Alterations in Thin Filament Regulation Induced by a Human Cardiac Troponin T Mutant That Causes Dilated Cardiomyopathy Are Distinct from Those Induced by Troponin T Mutants That Cause Hypertrophic Cardiomyopathy*

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We have compared the in vitro regulatory properties of recombinant human cardiac troponin reconstituted using wild type troponin T with troponin containing the ΔLys-210 troponin T mutant that causes dilated cardiomyopathy (DCM) and the R92Q troponin T known to cause hypertrophic cardiomyopathy (HCM). Troponin containing ΔLys-210 troponin T inhibited actin-tropomyosin-activated myosin subfragment-1 ATPase activity to the same extent as wild type at pCa8.5 (>80%) but produced substantially less enhancement of ATPase at lower pCa values (pCa4.5). The Ca2+ sensitivity of ATPase activation was increased (ΔpCa50 = +0.2 pCa units) and cooperativity of Ca2+ activation was virtually abolished. Equimolar mixtures of wild type and ΔLys-210 troponin T gave a lower Ca2+ sensitivity than with wild type, while maintaining the diminished ATPase activation at pCa4.5 observed with 100% mutant. In contrast, R92Q troponin gave reduced inhibition at pCa8.5 but greater activation than wild type at pCa4.5; Ca2+ sensitivity was increased but there was no change in cooperativity. In vitro motility assay of reconstituted thin filaments confirmed the ATPase results and moreover indicated that the predominant effect of the ΔLys-210 mutation was a reduced sliding speed. The functional consequences of this DCM mutation are qualitatively different from the R92Q or any other studied HCM troponin T mutation, suggesting that DCM and HCM may be triggered by distinct primary stimuli.

Dilated cardiomyopathy (DCM)1 is defined clinically by cardiac chamber dilatation with reduced contractile performance in the absence of underlying coronary artery disease. The heart appears thin walled and distended and, at the microscopic level, there is moderate myocyte hypertrophy and death, along with replacement fibrosis. Echocardiographic screening of relatives of affected individuals suggests that ~25–35% of cases are familial (1, 2). The disease is frequently inherited with an associated phenotype such as conduction disease, skeletal myopathy, or sensorineural hearing loss. To date, as many as 18 loci that cause DCM as the predominant phenotype have been identified, and in all but two of these, the disease is inherited in an autosomal dominant manner (3, 4). For 10 of the loci, the disease genes have been identified. These encode a diverse range of proteins, including components of the sarcomere: actin (ACTC) (5); β-myosin heavy chain (MYH7) (6); titin (TTN) (7); α-tropomyosin (TPM1) (8); and cardiac troponin T (TNNT2) (6).

In a recent report, Kamisago et al. (6) identified two mutations in β-myosin heavy chain and one in cardiac troponin T (the deletion of lysine 210) in kindreds having autosomal dominant dilated cardiomyopathy without conduction disease, skeletal muscle dysfunction, or other accompanying phenotypes. It was noteworthy that affected subjects did not have ventricular hypertrophy, and histology from one subject showed mildly increased interstitial fibrosis without the myocyte and myofibrillar disarray characteristic of hypertrophic cardiomyopathy (HCM). These mutations therefore appear to cause dilated cardiomyopathy directly and induce a phenotype that is distinctly different from HCM.

HCM is known to be caused by mutations in at least 10 genes, all but one of which encodes a sarcomeric protein (3, 9). In contrast to the contractile protein gene mutations that cause DCM, the functional consequences of the HCM mutations have been extensively characterized (reviewed in Refs. 3, 10, and 11). Most mutations in sarcomeric proteins have been found to increase maximum shortening speed and/or Ca2+ sensitivity in vitro, which may result in energetic compromise through increased cost of force production in vivo (9, 12–15). In HCM families, some individuals go on to develop a dilated cardiomyopathy phenotype, presumably through induction of apoptosis (3). Thus, one plausible hypothesis to explain how different mutations in the same gene can cause different cardiomyopathies is that DCM mutations produce similar, but more severe, perturbations of contractile protein function, sufficient to result in cell death. Alternatively, the DCM mutations in sarcomeric protein genes could initiate disease through qualitatively different perturbations of contractility (6, 16).

