Dilated and Hypertrophic Cardiomyopathy Mutations in Troponin and α-Tropomyosin Have Opposing Effects on the Calcium Affinity of Cardiac Thin Filaments

Paul Robinson, Peter J. Griffiths, Hugh Watkins, Charles S. Redwood

Abstract—Dilated cardiomyopathy and hypertrophic cardiomyopathy (HCM) can be caused by mutations in thin filament regulatory proteins of the contractile apparatus. In vitro functional assays show that, in general, the presence of dilated cardiomyopathy mutations decreases the Ca$^{2+}$ sensitivity of contractility, whereas HCM mutations increase it. To assess whether this functional phenotypes was a direct result of altered Ca$^{2+}$ affinity or was caused by altered troponin–tropomyosin switching, we assessed Ca$^{2+}$ binding of the regulatory site of cardiac troponin C in wild-type or mutant troponin complex and thin filaments using a fluorescent probe (2-[4′-iodoacetamido]aniline]-naphthalene-6-sulfonate) attached to Cys35 of cardiac troponin C. The Ca$^{2+}$-binding affinity ($pCa_{50} = 6.57 ± 0.03$) of reconstituted troponin complex was unaffected by all of the HCM and dilated cardiomyopathy troponin mutants tested, with the exception of the troponin I Arg145Gly HCM mutation, which caused an increase ($ΔpCa_{50} = +0.31 ± 0.05$). However, when incorporated into regulated thin filaments, all but 1 of the 10 troponin and α-tropomyosin mutants altered Ca$^{2+}$-binding affinity. Both HCM mutations increased Ca$^{2+}$ affinity ($ΔpCa_{50} = +0.41 ± 0.02$ and $+0.51 ± 0.01$), whereas the dilated cardiomyopathy mutations decreased affinity ($ΔpCa_{50} = −0.12 ± 0.04$ to $−0.54 ± 0.04$), which correlates with our previous functional in vivo assays. The exception was the troponin T Asp270Asn mutant, which caused a significant decrease in cooperativity. Because troponin is the major Ca$^{2+}$ buffer in the cardiomyocyte sarcoplasm, we suggest that Ca$^{2+}$ affinity changes caused by cardiomyopathy mutant proteins may directly affect the Ca$^{2+}$ transient and hence Ca$^{2+}$-sensitive disease state remodeling pathways in vivo. This represents a novel mechanism for this class of mutation. (Circ Res. 2007;101:1266-1273.)

Key Words: cardiomyopathy ■ calcium ■ troponin ■ signaling

The diseases hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM) can both be caused by autosomal dominant inheritance of mutations in genes encoding components of the cardiac sarcomere. HCM, a disease characterized by thickening of the left ventricle and septum in the absence of other cardiac or systemic disease, is caused by mutations to at least 9 genes encoding cardiac contractile proteins.1 The most commonly reported mutations occur in the thick filament proteins β-myosin heavy chain and cardiac myosin-binding protein-C.2 However, HCM mutations have also been identified in the genes encoding the thin filament regulatory proteins α-tropomyosin,3,4 cardiac troponin (cTnT),3,4 and cTnI,5 and to date, at least 62 HCM mutations in these genes have been identified (DNA Mutation Database: Familial Hypertrophic Cardiomyopathy; http://www.angis.org.au/Databases/Heart). More recently, mutations in thin filament regulatory protein genes have also been shown to cause the phenotypically distinct cardiac disorder, DCM. DCM is characterized by increased left ventricular chamber volume and decreased systolic function in the absence of underlying coronary artery disease. Approximately 20 loci for DCM have been characterized, with disease genes encoding cytoskeletal and costameric proteins as well as cardiac contractile proteins.7 Among the latter, mutations in cTnI,8–10 α-tropomyosin,11 and cTnC10 have been described.

