, implying an affinity decrease of an order of magnitude. This indicates that the mutation had a large effect on the binding of whole troponin to the thin filament and not merely an effect on the isolated troponin tail. Furthermore, because the F110I/T104A mutation caused only a modest weakening of troponin-tropomyosin binding to actin (Fig. 5), the most significant effect of the mutation, and the basis for the $K_R << 1$ in Fig. 6, was impaired interactions between troponin and tropomyosin rather than changes in the actin affinity of the regulatory complex.

DISCUSSION

The results in this article substantially agree with the recent findings of Palm et al. (39). R92Q, T104V (or A104V), and F110I mutations in TnT have similar effects on protein folding when examined either in human cardiac TnT peptides TnT-(70–170) (39) or instead in our bovine construct TnT-(1–156). In particular, mutant residue Val-104 was destabilizing; Ile-110 was stabilizing, and Gln-92 had a small effect in both studies. Nevertheless, unique features of each report result in some differences that are worth noting, and overall assessment of the mutations requires considering both studies. Our data on the T104A substitution indicate that this residue has an unexpected importance in troponin folding, in both whole troponin and in the troponin tail, in both control and cardiomyopathic contexts. Further emphasizing the importance of this residue, cardiomyopathy mutation to Val at this same position weakens stability, shown in both reports by effects on $T_m$. In our study but not in Palm et al. (39) Val-104 disrupts the adoption of normal secondary structure at 0 °C (Fig. 2A), perhaps suggesting that residue 104 affects folding of residues 1–69 that are included in our constructs but not in TnT-(70–170). However, a definitive conclusion on this point would require more data on the structure of this region of troponin, and the more notable point is that the two reports agree qualitatively on the effects of the mutations on folding. As discussed below, the functional implications of Fig. 2 do not require that the troponin tail be unfolded in vivo, which cannot be determined from the present experiments.

In both studies the F110I mutation promoted protein folding but impaired interactions of the troponin tail with its targets, strongly suggesting that flexibility is important for this process. Interestingly, Palm et al. (39) found similar properties for mutations R92W, R92L, and R94L, not examined here. In the present report, the effects of the mutations on whole troponin were also examined, so the mutations could be understood in the context of the entire protein. One of several advantages is that the effects of whole troponin on tropomyosin binding to actin are very much larger (20–35-fold (25, 34)) than the 3-fold effect of TnT-(70–170) (39). Furthermore, the current results allow quantitative comparison of effects on two target interactions, binding of troponin to actin-tropomyosin versus tropinin-tropomyosin binding to the thin filament. We found that only the former process was greatly affected by the F110I mutation. Once troponin binds to tropomyosin, interaction with actin is little affected. This result indicates it is troponin tail-tropomyosin interactions that are most impaired for this mutant, relatively rigid troponin. Some impairment of these interactions has been demonstrated independently in the context of TnT-(70–170); the F110I mutation alters salt-dependent dissociation of the fragment from a tropomyosin affinity column and diminishes the effect of the peptide on the ellipticity of a tropomyosin N terminus plus tropomyosin C terminus solution (39). New F110I observations are that this effect contrasts with a much smaller effect on thin filament assembly once the troponin is bound to tropomyosin, occurs with whole troponin, and overall weakens troponin anchoring by an order of magnitude.

One possible interpretation of these findings is that the examined TnT mutations occur in a region (residues 92–110) that directly interacts with tropomyosin, as has been suggested (39). It is difficult to confirm or exclude this hypothesis without a high resolution structure of the troponin tail-tropomyosin complex. However, in our view it is more likely that highly conserved TnT residues 112–136 are the element that binds to the similarly conserved C-terminal region of tropomyosin. By comparison, residues 92–110 are much less conserved, 33% in contrast to 70% (Fig. 1). In further support of the current proposal, tropinin-thin filament binding is only modestly impaired by deleting 98 N-terminal residues of TnT from whole troponin but is profoundly diminished by deleting 122 such residues (25). Interestingly, peptide TnT-(1–135) by itself has no detectable interaction with tropomyosin and actin, but TnT-(1–156) interacts very strongly (25, 26). These results suggest that the entire 112–136 region is required for the troponin anchoring function, although this could reflect poor folding of the TnT-(1–135) fragment.