Functional analysis of different mutations within a single gene that produce the divergent phenotypes of HCM and DCM provides a valuable opportunity to investigate the triggers that discriminate between these two disease pathways. In this report, we have focused on the ΔLys-210 troponin T mutant that causes DCM. In common with the HCM troponin T mutations,
it is highly likely that this apparently subtle mutation acts in a dominant-negative manner and is incorporated into the thin filament, where it affects normal thin filament function. The deleted amino acid forms one of a stretch of four lysine residues in human cardiac troponin T (amino acids 207–210). These lie within the globular C-terminal T2 domain (residues 188–288).

**Fig. 1.** The regulation of reconstituted thin filaments by wild type and mutant troponin in actin-tropomyosin-activated myosin ATPase and in vitro motility assays. A, actin-tropomyosin-activated myosin ATPase rates at 37 °C, pH 7.0, expressed as a percentage of the rate in the absence of troponin (8.0 ± 0.3 s⁻¹). B, mean thin filament sliding velocity at 28 °C, pH 7.4, measured with 10–15 nM troponin expressed as a percentage of the velocity of actin-tropomyosin-wild type troponin filaments at pCa5.4 measured in the same experiment (3.83 ± 0.17 μm/sec). C, fraction of filaments motile. Activating conditions (pCa4.5 ATPases, pCa5.4 motility), open boxes; relaxing conditions (pCa8.5 ATPases, pCa9 motility), shaded boxes. Error bars show S.E. calculated from eight ATPase experiments using four different preparations of each troponin, eleven separate motility experiments for ΔLys210 troponin T, and five motility experiments for R92Q troponin T.
which binds to troponins I and C as well as to tropomyosin (17) and may therefore affect thin filament function by a variety of mechanisms.

We have compared the changes in thin filament function caused by the ΔLys-210 mutation with those caused by a mutation in troponin T that causes HCM. For the latter we have used the R92Q troponin T mutant, which has been extensively characterized in transgenic mouse models (13, 18, 19) and myofibrils (20, 21) but has not yet been examined in reconstituted thin filaments in vitro. We found that the ΔLys-210 mutation had distinctive effects upon thin filament function: the maximally activated ATPase activity and filament sliding speed were decreased, and Ca^{2+} activation became non-cooperative. This pattern of changes was quite unlike the effect of the R92Q HCM mutation (increased ATPase activity and sliding speed and higher Ca^{2+} sensitivity with unaltered cooperativity) but closely resembled the properties of troponin extracted from end stage failing human hearts studied by the same techniques (22, 23).

**MATERIALS AND METHODS**

**Purification and Preparation of Proteins**—Rabbit skeletal muscle actin, rabbit and human cardiac muscle α-tropomyosin, and subfragment-1 (S-1) derived by chymotryptic digestion of whole rabbit skeletal muscle myosin were prepared as previously described (22, 24). Recombinant wild type human troponin subunits were overexpressed in BL21(DE3)pLysS Escherichia coli and subsequently purified (24). pMW172 expression constructs encoding R92Q and ΔLys-210 troponin T were made, respectively, by subcloning from an existing plasmid cytomegalovirus construct (12) and using a two-step PCR protocol for site-directed mutagenesis.

Whole troponin complexes were formed using a development of our established protocol (25). The subunits were mixed in a ratio of 1.5 troponin C:1 troponin I:1 troponin T in 6 M urea, 1 M KCl, 10 mM imidazole, 50 μM CaCl₂, 1 mM dithiothreitol, 0.01% sodium azide, pH 7.0, and the concentrations of first urea and then KCl were reduced using a stepwise dialysis protocol to 0 and 200 mM, respectively. The mixtures were centrifuged (12,300 × g, 5 min) to remove insoluble material and intact troponin purified by gel filtration using a Sepharose
functional properties of wild type and mutant troponin T were compared by assay of thin filament activation of skeletal muscle myosin S-1 ATPase activity and by the in vitro motility assay using skeletal muscle heavy meromyosin. Actin cosedimentation assays carried out as previously described (25) showed that the binding of the wild type and both mutant troponins to actin-tropomyosin were indistinguishable (data not shown).

In ATPase experiments carried out under activating conditions, the addition of wild type troponin increased ATPase activity, reaching a plateau at 207 ± 3% of the ATPase rate obtained using actin-tropomyosin alone, whereas when troponin containing ΔLys-210 troponin T was added the maximum ATPase activation was only 167 ± 6% of the actin-tropomyosin rate. This difference was consistently seen in four different troponin preparations and was highly significant (p < 0.001) (Fig. 1A).

