Altered Regulatory Function of Two Familial Hypertrophic Cardiomyopathy Troponin T Mutants†

Poushali Mukherjea,‡,|| Lily Tong,‡,⊥ Jonathan G. Seidman,§ Christine E. Seidman,§ and Sarah E. Hitchcock-DeGregori*,‡

Department of Neuroscience and Cell Biology, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, New Jersey 08854, and Department of Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115

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ABSTRACT: Mutations in the gene encoding human cardiac troponin T can cause familial hypertrophic cardiomyopathy, a disease that is characterized by ventricular hypertrophy and sudden, premature death. Troponin T is the tropomyosin-binding subunit of troponin required for thin filament regulation of contraction. One mutation, a change in the intron 15 splice donor site, results in two truncated forms of troponin T [Thierfelder et al. (1994) *Cell 77*, 701−712]. In one form, the mRNA skips exon 16 that encodes the C-terminal 14 amino acids; in the other, seven novel residues replace the exon 15- and 16-encoded C-terminal 28 amino acids. The two troponin T cDNAs were expressed in *Escherichia coli* for functional analysis. Both C-terminal deletion mutants formed a complex with cardiac troponin C and troponin I that exhibited the same concentration dependence as wild-type for regulation of the actomyosin MgATPase. However, both mutants showed severely reduced activation of the regulated actomyosin in the presence of Ca^{2+}, though the inhibition in the absence of Ca^{2+} and the Ca^{2+}-dependence of activation were not altered. The C-terminal deletions reduce the effectiveness of Ca^{2+}-troponin to switch the thin filament from the "off" to the "on" state. Both mutant troponin Ts have reduced affinity for troponin I; the shorter mutant is at least 6-fold weaker than wild-type. The low level of activation of the ATPase would be consistent with reduced contractile performance, and the results suggest reduced troponin I affinity may be the molecular basis for the disease.

Familial cardiac hypertrophy (FHC)[1] is an autosomal dominant trait characterized by thickening of the ventricular walls with associated myocyte disarray and hypertrophy and is associated with sudden, premature death (reviewed in ref *1* and *2*). Missense mutations in genes encoding several cardiac sarcomeric proteins can cause this disease, including tropomyosin, troponin-T (TnT), and troponin I (TnI) in the actin-containing thin filament and β-cardiac myosin heavy chain and ventricular myosin light chains in the thick filament. The mechanisms by which these mutations cause hypertrophic cardiomyopathy, myocyte disarray, and sudden death are poorly understood. In the study reported here, we have investigated the function of two troponin-T mutants that are truncated at the C-terminal end (*3*).

In striated muscles, TnT forms a complex with TnI and troponin C (TnC) that, together with tropomyosin and myosin, is involved in the Ca^{2+}-dependent thin filament-linked regulation of contraction (reviewed in refs *4*−*7*). Striated muscle TnT (the form that has been extensively studied) binds to both TnI and TnC in the Tn complex as well as to tropomyosin, and possibly actin, on the thin filament. In a reconstituted thin filament, TnT is required for inhibition of the actomyosin ATPase by the Tn complex in the absence of Ca^{2+} as well as for the binding of the Tn complex to actin−tropomyosin and activation of the actomyosin ATPase in the presence of Ca^{2+} (*8*, *9*). The elongated shape of the Tn complex (*10*) and its position spanning the C-terminal third or more of tropomyosin (*11*, *12*) is because of TnT. The N-terminal fragment of TnT (residues 1−158 of rabbit skeletal TnT) binds near the C-terminus of tropomyosin, including the head-to-tail overlap region (*13*, *14*). The C-terminal fragment of TnT (residues 159−259) binds to TnI and TnC and to tropomyosin closer to the middle of the molecule in a Ca^{2+}-sensitive manner (*14*−*16*). Recent studies of recombinant TnT fragments have refined earlier peptide studies, showing that deletion of the C-terminal 47−72 residues impairs TnI binding (*17*) and reduces inhibition of the actin-myosin ATPase by a reconstituted Tn in the absence of Ca^{2+} (*18*).

Eleven mutations at nine different sites in the cardiac TnT gene (TNNT2) cause hypertrophic cardiomyopathy with

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|| Present address: Novo Nordisk Pharmaceuticals, Inc., 100 Overlook Center, Princeton, N.J. 18450.

