Investigation of a Truncated Cardiac Troponin T That Causes Familial Hypertrophic Cardiomyopathy

Ca\textsuperscript{2+} Regulatory Properties of Reconstituted Thin Filaments Depend on the Ratio of Mutant to Wild-Type Protein

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Abstract—Familial hypertrophic cardiomyopathy (HCM) is caused by mutations in at least 8 contractile protein genes, most commonly β myosin heavy chain, myosin binding protein C, and cardiac troponin T. Affected individuals are heterozygous for a particular mutation, and most evidence suggests that the mutant protein acts in a dominant-negative fashion. To investigate the functional properties of a truncated troponin T shown to cause HCM, both wild-type and mutant human cardiac troponin T were overexpressed in \textit{Escherichia coli}, purified, and combined with human cardiac troponins I and C to reconstitute human cardiac troponin. Significant differences were found between the regulatory properties of wild-type and mutant troponin in vitro, as follows. (1) In actin-tropomyosin–activated myosin ATPase assays at pCa 9, wild-type troponin caused 80% inhibition of ATPase, whereas the mutant complex gave negligible inhibition. (2) Similarly, in the in vitro motility assay, mutant troponin failed to decrease both the proportion of actin-tropomyosin filaments motile and the velocity of motile filaments at pCa 9. (3) At pCa 5, the addition of mutant complex caused a greater increase (21.7%) in velocity of actin-tropomyosin filaments than wild-type troponin (12.3%). These data suggest that the truncated troponin T prevents switching off of the thin filament at low Ca\textsuperscript{2+}. However, the study of thin filaments containing varying ratios of wild-type and mutant troponin T at low Ca\textsuperscript{2+} indicated an opposite effect of mutant troponin, causing enhancement of the inhibitory effect of wild-type complex, when it is present in a low ratio (10% to 50%). These multiple effects need to be taken into account to explain the physiological consequences of this mutation in HCM. Further, these findings underscore the importance of studying mixed mutant:wild-type preparations to faithfully model this autosomal-dominant disease. (Circ Res. 2000;86:1146-1152.)

Key Words: familial hypertrophic cardiomyopathy ▪ troponin T ▪ cardiac muscle ▪ in vitro motility

Familial hypertrophic cardiomyopathy (HCM) is an autosomal-dominant disorder that predisposes to cardiac hypertrophy, contractile abnormalities, and sudden death. It is a disease of the sarcomere with mutations described in β myosin heavy chain, both myosin light chains, cardiac myosin binding protein C, α-tropomyosin, cardiac troponin I (TnI), cardiac troponin T (TnT), and cardiac actin.\textsuperscript{1,2} Biochemical and physiological analyses of the disease-associated proteins have shown that some mutations result in diminished contractility, whereas others appear to produce enhancement of contractility or Ca\textsuperscript{2+} sensitivity in vitro, suggesting that the end-stage disease may result from numerous different effects.\textsuperscript{3}

A number of mutations have been identified in the human cardiac TnT gene.\textsuperscript{4,5} Most give rise to single amino acid substitutions, but 1 mutation within an intronic splice donor site results in the loss of the 28 C-terminal amino acids encoded by exons 15 and 16 and their substitution by 7 nonsense amino acids followed by a termination codon.\textsuperscript{4} Watkins et al\textsuperscript{5} showed that this truncated TnT could be expressed in quail myocytes and that it was incorporated into the myofibril. Thus, the mutation seems to act as a dominant-negative rather than a null allele. In this fiber model, the presence of the mutation reduced isometric contraction. In a transgenic mouse model, however, the situation seemed rather more complicated.\textsuperscript{5} No more than 10% of the mutant TnT could be found in viable heterozygous mice, and a higher proportion of the mutant protein (eg, in homozygous mice) always led to death shortly after birth, with massive myocyte disruption. The viable mice did not have an obvious hypotrichal cardiac phenotype; indeed, the most noticeable defect was an impairment of diastolic relaxation. Thus, from...
physiological experiments, the authors concluded that the mutation may have multiple effects depending on the quantity expressed.