The strikingly disparate effects of the three mutations examined in this report do not immediately suggest a common molecular theme for the pathophysiology of hypertrophic cardiomyopathy due to mutations in the troponin tail domain. Nevertheless, there are at least two reasons to believe that many of these mutations act by similar mechanisms. First, a high percentage of all identified TnT mutations are clustered N-terminal to the putative tropomyosin-binding element, 112–136. Second, although the mechanism is not understood, troponin tail mutations seem to have one similar effect; they raise the calcium sensitivity of thin filament activation (16, 18, 20,
The validity of this generalization is supported by the finding that increased calcium sensitivity is a typical consequence of hypertrophic cardiomyopathy-inducing mutations in two other thin filament proteins, tropomyosin and TnI (40–44). Interestingly, rat cardiac TnI was employed in a study with a rare contrary result, decreased calcium sensitivity (19) caused by the R92Q mutation. Rat cardiac TnT contains threonine rather than alanine at position 104. As the current report shows, this species difference can be critical. One contrary result reported skeletal muscles (15).

The present data concerning a few mutations permit one to infer more general pathophysiological mechanisms, albeit tentatively. The T104V and F110I mutations greatly alter protein folding stability, and so must also affect the overall properties of the troponin tail. Therefore, the cardiomyopathy mutations should be thought of as broadly altering the troponin tail domain, rather than as locally changing residues directly involved in binding to tropomyosin or actin. The troponin tail primarily interacts with tropomyosin, and as far as is known, its interactions with actin are indirect (5). We suggest that the common theme of cardiomyopathy mutations in the troponin tail is a change in the effects of troponin on tropomyosin. In some cases but not all (e.g., not R92Q), this involves weaker binding of troponin to tropomyosin and either sub-normal or super-normal folding stability. Furthermore, we suggest that a result of the mutations is that the troponin-tropomyosin strand moves more flexibly than normal, allowing it to shift its azimuthal position on the actin filament at lower Ca$^{2+}$ concentrations. In support of this idea, cardiomyopathy-inducing mutations in tropomyosin appear to act by this mechanism. D175N and E160G mutations increase thin filament Ca$^{2+}$ sensitivity and decrease tropomyosin folding stability (40, 43, 45). Tropomyosin mutations K70T, V95A, and A63V have the same combination of effects (46). Thus an emerging pattern for tropomyosin mutations is an increased Ca$^{2+}$ sensitivity and increased flexibility. Similarly, we suggest that the troponin tail mutations interact abnormally with tropomyosin, resulting in altered flexibility of the troponin-tropomyosin strand. An increasing body of experimental (26, 47–51) and theoretical (52–54) work indicates that the cooperative activation of the thin filament by myosin critically depends on the flexibility with which tropomyosin shifts its azimuthal position on actin (55–57). Although less well understood, this flexibility must also affect activation by combined effects of both myosin and Ca$^{2+}$.

Protein-protein interactions within allosteric complexes stabilize the assembly of those complexes, but equally significantly, they are essential to mechanism; activating and inactivating shifts in quaternary structure represent changes in inter-subunit interactions. In this context, it is not surprising that the troponin tail region, first identified as an anchoring or assembly domain (reviewed in Ref. 5), is now known to be an important modulator of thin filament function. Thin filament properties and actin-myosin interactions can be affected by troponin tail changes that are either isomorph shifts (32, 58, 59), missense mutations (14–18, 21–23), or truncations (60–62). In a recent report (26), we showed that bovine cardiac troponin tail peptide TnT(1–156) suppresses thin filament-myosin S1 ATPase activity, weakens myosin S1-thin filament binding, and affects tropomyosin-binding position on actin. The skeletal troponin tail exerts similar effects (51). It is intriguing therefore that the mutations, in an important sense, weaken the ability of troponin-tropomyosin to suppress contraction; dis-inhibition of contraction by calcium occurs at lower calcium concentrations. We suggest this is due to an increased flexibility of the troponin-tropomyosin strand. However, the details of both normal and abnormal troponin-tropomyosin structure remain to be elucidated at a level of resolution allowing precise functional understanding.

In summary, critical interactions between troponyosin and troponin are proposed to depend on highly conserved TnT residues 123–136. Three cardiomyopathy mutations in this region had contrasting effects, promoting or weakening folding and thermal stability. Cardiomyopathy site Ala-104 of normal human TnT particularly strengthened protein folding. Mutation F110I strongly stabilized the troponin tail but weakened troponin binding to actin-tropomyosin 9-fold, indicating that loss of flexibility was especially disadvantageous to the interactions of troponin with tropomyosin. Finally, the data suggest that cardiomyopathy mutations in the troponin tail alter muscle function indirectly, by perturbing interactions between tropo-

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Folding and Function of the Troponin Tail Domain

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