⊥ Present address: Memorial Sloane-Kettering Cancer Center, New York, NY.

* To whom correspondence should be sent. Telephone: (732) 235-5236. Fax: (732) 235-4029. E-mail: hitchcoc@umdnj.edu.

[1] FHC, familial hypertrophic cardiomyopathy; cTnT, cardiac troponin T; cTnC, cardiac troponin C; cTnI, cardiac TnI; Tm, tropomyosin; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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varying degrees of severity (reviewed in *1*). Although most of these are missense mutations in the tropomyosin-binding domain of TnT, one is a G→A mutation in the splice donor sequence of intron 15 before the terminal coding exon 16. Two transcripts, a shorter transcript (designated as cTnTInt15*S*) and a longer transcript (designated as cTnTInt15*L*), are produced by the splice donor mutated gene that differ at their 3′ ends (*3*). The shorter transcript encodes a truncated polypeptide in which seven novel residues replace the C-terminal 28 residues. The longer transcript encodes a premature termination signal after exon 15 and results in the loss of the C-terminal 14 amino acids. Since the quantity of the aberrant transcripts was comparable to that of the normal transcript in affected (heterozygous) individuals, Thierfelder et al. (*3*) speculated that these mutant gene products, if translated, would severely affect thin filament assembly and/or function. When a cDNA encoding the C-terminal deletion mutant (corresponding to cTnTInt15*S*) was transfected into quail myocytes, the mutant TnT was expressed and incorporated into sarcomeres, but there was diminished Ca^{2+}-dependent activation of force development (*19*). Here, we report that both C-terminal deletion mutants showed reduced activation of the actomyosin ATPase in the presence of Ca^{2+}, though the inhibition in the absence of Ca^{2+} and the Ca^{2+}-dependence of activation were not altered. Both proteins, especially cTnTInt15*S*, had reduced affinity for cardiac TnI. Portions of this work were previously reported (*20*).

MATERIALS AND METHODS

Clones and Vectors. The previously described wild-type and mutant cTnT cDNAs clones from normal heart tissue and from an affected member of Family AU bearing the $Int15^{G\rightarrow A}$ mutation (*19*) were used in this study. The cDNAs were modified to introduce a Nde I site for cloning at the initiating ATG. The wild-type cTnT was cloned into the pSBET vector (*21*) at the NdeI and BglII sites. The sBET vector contains the *E. coli* Arg U gene for translating rare Arg codons. The two mutant cDNAs were subcloned into the pET11a expression vector (*22*) at the NdeI and BamHI sites. The DNA sequences of the cDNAs in the expression vectors were confirmed by automated DNA sequencing.

For expression, the cTnT-containing vectors were transfected into BL21(DE3) cells (*22*). The vectors containing the mutants were cotransfected with pSBET and double transformants were selected on ampicillin + kanamycin plates and confirmed by restriction analysis of plasmid DNA. Troponin T expression was poor with the conventional pET vectors alone.

Expression and Purification of Proteins. The cTnTs were purified from *E. coli*, using modifications of published methods (*23*). Large-scale bacterial cultures were induced with 0.4 mM IPTG at $OD_{550} = 0.6-0.7$ and the cells sonicated after lysis. The supernatant after centrifugation was subjected to ammonium sulfate fractionation. The TnT-containing pellet from the 30−40% ammonium sulfate fractionation was dialyzed against 6 M urea, 50 mM Tris, pH 7.0, 1 mM EDTA, and 1 mM DTT for further purification on a Mono-Q column at 4 °C, eluted with a linear 0−0.4 M NaCl gradient. Fractions containing cardiac TnT (according to SDS-PAGE analysis) were pooled, dialyzed against 6M urea, 50 mM Pipes, pH 6.0, 1 mM EDTA, and 0.5 mM DTT and loaded onto a cation-exchange Mono-S column, eluted with a linear 0−1 M NaCl gradient. The deletion mutants behaved differently on the cation-exchange column and in some cases 50 mM MES buffer at pH 5.8 was used.