The functional domains of TnT have been investigated in the skeletal muscle isofrom. The N-terminal 191 amino acids (72 C-terminal amino acid deletion) interact with tropomyosin and have an activating effect that is independent of Ca$^{2+}$.[7] This is probably due to binding of this region to the overlap between tropomyosin molecules, which increases the size of the regulated unit.[8] The C terminus regions 157 to 216 and 217 to 263 were proposed to participate, respectively, in inhibition/thin filament binding and in Ca$^{2+}$-sensitive binding to TnI and troponin C (TnC).[7] Thus, the deletion of 28 amino acids from the C terminus in the truncated TnT mutant could have several effects, potentially interfering with Ca$^{2+}$ regulation, but also possibly with inhibition or activation.

Recent studies of the effect of the HCM-causing truncated TnT on the regulatory properties of the thin filament have indeed indicated that Ca$^{2+}$ regulation is impaired, but it has not been shown consistently whether this was due to impaired relaxation at low Ca$^{2+}$ or impaired activation at high Ca$^{2+}$.[9–11] These studies have been performed with a variety of recombinant peptides: human or bovine TnT, with or without the 7 nonsense amino acids. To determine precisely the effect of the truncation mutation in TnT, we have expressed the exact human protein in *Escherichia coli* together with the wild-type human cardiac isoforms of TnI, TnC, and TnT and have reconstituted human cardiac troponin. We have compared troponin reconstituted with truncated TnT with that reconstituted with wild-type protein in their ability to regulate both actomyosin ATPase and actin filament movement using the in vitro motility assay. We find that the truncated TnT is incorporated normally but that it prevents switching off of the thin filament at low Ca$^{2+}$. Surprisingly, study of the regulation of thin filaments containing varying ratios of wild-type and truncated TnT suggests that there is an opposite, relaxing effect of the mutant when it is present in a low ratio (10% to 50%). The contrasting results obtained by studying this more relevant state need to be taken into account to explain the physiological consequences of this mutation in HCM.

Materials and Methods

The expression plasmid construction and protein purification methods are described briefly here and in full in the online Materials and Methods (see http://www.circresaha.org). The assay methods are described briefly in each figure legend and in detail in the online data supplement.

Preparation of Recombinant Human Cardiac Troponin Subunits and Complex

pMW172[12] expression constructs encoding either wild-type human cardiac TnT (288 amino acids) or mutant truncated TnT (267 amino acids) were overexpressed in BL21(DE3)pLysS cells,[13] and the TnT was purified from the bacterial extract by successive cationic and anionic exchange chromatography.

A pMW172 construct encoding human cardiac TnI, made using cDNA (obtained as a gift from Dr P. Barton [Imperial College, London, UK]) and a pET11c construct encoding human cardiac TnC (obtained as a gift from Prof I.P. Trayer [University of Birmingham, UK]) were used to produce TnI and TnC, as previously described.[14] Human cardiac troponin complexes were made by mixing a 1:1:1 molar ratio of recombinant TnC, TnI, and TnT (for the motility assay, the ratio was 3:1:1) in 6 mol/L urea; 1 mol/L KCl; and, in mmol/L, CaCl$_2$ 0.1, MES (pH 6.0) 10, and DTT 5, followed by stepwise dialysis into assay buffer. To separate aggregated protein, the mixtures were centrifuged at 300 000g at 4°C for 15 minutes.

Protein Purification

Heavy meromyosin, subfragment-1 (S-1), and F-actin from rabbit fast skeletal muscle; α-tropomyosin from rabbit heart muscle; and α-actinin from chicken gizzard were prepared by standard methods. Native human cardiac tropomyosin and troponin were prepared as described previously.[15] An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

Ca$^{2+}$ Regulation of Actin-Tropomyosin–Activated Myosin S-1 ATPase by Wild-Type and Mutant Troponin