At pCa 5.4, troponin increased the speed of actin-tropomyosin filament sliding in the in vitro motility assay, reaching a plateau at 10–15 nm troponin (Fig. 2A). As previously observed (24, 27), wild type troponin increased sliding velocity to 125 ± 2.6% that of actin-tropomyosin filaments. However, filaments reconstituted with troponin containing ΔLys-210 troponin T gave a significantly reduced sliding speed (111 ± 2.7% of actin-tropomyosin), and this difference was highly reproducible (p < 0.0001, 11 separate experiments, Fig. 1B). Troponin containing ΔLys-210 mutant troponin T also induced a small but significant decrease (p < 0.05) in the fraction of filaments motile under activating conditions compared with wild type troponin (Figs. 1C and 2B). Gel electrophoresis of the reconstituted thin filaments showed that both wild type and mutant thin filaments contained a full complement of bound troponin subunits and tropomyosin (Fig. 2C).

Under relaxing conditions (pCa9), ATPase activation and filament motility of thin filaments containing wild type and ΔLys-210 mutant troponin T were indistinguishable. ATPase activity was inhibited by ~90% (Fig. 1A), and in vitro motility was switched off by troponin such that <10% of the filaments were moving, and the speed of the remaining moving filaments was about 40% slower than actin-tropomyosin filaments, in agreement with previous measurements (24, 29) using this system (Fig. 1, B and C). The maximum switch-off of motility required 10–15 nm troponin with both wild type and ΔLys-210 mutant troponin T. Comparing data from paired preparations of thin filaments, we found no significant difference in the fraction of filaments moving at saturating troponin concentrations (Fig. 1C) nor at partially saturating concentrations (2.5–5 nm troponin). These results demonstrate that neither the EC50 of inhibition caused by troponin containing ΔLys-210 mutant troponin T, nor the maximum inhibition of motility, was different from wild type.

Thin filaments reconstituted with troponin containing the HCM troponin T mutation R92Q reproducibly gave enhanced activation of ATPase activity (224 ± 5% of actin-tropomyosin activity compared with 207 ± 3% using wild type) and thin filament sliding velocity (Fig. 1, A and B). In addition, at pCa9 thin filaments containing R92Q troponin T reproducibly gave less inhibition of ATPase (79% compared with 90% using wild type troponin) and less inhibition of filament motility than wild type (p = <0.05; see Fig. 1, A and C).

We determined the Ca2+ concentration dependence of thin filament activation of S-1 ATPase activity and motility using concentrations of troponin that gave maximal inhibition of filament activity at pCa9 (1 μM for ATPase, 15 nm for motility assay; Fig. 3 and Table I). A consistent pattern of results was obtained with both techniques: the Ca2+-dependent curves for
ATPase activation, fraction of filaments motile, and filament velocity of thin filaments containing ΔLys-210 mutant troponin T were much less steep (i.e., less cooperative) than those obtained using wild type troponin, and 50% activation was shifted to lower Ca^{2+} concentrations. The data were fitted to the Hill equation: 
\[ \text{rate} = a + b/(1 + 10^{n_{H} [\text{Ca}^{2+}]/K_{D}}) \]
The ΔLys-210 mutation increased calculated pCa_{50} by 0.19 ± 0.03, 0.28 ± 0.04, and 0.31 ± 0.03 pCa units for ATPase, fraction of filaments motile, and sliding speed, respectively. The apparent Hill coefficients, n_{H}, derived from fits of the mutant troponin data were close to unity (n_{H} = 1.24 ± 0.06, 0.88 ± 0.19, and 0.90 ± 0.12, respectively) and the ATPase data were well fitted by a simple saturation equation (rate = V_{max} [Ca^{2+}] / (EC_{50} + [Ca^{2+}]) with EC_{50} = 0.25 ± 0.03 μM), indicating that these thin filaments were not cooperatively activated by Ca^{2+}.

In contrast, the R92Q troponin T mutation had a different effect; the Ca^{2+} sensitivity of ATPase regulation was increased with no change in cooperativity (Table I).

The disease caused by both the DCM and HCM troponin T mutations is autosomal dominant, and it is likely that the cardiac thin filaments of affected individuals contain similar proportions of wild type and mutant troponin. Our previous work has shown that the effect on thin filament function of mixtures of wild type and mutant troponin is not directly predictable from the functional properties of the mutant troponin alone (24, 25). Thin filaments were reconstituted using stoichiometric amounts (7:1:1, respectively) of actin, tropomyosin, and total troponin (either wild type, mutant, or 50:50 wild type/mutant). Filaments containing an equimolar mix of wild type and mutant gave activation relative to actin-tropomyosin type mutant. Filaments containing an equimolar mix of wild type and mutant troponin is not directly work has shown that the effect on thin filament function of proportions of wild type and mutant troponin. Our previous with wild type troponin (Fig. 4A).