An expression plasmid for chicken cardiac TnC, pET11cTnC, was obtained from Dr. J. Putkey, Baylor College of Medicine (*24*). The protein purification was based on published methods for TnC (*25*). After the initial phenyl-Sepharose column chromatography, the protein containing fractions were pooled and further purified on a Mono-Q column on the FPLC system in 50 mM Tris, pH 7.5, 1 mM EDTA, and 1 mM DTT.

A rat cardiac TnI cDNA (in pET3d) was obtained from Dr. John Solaro, University of Illinois at Chicago, and TnI was purified according to published methods (*26*), with the following modification. The presence of a high amount of nucleic acid in the bacterial lysate interfered with the performance of the cation-exchange column. Therefore, after centrifugation the clear lysate was initially applied to a Q-Sepharose anion exchange column and the cTnI-containing flowthrough was collected for further purification.

Reconstitution of the Ternary Troponin Complex. The wild-type and mutant cTnTs were combined with cTnI and cTnC in the presence of 4.6 M urea in a 1:1:1.3 molar ratio at 4 °C. The complex was then dialyzed extensively at 4 °C against 10 mM imidazole, pH 7.0, 0.1 mM $CaCl_2$, and 1 mM DTT, gradually changing the NaCl concentration from 1 M to 50 mM NaCl. The protein was centrifuged at 14 000 rpm in a microfuge to remove any uncomplexed insoluble TnT and TnI. The resulting complex was analyzed on a 12% SDS gel and then scanned on a Molecular Dynamics model 300A computing densitometer to estimate the stoichiometry of the Tn components. The relative amounts of the component proteins were comparable in the Tn complexes reconstituted with the wild-type and mutant TnTs even though the TnI affinity of the FHC mutant TnTs was lower than that of wild-type. The concentration of the reconstituted Tn complex was estimated for use in the ATPase assays ($\epsilon_{278} = 0.48$, $m_w = 78\,000$; *27*).

Actomyosin ATPase Assay. The actomyosin ATPase assays were carried out in a total volume of 75 μL in a thermo-equilibrated Molecular Devices Thermomax microtiter plate reader (Molecular Devices, *28*). Increasing concentrations of the reconstituted Tn complex were added to a reconstituted thin filament system containing actin (2.4 μM) and tropomyosin (1 μM). The final concentration of myosin was 0.7 μM, while the concentration of the Tn complex varied from 0 to 1.5 μM. The assays were carried out in 10 mM imidazole, pH 7.0, 40 mM NaCl, 0.5 mM $MgCl_2$ and either 0.1 mM $CaCl_2$ or 0.2 mM EGTA at 28 °C. The reaction was initiated by the addition of MgATP to a final concentration of 5 mM and terminated after 15 min by the addition of 13.4% SDS and 0.12 M EDTA. The inorganic phosphate was determined colorimetrically according to White (*29*) and was read with a 650 nm filter. The curves were fit to the data using the Hill eq below where v = specific activity, n = maximal specific activity ($+Ca^{2+}$) or minimal specific activity ($-Ca^{2+}$), [X] = [Tn], K_{app} = apparent K_a of Tn for reconstituted actomyosin, H = Hill coefficient, and C = specific activity at [Tn] = 0 (fitted).

The Ca^{2+}-dependence of the actomyosin ATPase was carried out under the same conditions, except at a saturating

concentration of reconstituted Tn (1 μM) and in the presence of 0.45 mM CaEGTA. The ratio of Ca^{2+}/EGTA, to obtain the desired free Ca^{2+}, was determined using titration curves based on published binding constants (*30*) and calculated according to Perrin and Sayce (*31*). The curves were fit using the Hill eq below where υ = specific activity, n = maximal activity, [X] = [Ca^{2+}], H = Hill coefficient, K_{app} = apparent K_a of Ca^{2+} for activation, and C = constant for activity at [Ca^{2+}] = 0.