In cardiac muscle, troponin and tropomyosin form the principal mechanism by which contractility is regulated in response to the Ca$^{2+}$ concentration surrounding the contractile apparatus.12 In systole, Ca$^{2+}$ binds to the single low-affinity, regulatory site of cTnC (site II); this brings about conformational changes within troponin that result in both the inhibitory effect of cTnI being removed and a change in the position of the tropomyosin, allowing productive myosin head interaction with actins not directly in contact with troponin and hence cooperative activation of the thin filament. Kinetic measurements have suggested that this mechanism involves 3 states: blocked (in the absence of Ca$^{2+}$, tropomyosin prevents productive myosin head binding),
closed (tropomyosin moves in response to Ca$^{2+}$ binding to allow partial myosin attachment), and myosin induced (in which myosin head binding has shifted tropomyosin to expose fully the interaction sites on actin).13

Functional studies of HCM troponin and α-tropomyosin mutants have, in general, shown that the Ca$^{2+}$ sensitivity of contractile regulation is increased compared with wild type.14 In strong contrast, our studies of DCM mutations in thin filament regulatory proteins using 5 cTnT mutants, 2 α-tropomyosin mutants, and a single cTnC mutant have shown that these cause the opposite effect, that is, a decrease in Ca$^{2+}$ sensitivity, suggesting a fundamentally different effect at the level of the sarcomere.15,16 The mechanism by which these Ca$^{2+}$ sensitivity changes are generated and whether in every case the measured ΔpCa$^{0}$ relates to the same change in the affinity of the regulatory Ca$^{2+}$ site is unclear. Resolving this question is important because modeling, in which it was assumed that the measured in vitro functional changes correlate with actual Ca$^{2+}$ affinity changes, has suggested that the peak and duration of the Ca$^{2+}$ transient will be significantly affected.17 Some cardiomyopathy mutations in α-tropomyosin lie in N- and C-terminal regions that directly interact with troponin (for example, Glu180Gly), whereas other affected residues do not (for example, Ala63Val); similarly some cTnT mutations are found in the N-terminal T1 region, which binds only to tropomyosin, and others lie in the T2 part, which forms the troponin “core domain” by interaction with both cTnC and cTnI. It also remains possible that some of the observed ΔpCa$^{0}$ values may result from changes in the equilibria governing the blocked$\rightarrow$ closed$\rightarrow$ myosin-induced transitions described by the 3-state steric blocking model.13 The extant data are restricted to HCM mutations, with increases in thin filament Ca$^{2+}$ affinity reported for a single cTnT mutant18 and 4 cTnI mutants.19

To investigate the mechanism by which the Ca$^{2+}$ sensitivity of regulation is altered by cardiomyopathy mutations, we measured the Ca$^{2+}$ binding of the regulatory site of cTnC in whole troponin and in reconstituted thin filaments. We performed this by using the change in spectrofluorescent intensity conferred by a 2-[4'-(iodoacetamido)aniline]-naphthalene-6-sulfonate (IAANS) label bound to Cys35 of recombinant human cTnC to give a direct indication of the state of its regulatory Ca$^{2+}$-binding site. We used this reagent to measure the effect of HCM and DCM troponin mutations on Ca$^{2+}$ affinity of the troponin complex and the Ca$^{2+}$ affinity of thin filaments reconstituted with α-tropomyosin and troponin mutants. We show that the alterations in Ca$^{2+}$ sensitivity observed in the functional studies closely correlate with measured changes in thin filament Ca$^{2+}$ affinity and suggest that these changes in myocyte Ca$^{2+}$ buffering may contribute to disease pathogenesis.