Interestingly, whereas 100% ΔLys-210 troponin T mutant gave an increase in Ca^{2+} sensitivity of ATPase activation of +0.23 pCa units, the ΔLys-210/wild type mixture resulted in a significant decrease in Ca^{2+} sensitivity compared with wild type; ΔpCa_{50} = −0.27 (p < 0.001; n = 4) (Fig. 4A). The apparent Hill coefficient for the 50:50 mixture was intermediate between 100% wild type and 100% mutant and was significantly different from that obtained with wild type troponin alone (p < 0.001). The same pattern of results was observed using in vitro motility assay: in thin filaments with a 50:50 mixture of wild type and ΔLys-210 troponin, the Ca^{2+} sensitivity of the fraction motile parameter was less than wild type by 0.07 pCa units (data not shown).

Filaments containing an equimolar mix of wild type and mutant troponin T gave similar levels of activation and inhibition as 100% wild type (Fig. 4B).

![Figure 4](image)

**FIG. 4.** Ca^{2+} control of thin filaments by 50:50 mixtures of wild type and mutant troponin. Thin filament activation of myosin S-1 MgATPase activity was measured as a function of Ca^{2+} concentration using 0.5 μM S-1, 3.5 μM actin, 0.5 μM rabbit cardiac tropomyosin, and 0.5 μM (total) human cardiac troponin to compare the effect of 100% wild type, 100% mutant, and 50:50 wild type/mutant troponin mixtures. These measurements were obtained by pH 7.4. Data from individual experiments were fitted to the Hill equation, and the means ± S.E. of the derived parameters, pCa_{50} and n_{H}, from n separate experiments are given in the table.

### Table I

|                  | ATPase pCa_{50} | Sliding velocity pCa_{50} | Motile fraction pCa_{50} | n_{H} |
|------------------|----------------|--------------------------|--------------------------|------|
| Wild type troponin T | 6.48 ± 0.02    | 2.98 ± 0.18              | 1.65 ± 0.05              | 1.57 ± 0.13 |
| ΔLys-210 troponin T  | 6.67 ± 0.01    | 1.24 ± 0.06              | 0.90 ± 0.12              | 0.88 ± 0.19 |
| R92Q troponin T     | 6.72 ± 0.03    | 2.97 ± 0.38              | ND*                     | ND*  |

*Not determined.*
R92Q troponin T produced an increase in the Ca\(^{2+}\) sensitivity of regulation of ATPase activation that was intermediate between pure wild type and pure mutant filaments with no change in cooperativity (\(\Delta pC_{50} = +0.10\) for 50:50 wild type/R92Q compared with \(\Delta pC_{50} = +0.18\) for R92Q) (Fig. 4B).

**DISCUSSION**

The deletion of lysine 210 in cardiac troponin T has been reported to be a cause of inherited dilated cardiomyopathy (6). When human cardiac troponin T with this mutation was incorporated into reconstituted thin filaments, we found a pattern of functional changes in vitro that was distinctly different from the changes previously observed with hypertrophic cardiomyopathy mutations. The Ca\(^{2+}\)-activated rate of actomyosin ATP hydrolysis and the thin filament sliding speed were reduced compared with wild type troponin, the Ca\(^{2+}\) activation curve became non-cooperative, and \(pC_{50}\) was increased. It is particularly noteworthy that a 50:50 mixture of wild type and mutant troponin T, which is likely to reflect the situation in vivo, still reduced the maximally activated actomyosin ATPase and filament sliding speed to the same level as 100% mutant troponin T but gave Ca\(^{2+}\) sensitivity significantly lower than wild type.