Binary Interactions of cTnT with cTnI. Complex formation between wild-type and mutant TnTs with cTnI was studied using HPLC (*17*) with two Waters Protein Pak 300SW gel filtration columns (0.5 × 30 cm) connected in tandem. Cardiac TnT and TnI were combined at molar ratios ranging from 0.4:1 to 2.8:1 in 10 mM Tris (pH 7.0) and 0.5 M NaCl and chromatographed at a flow rate of 1 mL/min. The TnI concentration of the samples loaded was kept constant at 15 μM, while total TnT added ranged from 5 to 40 μM. The total volume of sample applied was kept constant for each series of measurements (100−200 μL, depending on the experiment) and absorbance at 225 nm was monitored. Under the conditions of the experiment, cTnT eluted between 18.3 and 19.1 min and cTnI between 12.5 and 13 min in different experiments. Wild-type and mutant TnT's coeluted with the TnIT complex. Therefore, the fraction of cTnT bound was quantitated by measuring the loss of the free cTnI. Peak heights for total cTnI and free cTnI in a complex were measured manually for each chromatogram after making necessary baseline adjustments. Even at saturating amounts of TnT, the TnI peak was never completely lost and because of the presence of a residual shoulder the calculated maximum TnI bound was only 70%. The concentration of TnT bound was calculated based on the assumption that it forms a 1:1 complex with TnI and used to calculate the free TnT. The curves were fit to the data using the Hill eq below where υ = fraction TnI bound, n = maximal fraction TnI bound, [X] = [free TnT], H = Hill coefficient, K_{app} = apparent K_a of TnT for TnI, and C = 0.

Actin Binding Assays. The reconstituted cardiac Tn complex (1 μM) was combined with F-actin (2.5 μM) and tropomyosin (1 μM) in actin binding buffer (100 mM NaCl, 10 mM imidazole, pH 7.0, 2 mM $MgCl_2$, 0.5 mM DTT, and 0.2 mM $CaCl_2$ or 2 mM EGTA). The Tn complex was combined with tropomyosin and prespun to remove insoluble protein. After addition of actin, the thin filament complex was centrifuged for 25 min at 60 000 rpm in a Beckman model TL-100 ultracentrifuge in a TLA-100 rotor. The pellets and supernatants were analyzed on SDS-PAGE, visualized using Coomassie Blue.

General Methods. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (*32*). Contractile proteins, other than the recombinant cardiac Tns were prepared from chicken pectoral muscle using methods that have been published. Skeletal actin was purified from an acetone powder (*33*). Native skeletal chicken Tn was prepared according to Potter (*34*) with the modification that protease inhibitors were added during extraction. α,α-Tropomyosin was purified from the isoelectric precipitation of Tn preparation (*35*). Myosin was prepared as described by Margossian and Lowey (*36*). Protein concentrations were determined by measuring the tyrosine difference absorption spectrum at 295 nm (*37*, *38*). The Hill equation was used to fit curves to the data with SigmaPlot (Jandel Scientific):

$$\upsilon = (n[X]^H \times K_{app}H/1 = [X]^H \times K_{app}H) + C$$

C-Terminal Sequences of cTnT and Two FHC Mutants

Wildtype

Exon 15 / Exon 16

INVLRNRINDNQKV / SKTRGKAKVTGRWK**

d14 Mutant

Exon 15

INVLRNRINDNQKV**

d21 Mutant

Exon 15

LQDPREG**

FIGURE 1: The C-terminal amino acid sequences of wild-type and the two FHC troponin T mutants. The amino acid sequence encoded by exon 15 and exon 16 is shown for the wild-type human cTnT. The cTnTInt15*L* mutant has lost the 14 C-terminal residues encoded by exon 16, while in the cTnTInt15*S* mutant, 28 C-terminal amino acids encoded by exons 15 and 16 have been replaced by 7 novel residues. (ref *3*).