Troponin complexes were reconstituted as described using purified recombinant TnI, TnC, and either wild-type or mutant TnT. The subunit compositions of wild-type and mutant complex were the same as determined by SDS-PAGE and scanning densitometry (TnT:TnI:TnC was 1:1.05:0.90 for wild-type [n=4] and 1.11:0.94 for mutant [n=3]). In activating Ca$^{2+}$ conditions (pCa 4), addition of wild-type complex to myosin S-1–actin–α–tropomyosin caused an increase of up to 80% in the ATPase rate at 37°C; addition of troponin containing mutant TnT gave an indistinguishable activation (Figure 1A). At pCa 9, wild-type troponin inhibited the actin-tropomyosin–activated myosin S-1 ATPase by 80%; however, under these conditions, the mutant complex did not give any substantial inhibition of ATPase (Figure 1A). This marked decrease in the ability of the mutant complex to inhibit actin-tropomyosin–activated myosin S-1 ATPase in EGTA was also observed at 25°C (data not shown). The Ca$^{2+}$ dependence of actin-tropomyosin–activated myosin S-1 ATPase was measured (Figure 1B). The transition from inhibited to activated ATPase occurred at a higher pCa$_{50}$ with the mutant troponin complex than with the wild-type complex (6.69 compared with 6.26).

The binding of troponin to actin-tropomyosin, measured by cosedimentation, under ATPase conditions at both pCa 5 and pCa 9, was the same for both wild-type and mutant complex. With 1 μmol/L added troponin at pCa 9, both the amount of troponin bound (tropomyosin:TnT 1:0.88 for wild-type [n=4], 1.09 for mutant [n=3]) and the subunit composition of the bound troponin (TnT:TnI:TnC 1:1.09:0.93 for wild-type [n=4], 1:1.12:0.95 for mutant [n=3]) were the same.

Ca$^{2+}$ Control of In Vitro Motility by Reconstituted Human Cardiac Troponin

Comparison Between Native and Reconstituted Recombinant Human Cardiac Troponin

We have previously used the in vitro motility assay to investigate regulation of the movement of rhodamine-phalloidin–labeled actin-tropomyosin filaments over a bed of immobilized heavy meromyosin by native human cardiac troponin and by rabbit skeletal muscle troponin reconstituted from purified subunits.[15–17] In this study, we have for the first time examined regulation of motility conferred by human
cardiac muscle troponin reconstituted from purified individual subunits expressed in *E. coli*. This was compared with native troponin extracted from human hearts. At pCa 5, native and recombinant troponin increased the velocity of actin-tropomyosin filaments by 45% and 12%, respectively. At pCa 9, saturating concentrations of both troponins reduced the fraction of filaments motile by a similar degree (Figure 2A), and the velocity was reduced by 19%. We conclude that the reconstituted recombinant preparation is qualitatively the same as native troponin and is a suitable background in which to study the functional effects of troponin mutants. The quantitative differences may indicate imperfect folding of peptides, imperfect assembly into the troponin complex, or post-translational modifications.

**Ca**<sup>2+</sup> Control of In Vitro Motility by Troponin Containing Wild-Type and Truncated Mutant TnT

The regulatory effects of troponin reconstituted with wild-type and truncated mutant TnT were compared in 17 paired experiments. In contrast to wild-type troponin, at pCa 9 the mutant complex did not reduce the fraction of filaments motile (Figures 2C and 3) and, instead of a decrease in filament velocity, the mean velocity actually increased by 31% (Figures 2B and 3). The binding, determined by cosedimentation, of wild-type and mutant troponin to actin-tropomyosin under these conditions was indistinguishable (data not shown). The frequency histograms of filament velocity (Figure 2A) indicated that nearly all of the filaments