**Materials and Methods**

**Proteins**

To produce cTnC with a single cysteine residue (Cys35), the Cys84Ser mutation was introduced into existing wild-type and Gly159Asp cTnC pMW172 expression constructs using a 2-step PCR mutagenesis protocol. Recombinant human wild-type and mutant cardiac troponin subunits and Ala-Ser α-tropomyosin were overexpressed in BL21 (DE3) pLysS *Escherichia coli* and subsequently purified according to our established protocols.20,21 Actin was extracted from rabbit skeletal muscle as previously described.22

**Labeling**

A 5-fold molar excess of IAANS was added to Cys84Ser cTnC (2 mg/mL) in 6 mol/L urea, 150 mmol/L KCl, 50 mmol/L Tris HCl (pH7.5), and 0.2 mmol/L DTT and incubated in the dark at 25°C for 5 hours. The reaction was quenched with 10 mmol/L L-cysteine, and free label was removed by extensive dialysis. The amount of label incorporated was determined by the absorbance at 325 nm using an extinction coefficient of 24 900 adjusting for the absorbance of unlabeled cTnC.

**Troponin and Thin Filament Reconstitution**

Wild-type and mutant troponin complexes were reconstituted from individual subunits using stepwise dialysis and gel filtration as previously described.16 Thin filaments were reconstituted at an actin, tropomyosin, and troponin ratio of 7:1:1 respectively. Levels of free troponin were ascertained by centrifugation of thin filaments at 325 000g for 10 minutes and analysis of the total, supernatant, and pellet fractions on 12.5% sodium dodecyl sulfate polyacrylamide gels.

**Fluorescence Measurements**

For measurement of Ca$^{2+}$-dependent fluorescence of IAANS-troponin in the absence of actin-tropomyosin, 3 μmol/L troponin was used; for analysis of thin filament Ca$^{2+}$ affinity, thin filaments were reconstituted with 21 μmol/L actin, 3 μmol/L Ala-Ser α-tropomyosin, and 3 μmol/L labeled troponin. The final buffer concentration of EGTA was 1 mmol/L, and the free Ca$^{2+}$ concentration was set using the appropriate concentration of CaCl$_2$, as calculated by WINMAXC version 2.0. Steady-state fluorescence measurements (excitation 325 nm, emission 455 nm) were made using a RF-1501 spectrofluorometer (Shimadzu). The change in fluorescence was monitored as the Ca$^{2+}$ was titrated with final ΔF values adjusted for the difference in assay mix volume following each incremental addition of 10 mmol/L CaCl$_2$. The adjusted and normalized ΔF was plotted as a function of Ca$^{2+}$ concentration and the resultant curves fitted to the Hill equation.

**Statistical Analysis**

The data are expressed as the average of n experiments±SEM (SE). Statistically significant differences were determined using an unpaired Students t test (InStat, GraphPad Software), with significance values defined as *P*<0.05.

**Results**

**Use of IAANS-Cys35-cTnC to Measure the Ca$^{2+}$ Affinity of Human Cardiac Troponin and Thin Filament**

To measure Ca$^{2+}$ binding at the regulatory site of cTnC in whole troponin and in reconstituted thin filaments, we modified the approach described by Putkey et al for chicken cTnC.23 We expressed and purified human cTnC with the cysteine at residue 84 mutated to serine and then modified the remaining cysteine at position 35 with IAANS (IAANS-Cys35-cTnC). As measured using our standard actin-activated myosin subfragment-1 ATPase assay,24 the Cys84Ser mutation did not affect Ca$^{2+}$ regulation (wild type: pCa$^{0}$=6.38±0.01, n=5; Cys84Ser: pCa$^{0}$=6.39±0.03, n=5; *P*=NS), and the introduction of the IAANS group had only a small effect on troponin function (wild type: pCa$^{0}$=6.38±0.01, n=5; IAANS-Cys35, pCa$^{0}$=6.55±0.02, n=5; *P*<0.05). The fluorescence emission spectrum of
IAANScys35-cTnC on excitation at 325 nm is relatively insensitive to Ca$^{2+}$ (Figure 1). When IAANScys35-cTnC is reconstituted as whole troponin with recombinant human cTnI and cTnT, the fluorescence emission decreases $\approx 58\%$ from EGTA to high Ca$^{2+}$ solution, and similarly, when IAANScys35-cTnC is part of a fully reconstituted thin filament with human recombinant Ala-Ser $\alpha$-tropomyosin and native rabbit skeletal actin, the fluorescence decreases by 43\% (Figure 1).