RESULTS

Regulation of Actomyosin ATPase. The main goal of the present study was to gain insight into the mechanism by which the C-terminal deleted forms of TnT cause hypertrophic cardiomyopathy. To address this question, we have assessed the in vitro functional differences between the wild-type and mutant TnTs. The ability of the mutants to activate the regulated thin filament in a Ca^{2+}-dependent fashion was assayed using an actomyosin MgATPase assay. Cardiac Tn was reconstituted with wild-type cTnT, or the cTnTInt15*L* or cTnTInt15*S* cTnT mutants, and cardiac TnC and TnI. Figure 2 (Table 1) shows that the Tn complex reconstituted with wild-type TnT activated the actomyosin ATPase to a rate that was 2.4 ± 0.05-fold over unregulated actin−tropomyosin. The complexes with cTnTInt15*L* and cTnTInt15*S* activated only 1.7± 0.06-fold and 1.5 ± 0.03-fold, respectively, in the presence of Ca^{2+}. Inhibition in the presence of EGTA was comparable for the wild-type and mutant Tn complexes. The amount of complex needed for half-maximal activation, a measure of the affinity of the Tn complex for actin−tropomyosin, was unaltered by the mutations (Table 1). These results indicate that the loss of the conserved C-terminal amino acid residues of cTnT severely reduces Ca^{2+}-dependent activation of the thin filament, the switch of the thin filament from the "off" to the "on" state.

One possible explanation for the failure to activate in the presence of Ca^{2+} would be a reduced amount of Tn or TnT in the thin filament. Analysis of actin cosedimented with Tm−Tn (with wild-type or cTnTInt15*S*) with and without

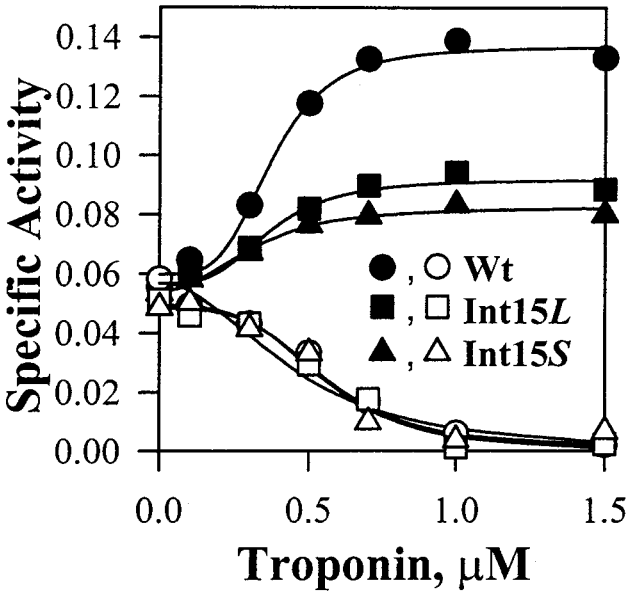

FIGURE 2: Regulation of the actomyosin ATPase by wild-type cTnT and the cTnTInt15*L* and cTnTInt15*S* mutants. Increasing concentrations of reconstituted cardiac Tn containing wild-type or mutant TnT were added to actin−tropomyosin and myosin (see Materials and Methods). Reactions were carried out either in the presence of 0.1 mM $CaCl_2$ (filled symbols) or in 0.2 mM EGTA. Specific activity is expressed as μmol P_i/mg myosin/min. The curves were fit to the data using the Hill equation. Symbols: ●, ○, wild-type cTnT; ■, □, cTnTInt15*L*; ▲, △, cTnTInt15*S*.

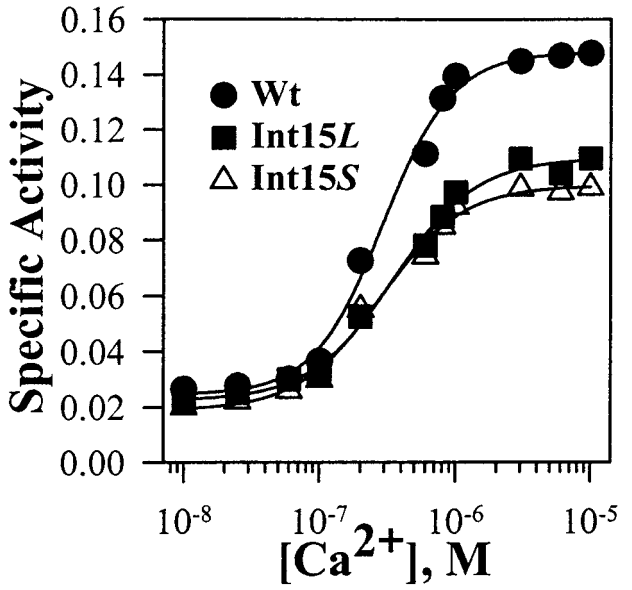

FIGURE 3: Ca^{2+}-dependence of the regulated actomyosin ATPase with wild-type cTnT and the cTnTInt15*L* and cTnTInt15*S* mutants. The reactions were carried out as described for Figure 2, except at a saturating cardiac Tn concentration (1 μM in this experiment) and with 0.45 M CaEGTA. The free Ca^{2+} concentration was controlled using a CaEGTA buffer (see Materials and Methods). Specific activity is expressed as μmol P_i/mg myosin/min. The curves were fit to the data using the Hill equation. Symbols: ●, wild-type cTnT; ■, cTnTInt15*L*; △, cTnTInt15*S*.