The deleted amino acid forms one of a stretch of four lysine residues in human cardiac troponin T (amino acids 207–210). These amino acids lie within the C-terminal chymotryptic T2 fragment known to bind tropomyosin, troponin C, and troponin I (17). Studies of peptides and deletions within this region have indicated that troponin I binds to heptad repeat sequences C-terminal to these four lysines (approximately residues 229–268) (30), whereas both troponin C and tropomyosin may interact close to or directly with these residues (31). The recently reported (32) crystal structure of the T2 fragment in complex with troponins I and C shows that residue 210 forms part of a short \(\alpha\)-helix N-terminal to the separate helix involved in a coiled coil interaction with troponin I that has no direct association with any other troponin subunit. The change in maximum sliding speed and ATPase is compatible with experiments that have shown that one function of troponin T is to determine the cross-bridge turnover rate (12, 26, 33); the remarkable uncoupling of cooperative activation could be related to the role of troponin T in determining the size of the thin filament cooperative unit through its interaction with tropomyosin (34, 35). The recent report of a DCM mutation within the N-terminal T1 domain of troponin T, which only binds to tropomyosin (36), and two mutations in tropomyosin itself (8) also suggest that alterations in tropomyosin-troponin T interactions may be responsible for the appearance of the DCM phenotype (reviewed in Ref. 16).

The decreased Ca\(^{2+}\) sensitivity observed with 50:50 wild type/\(\Delta\)Lys-210 mixtures was surprising given the significant increase in Ca\(^{2+}\) sensitivity obtained in experiments using 100% DCM mutant troponin T. This emphasizes our previous findings that the regulatory properties of a 50:50 wild type/mutant troponin mixture are often quite different from both wild type and mutant troponin in a way that could not be predicted from the functional properties of the mutant troponin alone (24, 25, 29). The laboratory of Morimoto has recently reported a similar decreased Ca\(^{2+}\) sensitivity in rabbit heart trabeculae in which endogenous troponin was displaced upon incubation with human troponin T (either wild type or mutant) and human complex reconstituted in situ by the addition of human troponins I and C (37). It appears that these treated trabeculae contained about 50% human mutant troponin T and 50% endogenous rabbit troponin T. Decreased Ca\(^{2+}\) sensitivity together with the decreased cross-bridge turnover rate and reduced cooperativity suggest that the expression and incorporation of this DCM troponin T mutant in vivo may result in myocardial failures that are markedly less responsive to Ca\(^{2+}\), contract more slowly, and thus are unable to produce sufficient force during activation.

Interestingly, the changes in contractility predicted to be caused by the DCM troponin T mutant closely resemble the alterations in myofilament regulation identified in end stage failing human hearts. Investigations using tissue from hearts affected by a number of aetiologies (including non-familial DCM) have shown a consistent pattern of reduced unloaded shortening speed and reduced myofibrillar ATPase activity (38–40). Reports are less clear over possible changes in Ca\(^{2+}\) sensitivity that has been suggested to be increased or unaltered; however, recent work suggests that additional factors such as sarcomere length and phosphate concentration are involved in determining which way the Ca\(^{2+}\) sensitivity will change (41–43). The reduced unloaded shortening speed correlates with the reduced ATPase and sliding speed caused by the \(\Delta\)Lys-210 mutation in troponin T. The effects of the \(\Delta\)Lys-210 mutation also correlate with the altered properties of troponin extracted from failing hearts. Failing heart troponin was found to confer a slower maximum sliding speed and a higher Ca\(^{2+}\) sensitivity with reduced cooperativity (22, 23). Thus it is possible that a reduced maximum rate of cross-bridge turnover is a common causative feature of end stage heart failure, because a defect of this kind could not be fully compensated for by increasing Ca\(^{2+}\) sensitivity or modifying Ca\(^{2+}\) handling (44). It remains unclear whether these contractility changes in end stage failing hearts mediate the progress of heart failure or whether they are compensatory epiphenomena. However, the data presented here that show a troponin T mutant that is responsible for inherited DCM causing similar changes in contractility supports the notion that alterations to troponin may contribute directly to the progression of acquired forms of DCM.

The functional alterations caused by the \(\Delta\)Lys-210 troponin T mutation are in many respects opposite from the data presented here for the R92Q HCM mutant and from our analyses of other HCM troponin T mutants (Ref. 24). Furthermore, this combination of properties is distinct from any other reported in vitro biochemical study on HCM troponin T mutants (21, 45, 46), suggesting that the reduced maximal activation, depressed ATPase, and reduced Ca\(^{2+}\) sensitivity or modifying Ca\(^{2+}\) handling (44). It remains unclear whether these contractility changes in end stage failing hearts mediate the progress of heart failure or whether they are compensatory epiphenomena. However, the data presented here that show a troponin T mutant that is responsible for inherited DCM causing similar changes in contractility supports the notion that alterations to troponin may contribute directly to the progression of acquired forms of DCM.

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