It was found that titration of the fluorescence change as a function of Ca$^{2+}$ concentration for wild-type troponin complex produced a $p_{Ca_{50}}$ of 6.57 $\pm$ 0.03 (Figure 2A), equivalent to a $K_d$ of $2.8 \times 10^{-7}$ mol/L and with a Hill coefficient, $n_H$ (a measure of cooperativity), of 1.12 $\pm$ 0.13. This is consistent with values reported previously and demonstrates that the probe detects Ca$^{2+}$ binding exclusively at the regulatory site II and not the higher affinity, structural sites III and IV. We found that the affinity was decreased ($p_{Ca_{50}}$ = 6.72 $\pm$ 0.02), whereas the cooperativity was increased ($n_H$ of 1.78 $\pm$ 0.09), when measuring the Ca$^{2+}$ binding to fully reconstituted thin filaments compared with isolated troponin (Figure 2B). These data are in excellent agreement with previously published thin filament binding affinities$^{19,25}$ and show that the IAANScys35-cTnC molecules on the reconstituted thin filament are binding Ca$^{2+}$ in a cooperative fashion.

Ulacentrifugation of our assembled thin filament preparations and densitometry analysis of SDS-PAGE gels of the supernatant and pellet fractions showed that 98.4\% of the troponin was actin bound under our assay conditions (Figure 2B, inset). Therefore we can be confident that the decrease in fluorescent emission is reporting cooperative Ca$^{2+}$ binding to the regulated thin filament and that free troponin is not interfering with our assay measurements.

There have been 2 other attempts to measure Ca$^{2+}$ affinity of reconstituted thin filaments using the IAANS fluorescent probe at Cys84$^{19}$ and Thr53. $^{26}$ Ca$^{2+}$ affinity values for both isolated troponin and reconstituted thin filaments from both studies are in excellent agreement with our data. We performed preliminary studies to compare IAANS labeling of cTnC at Cys35, Cys84, and Cys35/Cys84 and also dansylaziridine-labeled cTnC; in these experiments, only IAANScys35-cTnC gave a suitably large Ca$^{2+}$-dependent fluorescence change that was both sensitive to binding at the regulatory site II of TnC and insensitive to binding at the structural sites III and IV (data not shown).
Figure 3. Representative data for the changes in Ca$^{2+}$ affinity caused by HCM mutations in troponin complex (A) and thin filaments (B). The change in relative fluorescence emission intensity is plotted as a function of Ca$^{2+}$ concentration. Troponin complexes were either wild type (closed circles) or reconstituted with cTnT Arg92Gln (closed triangles) or cTnI Arg145Gly (closed squares). The points represent means ± SEM (n=3), and the curves are fits to the Hill equation.

Measurement of the Ca$^{2+}$ Affinity of Troponin Reconstituted With HCM Troponin Mutants

We analyzed the Ca$^{2+}$-binding properties of reconstituted troponin complex containing HCM mutations in cTnT (Arg92Gln) and cTnI (Arg145Gly). These mutations have both been previously characterized in in vitro functional studies in our laboratory.\textsuperscript{16,20} The Arg92Gly mutation in cTnT was chosen as a typical HCM mutation in this gene for comparison with DCM TnT mutations; the mutated residue lies within the extended N-terminal T1 region of cTnT and is not predicted to be in direct contact with cTnC or cTnI. The cTnI Arg145Gly mutation alters a residue within the inhibitory region and is in close proximity to site II of troponin C.\textsuperscript{27}

Figure 3A shows that the Arg92Gln mutation has no significant effect on the Ca$^{2+}$ affinity of the regulatory binding site with a pCa$_{50}=6.63 \pm 0.04$ whereas, the cTnI Arg145Gly mutation significantly increases to pCa$_{50}=6.99 \pm 0.05$.