Table 1: Properties of Wild-type and Mutant CTnT

	Wt cTnT	cTnT InT 15 *L*	cTnT Int 15 *S*
fold activation ($+Ca^{2+}$)[a]	2.4 ± 0.05	1.7 ± 0.06	1.5 ± 0.03
K_{Tn} for activation[b] (μM)	0.37 ± 0.03	0.36 ± 0.06	0.31 ± 0.05
$K_{Ca^{2+}}$ for activation[c] (10^{-7} M)	2.9 ± 0.3	3.5 ± 0.3	2.8 ± 0.2
$K_{TnT-TnI}$ (apparent)[d] (μM)	7.9 ± 0.7	15.0 ± 1.2	49 ± 2

[a] Activation is the maximal ATPase with Tn relative to the actomyosin ATPase without Tn (± s.e.). [b] The Tn concentration required for half-maximal activation of the actomyosin ATPase ($+Ca^{2+}$ ± s.e.). [c] The Ca^{2+} concentration required for half-maximal activation of the actomyosin ATPase (± s.e.). [e] The concentration of TnT for half-maximal binding to TnI (± s.e.). The value for Int15*S* was based on a curve in which the maximal binding was constrained to that of wild-type.

Ca^{2+} verified the presence of TnT in both pellets. The stoichiometry could not be determined because the wild-type cTnT comigrated with actin and was identified using an immunoblot. However, the Tm/TnI/TnC ratios were similar in all pellets inferring the presence of a comparable amount of bound TnT. In contrast, a complex of skeletal TnI and TnC with chicken skeletal TnT with C-terminal deletions of 47 or 72 residues fails to activate the actomyosin ATPase because Tns containing the mutant TnTs do not bind to actin−tropomyosin in the presence of Ca^{2+} (*18*).

The C-terminus of TnT is in a region of Tn which exhibits Ca^{2+}-sensitive binding to the thin filament (*15*). It was expected that the C-terminal mutations might have an effect either on the apparent Ca^{2+}-affinity of the Tn complex or on the cooperativity of activation. Figure 3 (Table 1) shows that in the presence of saturating Tn, the Ca^{2+}-concentration required for half-maximal activation was comparable with Tn complexes containing wild-type and mutant TnTs. Our results, therefore, indicate that the mutant Tn complexes do not show a significant change in Ca^{2+}-sensitivity, although the Ca^{2+}-dependent activation is severely diminished.

Binary Interactions with cTnI. In the Tn complex, TnT binds to both TnC and TnI. The C-terminus of TnT contains binding sites for TnC and TnI (reviewed in *4*, *6*). Binding of cTnI to the wild-type and mutant cTnTs was studied by HPLC (*17*) using a Waters Protein Pak 300SW gel filtration column. Increasing amounts of TnT were added to a constant amount of cTnI (15 μM) such that the TnT/TnI molar ratios ranged from 0 to 2.8:1. The free TnT was calculated as discussed in the Materials and Methods.