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**Figure 1.** Regulation of actin-tropomyosin (TM)–activated myosin S-1 ATPase by wild-type and mutant troponin. A, Titration of wild-type and mutant troponin at high and low pCa. Actin-tropomyosin–activated myosin S-1 ATPase rates were measured using (in μmol/L) rabbit skeletal muscle myosin S-1 0.5, rabbit skeletal muscle actin 7, and α-tropomyosin 1 in a solution containing (in mmol/L) PIPES 5, MgCl<sub>2</sub> 3.87, DTT 1, and ATP (pH 7.0) 3 at 37°C in the presence of 0 to 2 μmol/L troponin complex and either 0.1 mmol/L CaCl<sub>2</sub> (pCa 4) or 1 mmol/L EGTA (pCa 9). The uninhibited actin-tropomyosin–activated myosin S-1 ATPase rate was 7.8/second. Data are mean ± SE from 4 experiments. ■, Troponin containing wild-type TnT, pCa 4; ○, troponin containing wild-type TnT, pCa 9; □, troponin containing mutant truncated TnT, pCa 4; and ▼, troponin containing mutant truncated TnT, pCa 9. B, Ca<sup>2+</sup> dependence of regulation by wild-type and mutant troponin. Actin-tropomyosin–activated myosin S-1 ATPase rates were measured as above. Data are mean ± SE from 3 experiments. Lines drawn are best fits of the data to the Hill equation using Kaleidagraph (Synergy Software). Derived parameters for wild-type troponin are pCa<sub>50</sub> = 6.26 ± 0.02, Hill coefficient = 1.30 ± 0.07; for mutant troponin, pCa<sub>50</sub> = 6.69 ± 0.07, Hill coefficient = 1.11 ± 0.21. ■, troponin containing wild-type TnT; ○, troponin containing mutant truncated TnT.

**Figure 2.** In vitro motility study of cardiac muscle thin filaments reconstituted with native, wild-type, and mutant troponin at pCa 9. A, Analysis of movement of reconstituted cardiac muscle thin filaments over skeletal muscle heavy meromyosin at pCa 9. Movement of fluorescent actin–filaments over a microscope slide coated with 100 μg/mL skeletal muscle heavy meromyosin in 1 mmol/L MgATP during a 0.6-second time interval was recorded and analyzed. One thousand filament vectors were obtained and plotted as a frequency histogram. Thin filaments reconstituted with 10 nmol/L actin, 30 nm human cardiac troponin and 20 nmol/L wild-type TnT and native troponin were switched off, with most of the filaments not moving. In contrast, thin filaments reconstituted with truncated TnT were nearly all moving, and the velocities were widely distributed. B and C, Titration of troponin reconstituted with wild-type and truncated mutant TnT. Graphs show results from a single experiment in which increasing concentrations of troponin were mixed with 10 nmol/L actin–filaments and 30 nmol/L human cardiac tropomyosin. Points are mean ± SE for 4 measurements of velocity (B) and fraction of filaments motile (C) made at different locations within the same motility cell. ●, Thin filaments reconstituted with wild-type TnT; ○, thin filaments reconstituted with truncated TnT.
were moving and that the greater average speed of filaments containing mutant troponin was accompanied by an increased spread of velocity vectors, derived from either the presence of multiple populations of filaments with different velocities or increased variability of velocity. At pCa 5, both wild-type and mutant troponin caused an increase in filament velocity (Figure 3), reaching a maximum by 20 nmol/L. The increase in velocity (21.7%) of actin-tropomyosin filaments incorporating the mutant troponin was significantly greater than that seen with wild-type troponin (12.3%) \((P=0.028 \text{ by paired } t \text{ test, 11 experiments})\). The distribution of velocities at pCa 5 was similar for wild-type and mutant troponins.

Properties of Wild-Type/Mutant Truncated TnT Mixtures

In vivo, it is likely that the wild-type and mutant truncated TnT are both expressed and incorporated into troponin, and so there is the potential for interaction between the 2 species. To examine this, we have studied the regulatory effects of troponin containing different ratios of wild-type and truncated TnT at a constant total troponin concentration. In actin-tropomyosin–activated myosin S-1 ATPase assays performed under the same conditions as those in Figure 1 using 2 \(\mu\text{mol/L}\) total troponin, changing the proportion from 100% wild-type TnT to 100% truncated TnT resulted in a straightforward linear increase in rate at pCa 9 (Figure 4A). However, a markedly different result was obtained using the in vitro motility assay. We carried out 8 titrations of mixtures at pCa 9, and in each case the pattern of results was the same. Figures 4B and 4C showed the pooled data from 3 of these experiments in which the fraction of filaments motile was reduced to \(\approx 45\%\) by wild-type troponin. There is a clear biphasic effect; mixtures containing 10% to 50% truncated TnT actually reduced the fraction of filaments motile and filament velocity, whereas \(70\%\) or more truncated TnT resulted in a progressive switching on of the filaments (high fraction motile and high velocity). Horizontal lines show the average velocity and fraction motile of actin-tropomyosin filaments. ○, Actin-tropomyosin; ●, actin-tropomyosin-troponin.
mixture (pCa_{50} for fraction of filaments motile was 6.31±0.02 for wild-type and 6.35±0.03 for the 25% mutant mixture).