Measurement of the Ca$^{2+}$ Affinity of Thin Filaments Reconstituted With HCM Mutants of Troponin

The cTnI Arg145Gly and cTnT Arg92Gln HCM mutants were reconstituted in the regulatory thin filaments and the IAANS fluorescence titrated with Ca$^{2+}$. Thin filaments containing either cardiac troponin I Arg145Gly (pCa$=7.23 \pm 0.03$ $P<0.001$) or cTnI Arg92Gln (pCa$=7.13 \pm 0.04$ $P<0.001$) were found to increase significantly Ca$^{2+}$ affinity compared with wild-type thin filaments (pCa$_{50}=6.72 \pm 0.02$) (Figure 3B). Thin filaments were also reconstituted with 50% wild-type/50% mutant mixtures of protein to more accurately reflect the expected in vivo ratios; under these conditions, both Arg145Gly (pCa$=7.01 \pm 0.03$ $P<0.001$) and Arg92Gln (7.18±0.05 $P<0.001$) mutants still had significantly enhanced Ca$^{2+}$ affinities.

Measurement of the Ca$^{2+}$ Affinity of Troponin Reconstituted With DCM Troponin Mutants

We also reconstituted troponin complex using 5 cTnT mutants (Arg131Trp, Arg141Trp, Arg205Leu, ΔLys210, and Asp270Asn) and 1 cTnC mutant (Gly159Asp) that have been shown to cause DCM. These proteins have previously been characterized in in vitro functional assays.\textsuperscript{15,16} It was seen that, as with the cTnT Arg92Gln HCM mutant, all of the DCM mutants in cTnT and cTnC had no effect on the Ca$^{2+}$ affinity of isolated troponin, as illustrated by the representative binding curves for cTnT Arg131Trp, cTnT Asp270Asn, and cTnC Gly159Asp (Figure 4A). These data indicate that all the disease-causing mutants studied with the exception of cTnI Arg145Gly have no direct affect on the structure of the regulatory Ca$^{2+}$-binding site on cTnC.

Measurement of the Ca$^{2+}$ Affinity of Thin Filaments Reconstituted With DCM Mutants of Troponin and α-Tropomyosin

The six DCM cTnT and cTnC mutants were reconstituted into thin filaments to assess their effect on cooperative Ca$^{2+}$ binding in comparison to wild-type filaments. Representative Ca$^{2+}$-binding curves for cTnT Arg131Trp, cTnT Asp270Asn, and cTnC Gly159Asp are shown in Figure 4B. All DCM mutations studied were also reconstituted as 50% wild-type/50% mutant mixtures in thin filaments (all pCa$_{50}$ data summarized in Figure 5A). In general, it was found that the DCM troponin mutations lowered the Ca$^{2+}$ affinity of reconstituted thin filaments. An exception to this was the cTnT Asp270Asn mutant, which showed unaltered pCa$_{50}$ but significantly reduced cooperativity of Ca$^{2+}$ binding ($\Delta n_I=0.70 \pm 0.02$), a property unique in this study (Figure 4B). The cTnT ΔLys210 mutant displayed enhanced Ca$^{2+}$ affinity when present in thin filaments as the sole cTnT species but reduced Ca$^{2+}$ binding when present at the more physiologically relevant
1:1 ratio with wild-type cTnT (Figure 5A); this mirrored the behavior of this mutant in the regulation of ATPase activity (Figure 5B).

In addition to the troponin mutants, the effect of 2 previously reported DCM mutations in $\gamma$/H925 -tropomyosin, Glu40Lys, and Glu54Lys were also tested to investigate the complete cohort of DCM mutations studied in Mirza et al. The Glu40Lys mutant affected thin filament Ca$^{2+}$/H1100 affinity in a similar manner to the typical DCM troponin mutants (Figure 5A). The Glu54Lys mutant, however, caused an

Figure 4. Representative data for the changes in Ca$^{2+}$/H1100 affinity caused by DCM mutations in troponin complex (A) and thin filaments (B). The change in relative fluorescence emission intensity is plotted as a function of Ca$^{2+}$/H1100 concentration. Troponin complexes were either wild type (closed circles) or reconstituted with cTnT Arg131Trp (open triangles), cTnT Asp270Asn (open squares), or cTnC Gly159Asp (half-filled squares). The points represent means ± SEM (n=3), and the curves are fits to the Hill equation.