Figure 4 shows that cTnI binding to wild-type cTnT reached saturation around 1:1 TnT/TnI. The cTnTInt15*L* mutant shows saturation at higher ratios of TnT/TnI, while the cTnI binding ability of the shorter cTnTInt15*S* mutant was even more compromised. The apparent K_d for the wild-type protein was about 8 μM, while that for the cTnTInt15*L* mutant was 2-fold weaker at 15 μM (Table 1). The binding affinity for cTnTInt15*S* was too weak to be directly measured, but when the plateau value was constrained to that of wild-type, an apparent K_d of 49 μM was reported. The apparent K_ds were calculated based on the concentration in the TnI−TnT mixture applied to the column and not on the concentration once it was on the column. Since our measurements may not be at equilibrium, we cannot know if the behavior of the mutant TnTs reflects changes in the on or off rates, or both. For these reasons, we refer to the K_ds as "apparent". The apparent K_d for wild-type cTnT−cTnI is higher than that obtained using a biosensor assay at higher ionic strength (K_d = 2.2 μM, *39*). Our results, however, show that the loss of the C-terminal amino acid residues of cTnT conclusively reduces TnI affinity. While the functional differences could reflect improper folding, the present findings with cTnT are consistent with Jha et al. (*17*)

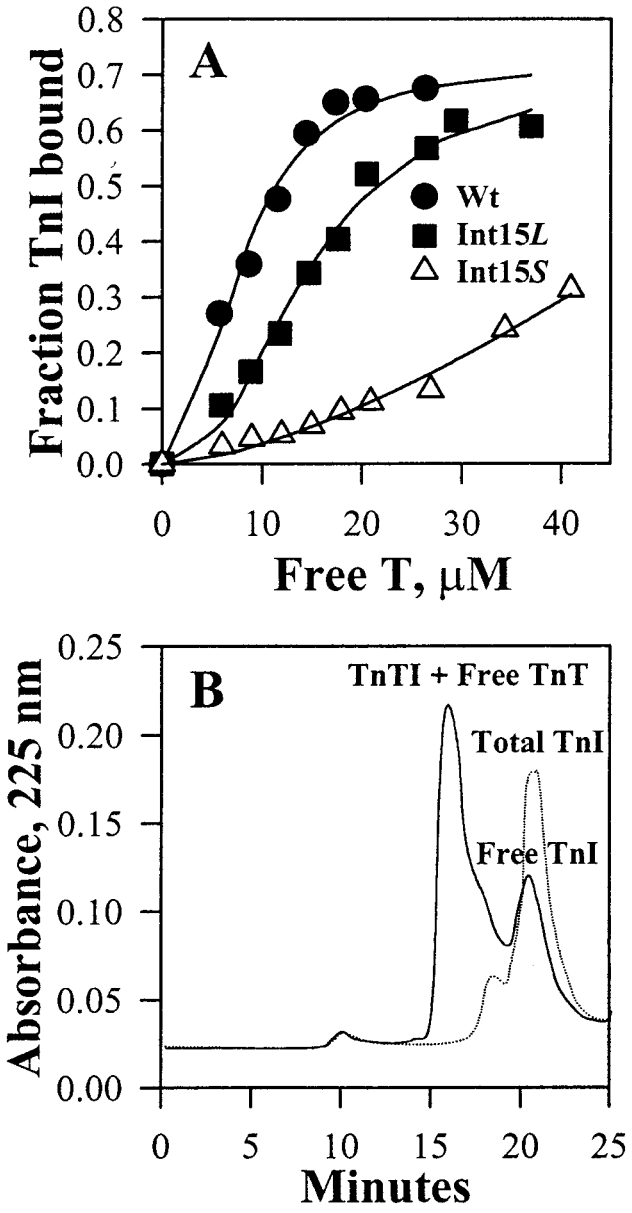

FIGURE 4: Binding of cTnI to wild-type and mutant cTnTs. Cardiac TnT and cTnI were combined and chromatographed on a HPLC gel exclusion column. A. Fraction of TnI bound to TnT as a function of the TnT concentration. The curves were fit to the data using the Hill equation. Symbols: ●, wild-type cTnT; ■, cTnTInt15*L*; △, cTnT Int15*S*. The fractional TnI binding was calculated as described in Materials and Methods. B. The elution profile of cTnI compared to that of a wild-type cTnT−cTnI complex. Solid line, TnT+TnI; dotted line, TnI alone.

who reported that C-terminal residues 202−258 of skeletal TnT (which correspond to residues 232−288 of cTnT) contain the interaction site for TnI.

We have not been able to study the interactions of the cTnT mutants with cTnC. Although, skeletal TnT−TnC complex formation has been studied on nondenaturing polyacrylamide gels (*25*, *40*), cTnT and cTnC did not form a complex in these conditions.