In these admixture experiments, both complexes appeared to bind equally well to actin-tropomyosin; in sedimentation assays using a 50/50 troponin mixture, equal amounts of wild-type and mutant TnT were detected in the pellet (wild-type:mutant=1:1.05 [n=2]).

Effect of Mutation on Isometric Force
A load can be imposed on actin filaments in the in vitro motility assay by including the actin binding protein, α-actinin, bound to the siliconized cover glass with the immobilized motor protein. Above a critical concentration of α-actinin, filament velocity rapidly diminished to 0, whereas the fraction of filaments motile decreased linearly with α-actinin concentration. We defined an empirical parameter, index of retardation, as the concentration of α-actinin needed to stop all filaments moving (~0.8 μg/mL with actin). The index of retardation is very consistent both within and between experiments, and we have previously demonstrated that it is a measure of isometric force.18 We used this technique to study thin filaments containing native, wild-type, and truncated TnT with saturating troponin and tropomyosin at pCa 5 (Figure 5). As in previous experiments, the addition of wild-type or native troponin-tropomyosin increased the index of retardation 3-fold. Thin filaments containing truncated TnT were indistinguishable from those containing wild-type troponin. We also compared the index of retardation of actin–β-tropomyosin filaments containing wild-type troponin or a troponin containing a mixture of 75% wild-type and 25% mutant TnT, because this ratio gave the maximum effect on fraction of filaments motile (Figure 4). The 2 plots were indistinguishable from each other.

Discussion
Previous studies have indicated that the majority of mutant contractile proteins that cause hypertrophic cardiomyopathy do so through a dominant-negative mechanism. Individuals with the disease are heterozygous and thus have 1 mutant and 1 wild-type copy of the gene. For the β-myosin heavy chain, α-tropomyosin, and cardiac TnT genes, there are direct, experimental data indicating that the mutant peptide does incorporate into the sarcomere.19–21 It is for this reason that biochemical and biophysical analysis of the mutant peptide is a logical way to begin to understand the disease etiology. It is important in such studies that the system investigated be as similar as possible to human heart muscle. The regulatory properties of thin filaments depend critically on all components of the thin filament, as is shown by our observation of different effects of HCM mutations in tropomyosin using either rabbit skeletal muscle or human cardiac muscle tropinin.22 We have therefore taken care to use cardiac muscle tropomyosin and recombinant human cardiac TnT, TnI, and TnC in these experiments. We have also compared our reconstituted troponin with native human cardiac troponin. Recent publications have studied the same truncated TnT mutation in heterologous systems and have found that the mutant troponin is inhibitory at all pCa, in contrast to our observation that mutant troponin is active at all pCa.9–11 A comparative analysis of these experiments is presented in the online data supplement (see http://www.circresaha.org). We think it likely that the differences arise from the different TnI and TnC and tropomyosin used. Truncation mutants of skeletal muscle TnT with C-terminal deletions of 57 and 72 amino acids have been shown to decrease the inhibitory activity of troponin while not affecting its activating property.7,23

In the main, in vitro studies of mutant proteins that cause HCM have focused on pure preparations of the mutant peptide, rather than the situation that occurs in the disease state with a mixture of both mutant and wild type. An important finding from the current work is that this truncation allele of TnT is associated with a failure of inhibition of the thin filament when studied alone, but with enhanced efficiency of inhibition (ie, switching off) when a minority component of filaments also containing wild-type peptide. Presumably the drop in fraction of filaments motile observed with low concentrations of pure mutant troponin and the wide distribution of velocities at pCa 9 (Figure 2) are consequences of the same phenomenon. This biphasic response illustrates that it is not sufficient to extrapolate from observations of pure mutant protein when trying to model pathogenesis in HCM.