Figure 5. The effects of HCM or DCM mutations in thin filament regulatory proteins on thin filament Ca$^{2+}$/H1100 affinity compared with previously reported changes in the Ca$^{2+}$/H1100 sensitivity of thin filament–activated myosin ATPase. The mean differences in pCa$_{50}$ of Ca$^{2+}$/H1100 binding between wild-type and mutant troponin or tropomyosin measured in 3 paired experiments are plotted as a histogram (A). Differences in pCa$_{50}$ of ATPase regulation are shown from paired experiments performed in our laboratory and previously published reports (B). Solid bars indicate 100% mutant protein; open bars indicate equimolar mutant:wild-type mixes. Error bars show SEM; statistical significance is denoted as: ***P<0.001; **P<0.01; *P<0.05; NS; P>0.05.
increase in Ca\(^{2+}\) affinity (although no significant change from wild type in 1:1 wild-type/mutant filaments).

Discussion
The data described here are the first to report the direct effects of DCM-causing mutations in troponin and tropomyosin on thin filament Ca\(^{2+}\) affinity and to compare these changes with those produced by HCM mutations. These data illustrate that, in general, DCM and HCM mutations in thin filament regulatory proteins have opposite effects on the Ca\(^{2+}\) affinity of reconstituted thin filaments and that these changes (with the exception of the Arg145Gly cTnI HCM mutant) are mediated without altering the Ca\(^{2+}\) affinity of the isolated troponin. Furthermore, the direction of these changes are shown in nearly every case to match the shifts in Ca\(^{2+}\) sensitivity reported in functional assays (Figure 5).

The HCM-causing mutations cTnT Arg92Gln and cTnI Arg145Gly both cause increased thin filament Ca\(^{2+}\) affinity and the cTnT mutant, but not cTnT Arg92Gln, also enhanced the Ca\(^{2+}\) binding of troponin complex. Both have been shown previously to cause an increase in Ca\(^{2+}\) sensitivity of regulation of in vitro thin filament–activated myosin ATPase and motility of regulated filaments as well as isometric force development of skinned myofibers.16,20,28 Arg92 of cTnT is present in the extended T1 N-terminal tail and hence not predicted to interact directly with the other 2 troponin subunits. The mutation, however, could indirectly affect the Ca\(^{2+}\) affinity of the thin filament via altering cooperative conformational communication by disrupting interactions between the troponin T1 and tropomyosin. Arg145 is located in the inhibitory region of cTnI, and structural studies have suggested that it lies close to site II of cTnC.29 Previous binding studies performed using skeletal TnI peptides suggest that arginine 112 (equivalent to cardiac residue 145) is an important interactor with TnC and that if glycine is substituted at this residue, the binding interaction is decreased.30 It thus appears that the decreased binding of cTnC to cTnI results in increased Ca\(^{2+}\) affinity. Increases in thin filament Ca\(^{2+}\) affinity have been previously reported for a single cTnT HCM mutant18 and 4 cTnI mutants.19 The latter study analyzed the Arg145Gly mutant and failed to detect a change in Ca\(^{2+}\) affinity of the isolated troponin complex, in contrast with our result with this mutant (Figure 3A); this may be attributable to the use of mouse recombinant troponin subunits rather than the human proteins used in our study.

In general, DCM mutations decreased thin filament Ca\(^{2+}\) affinity, in direct contrast to the increases caused by the 2 HCM mutations studied. Similar to the cTnT Arg92Gln HCM mutation, the DCM mutations did not affect the Ca\(^{2+}\) affinity of isolated troponin and only altered Ca\(^{2+}\) binding in fully reconstituted thin filaments. An exception to this trend was Asp270Asn cTnT; this mutant showed reduced cooperativity of Ca\(^{2+}\) binding but with no change in affinity (Figure 4B).