DISCUSSION

Troponin T, through its interactions with TnI, TnC, tropomyosin, and actin makes the striated muscle thin filament a Ca^{2+}-sensitive regulatory unit. In the absence of Ca^{2+}, TnT is required for inhibition of actin−myosin interaction; in the presence of Ca^{2+}, it allows for activation and facilitates the switch of the thin filament to the "on" state. Analysis of TnTs containing disease-causing mutations has given insight into the molecular basis of disease as well as the basic mechanism of TnT function. The two FHC TnT mutants in which the C-terminus has been deleted (cTnTInt15*L*, cTnTInt15*S*) are deficient in regulatory function in that reconstituted Tn complexes activate the actomyosin MgATPase poorly compared to wild-type. Inhibition in the absence of Ca^{2+} (with EGTA) is unaffected. When quail pectoralis myocytes were transfected with cTnTInt15*S*, the TnT incorporated into well-organized sarcomeres in the majority of fibers, but the maximal force generation was well below that of wild-type, on average, consistent with our in vitro results (*19*). Expression of the truncated TnT in a transgenic mouse, however, resulted in smaller hearts with diastolic dysfunction or was lethal, depending on the level of expression, implying altered myofibrillar assembly (*41*).

Even though both mutants have reduced affinity for TnI, the ternary complex remains intact and binds to actin-tropomyosin both in the presence and absence of Ca^{2+}. Therefore, the failure to activate fully the actomyosin ATPase is not caused by lack of TnIC in the reconstituted thin filament. By comparison, skeletal TnTs with larger C-terminal deletions also have reduced TnI affinity (*17*) and form ternary Tn complexes that inhibit poorly in the absence of Ca^{2+} and where the TnIC is released from actin−tropomyosin in the presence of Ca^{2+} (*18*). Also, the skeletal TnT deletions make the actomyosin ATPase more Ca^{2+} sensitive where as the FHC TnT deletions reported here have no effect. Further comparison of the skeletal and cardiac mutants is difficult because the C-terminal sequences, except for the last seven residues, are not conserved, though it is clear that the C-terminal regions of both isoforms are required for TnI binding (e.g., *17*, *18*, *42*, *43*, and the present work), in contrast to an earlier study on cardiac TnT (*44*). Taking the cardiac and skeletal results together, we can conclude that normal TnI binding to TnT is required for inhibition of the actomyosin ATPase in the absence of Ca^{2+} as well as for activation in the presence of Ca^{2+}. While the activation by cTnTInt15*S* is only slightly lower than that by cTnTInt15*L*, they differ in affinity for TnI by least 3-fold indicating, not surprisingly, that activation cannot be explained simply in terms of TnI−TnT affinity.

Assays of contractile function of these TnT deletion mutants (*19*, *41*) and other FHC mutants (reviewed in *1*) are consistent with the general conclusion that FHC mutations result in impaired cardiac myocyte contractile performance. The low level of activation of the actomyosin ATPase observed here with the TnT C-terminal truncations would be consistent with reduced contractile performance resulting from incomplete "turning on" of the thin filament, implying reduced TnT−TnI affinity as the basis for the disease mechanism. What we cannot know, however, from the in vitro analyses is the effect of the deletion on proper myofibril assembly in cardiac myocytes. The highly deleterious effect of the mutation on mouse cardiac myocyte formation (*40*) and the presence of disordered sarcomeres in some transfected skeletal myocytes (*19*) suggests the mutation may also affect cardiac function at an organizational level, in addition to influencing the basic contractile mechanism and its regulation.

At this time, there can be no generalization as to the effect of FHC-associated TnT mutations on assays of contractile function. The I79N and R92Q mutations have been the most extensively studied, in a variety of experimental systems. The mutations have no effect on the actin−myosin S1 or myofibrillar ATPase or maximal tension and either have no effect, increase, or decrease the Ca^{2+}-sensitivity, depending on the assay (*45*−*49*). Unlike the Int15*S* mutation, I79N and R92Q have no effect on sarcomeric structure (*47*, *48*). While some TnT mutations have been reported to increase the level of activation of contractile function, depending on the assay (*45*, *47*, *49*), the C-terminal deletions are the only ones to impair activation (this report, *19*).

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