Because of difficulties obtaining myocardium from genotyped individuals with HCM and the difficulties in resolving wild-type from mutant protein (as the mutations are usually subtle, typically missense), the actual ratio of mutant and wild-type peptide in the heart in HCM is often not known. For the truncated TnT allele analyzed in this study, even mRNA data from myocardium are lacking. Data regarding the anomalous splice isoforms that result from the intronic mutation have been obtained from studies in leukocytes in which 2 aberrant splice forms are equally abundant and, together, approximately equal the amount of wild-type transcript.4 Despite this uncertainty, however, we can be confident that the mutant peptide is present within the range of a few percent up to 50% and is therefore expected to show the increased efficiency of switching off in low calcium that characterizes this part of the biphasic response (Figures 4).

Such a biphasic response arising from different ratios of mutant to wild-type peptide has not been seen before in
HCM. In the 1 previous in vitro study looking at the effect of admixture, Arg403Gln β-myosin heavy chain exerted a dominant effect over the wild-type peptide, such that a 50/50 mix had the same phenotype as the mutant peptide alone. In contrast, a 50/50 mix of the truncated TnT behaved more closely to the wild type, whereas lesser amounts have characteristics opposite those of the pure mutant. The biphasic response may perhaps be explained by considering the different functional domains of the protein and the way in which these may direct cooperative regulation with the thin filament. TnT has been divided into 2 functional domains. The N-terminal domain, T1, is largely α-helix and binds strongly to tropomyosin, where it influences the end-end interaction and cooperativity. The C-terminal domain, T2, binds to tropomyosin, TnI, and TnC. The presence of TnC complexed with the T2 domain makes its interactions with TnI and tropomyosin Ca2+-sensitive. In this model, TnT has 3 domains: an activator domain (1 to 191 in chicken cardiac actin is COOH-terminal domains of troponin T: ATPase activation and binding to tropomyosin, because actin monomers markedly outnumber attached cycling myosin heads.19 In vivo, and in assays involving transfected myocytes or reconstituted skinned fibers, myosin heads are relatively abundant, allowing cooperative recruitment by myosin. It remains unknown whether the different findings with regard to force reflect this difference or whether the force generation is indeed normal at the level of the cross-bridge, but is abnormal in the sarcomere because of structural abnormalities consequent on incorporation of truncated TnT, as was seen in the transgenic mouse model.6

The abnormalities in relaxing conditions that characterize thin filaments with a mixed population of mutant and wild-type TnT, and the increased velocity in activation conditions, are qualitatively similar to abnormalities described for missense mutations in TnT that cause HCM. The increase in filament velocity at pCa 5 seen when changing from 100% wild-type to a 50:50 mix, and then to 100% mutant TnT, appears to mirror the mechanisms leading to the increased unloaded shortening velocity observed with missense TnT peptides. This parameter of the motility assay can reflect changes either in step size (which seem unlikely in this context) or in the crossbridge cycling rate. Previous work has demonstrated that the cycling rate is modulated by TnT. Increases in crossbridge cycling rate are most simply explained by proposing an increase in the rate of detachment, which implies a shorter duty cycle. These changes would seem likely to lead to increased cost of force production and consequently to an energy imbalance in the myocardium.

The enhanced efficiency of switching off the thin filament by a mixture of wild-type and mutant TnT is observed when thin filaments are only partially switched off. It is notable that this change is opposite that which would have been predicted if only 100% mutant TnT peptides had been studied. These findings underscore the need to study mixed wild-type and mutant preparations to model this autosomal-dominant condition.

**Acknowledgments**

This work has been supported by the British Heart Foundation and the Wellcome Trust.

**References**


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Circ Res. 2000;86:1146-1152
doi: 10.1161/01.RES.86.11.1146

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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