The Ca\(^{2+}\)-saturated crystal structure of the core domain of cardiac troponin shows that Asp270 forms hydrogen bonds with both Tyr111 and Arg147 of cTnC, these residues being located in the Mg\(^{2+}\) saturated structural Ca\(^{2+}\)-binding sites III and IV, respectively, of cTnC.27 The aspartate-to-asparagine mutation removes the ability of the residue to form 1 of these hydrogen bonds, and this predicted loss of communication between cTnC and cTnT may underlie the unique effects of the mutation on Ca\(^{2+}\) binding. The remaining DCM mutations in cTnT are clustered in 2 distinct regions. The Arg131 and Arg141 mutations lie within or adjacent to the TnT1-TM overlap region,31 and the missense mutation to a tryptophan residue may disrupt propagation of thin filament activation on Ca\(^{2+}\) binding, thus reducing the affinity of Ca\(^{2+}\) binding to subsequent troponins along the filament. The Arg205Leu and ΔLys210 DCM mutations are both found on an α-helix adjacent to the IT arm at the N terminus of the core domain residues of TnT.27 Apart from suggestions made from a solution NMR study before the publication of the crystal structure (which predicts that these residues may interact with TnC\(^{2+}\)), it is not thought that either of the mutated residues are involved in subunit–subunit interactions. The experiments detailed here suggest an indirect cooperative effect of these 2 mutations on Ca\(^{2+}\) affinity, thereby suggesting a stereospatial effect of the mutations on the conformational changes that take place in troponin as the thin filament activates. A further layer of complexity has recently been revealed in that the Gly159Asp cTnC DCM mutation has been shown to blunt the effects of troponin phosphorylation.33,34 Because phosphorylation of troponin directly modulates Ca\(^{2+}\) affinity,35 alteration of troponin phosphorylation status may provide another mechanism by which cardiomyopathy mutations alter thin filament Ca\(^{2+}\) binding.

Because the cardiomyopathy-causing mutations act in an autosomal dominant manner, we compared the Ca\(^{2+}\) affinity of wild-type thin filaments with thin filaments composed of mutant protein and also of 50% mutant/50% wild-type mixture to more accurately reflect the predicted in vivo protein ratios. Our previous functional studies of filaments containing wild-type/mutant mixtures have revealed some unexpected results not necessarily predictable from the difference between wild type and mutant alone.15,16,20,24 Most marked of these is the ΔLys210 cTnT DCM mutant, which surprisingly causes an increase in the Ca\(^{2+}\) sensitivity of myosin ATPase regulation but, when present in equimolar ratio with wild-type cTnT, produces a reduction in Ca\(^{2+}\) sensitivity (and is thus similar to other DCM mutants).16 This pattern was repeated with our analysis of thin filament Ca\(^{2+}\) affinity (Figure 5) and underscores the strong qualitative correlation between measured Ca\(^{2+}\) affinity and in vitro functional regulation. This anomalous behavior is not readily explainable but is likely to be caused by unusual propagation of the Ca\(^{2+}\) signal in mosaic filaments composed of troponin complexes with different properties (see also discussion by Robinson et al16).

Thin filaments reconstituted using the α-TM Glu54Lys DCM mutant showed a marked increase in Ca\(^{2+}\) affinity compared with wild type, although no significant change was detected in filaments containing an equimolar mixture of wild type and mutant. This mutant has also exhibited anomalous behavior in a number of structural and functional analyses we have previously performed.15,36 Glu54 occupies the same position on the heptad repeat sequence as Glu40, but it is in a different region in the 19 amino acid pseudorepeat sequence thought to mediate the rolling of troponymosin on the thin filament.
filament in response to Ca\(^{2+}\).\(^{37}\) This difference in position relative to the underlying actin monomer could account for the unique behavior of the Glu54Lys mutant in our studies.

It would be intriguing to examine the effects of the mutants on Ca\(^{2+}\) affinity when present in an intact myofilament lattice in which the cooperativity of Ca\(^{2+}\) binding is significantly higher and variables such as sarcomere length could be examined. We attempted to test this in porcine skinned cardiac fibers in which endogenous troponin was exchanged with IAANScys35-cTnC troponin. However, it was found that the fluorescence of the probe in this environment was essentially insensitive to Ca\(^{2+}\) (data not shown), in agreement with the notion that the properties of these fluorescent reporters are exquisitely dependent on the milieu into which they are incorporated (Figure 1).\(^{23}\)

cTnC is thought to be the predominant chelator of Ca\(^{2+}\) in the cardiomyocyte sarcoplasm,\(^{38}\) and therefore any marked change in its affinity may result in significant perturbations of intracellular Ca\(^{2+}\) change in its affinity may result in significant perturbations of intracellular Ca\(^{2+}\) in the cardiac fibers in which endogenous troponin was exchanged for IAANScys35-cTnC troponin. However, it was found that the fluorescence of the probe in this environment was essentially insensitive to Ca\(^{2+}\) (data not shown), in agreement with the notion that the properties of these fluorescent reporters are exquisitely dependent on the milieu into which they are incorporated (Figure 1).\(^{23}\)

Contractile protein mutations suggest that significant decreases in sarcomeric Ca\(^{2+}\) buffering are likely and that these may stimulate progression toward the dilated phenotype. Recent studies have suggested that changes in the duration or amplitude of the Ca\(^{2+}\) transient may stimulate apoptotic signaling that could ultimately lead to DCM. Mice overexpressing a mutant, inactive Na\(^{+/}Ca\(^{2+}\) exchanger show a marked increase in Ca\(^{2+}\) transient duration and increased occurrence of apoptosis.\(^{40}\) Also, the Arg9Cys mutation in phospholamban that causes DCM has been shown to increase inhibition of SERCA2A; this gain-of-function effect was postulated to increase cytosolic Ca\(^{2+}\) in cardiomyocytes and link directly to the macroscopic tissue remodeling in the DCM disease phenotype.\(^{41}\) Analysis of a gene-targeted ΔLys210 cTnT mouse model has shown that the amplitude of the Ca\(^{2+}\) transients was greater in mutant mice than controls and that the mutant hearts were more prone to arrhythmogenesis,\(^{32}\) again suggesting that contractile protein mutations affect Ca\(^{2+}\) transient amplitude and pattern. Our data indicate that this can result directly from altered cTnC Ca\(^{2+}\) buffering, although mechanisms to compensate for this intrinsic change to cytofilament Ca\(^{2+}\) buffering may also be significant.

It is becoming increasingly clear that intracellular Ca\(^{2+}\) imbalance and the subsequent aberrant signaling that may result from this must be considered when attempting to explain how mutations in sarcomeric proteins can cause the global cardiac remodeling observed in both DCM and HCM. The data presented in this study, as well as those discussed above, show that this may be initiated by opposing, direct effects on buffering of the Ca\(^{2+}\) transient that likely contribute to the diverging disease pathways of these contrasting cardiomyopathies.

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**References**


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20. Elliott K, Watkins H, Redwood CS. Altered regulatory properties of human cardiac troponin I mutants that cause hypertrophic cardiomyo-
21. Bottinelli R, Covello DA, Redwood CS, Pellegrino MA, Maron BJ, 
Spirito F, Watkins H, Reggiani C. A mutant tropomyosin that causes 
hypertrophic cardiomyopathy is expressed in vivo and associated with 
22. Pardee JD, Spudich JA. Purification of muscle actin.
Spirito P, Watkins H, Reggiani C. A mutant tropomyosin that causes 
hypertrophic cardiomyopathy is expressed in vivo and associated with 
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Spirito P, Watkins H, Reggiani C. A mutant tropomyosin that causes 
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