A Dilated Cardiomyopathy Troponin C Mutation Lowers Contractile Force by Reducing Strong Myosin-Actin Binding*

Received for publication, September 8, 2009, and in revised form, March 9, 2010. Published, JBC Papers in Press, April 6, 2010, DOI 10.1074/jbc.M109.064105

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In this study we explore the mechanisms by which a double mutation (E59D/D75Y) in cardiac troponin C (CTnC) associated with dilated cardiomyopathy reduces the Ca\(^{2+}\)-activated maximal tension of cardiac muscle. Studying the single mutants (i.e. E59D or D75Y) indicates that D75Y, but not E59D, causes a reduction in the calcium affinity of CTnC in tropinin complex, regulated thin filaments (RTF), and the Ca\(^{2+}\) sensitivity of contraction and ATPase in cardiac muscle preparations. However, both D75Y and E59D are required to reduce the actomyosin ATPase activity and maximal force in muscle fibers, indicating that E59D enhances the effects of D75Y. Part of the reduction in force/ATPase may be due to a defect in the interactions between CTnC and cardiac troponin T, which are known to be necessary for ATPase activation. An additional mechanism for the reduction in force/ATPase comes from measurements of the binding stoichiometry of myosin subfragment-1 (S-1) to the RTF. Using wild type RTFs, 4.8 mol S-1 was bound per mol filament (seven actins), whereas with E59D/D75Y RTFs, the number of binding sites was reduced by ~23% to 3.7. Altogether, these results suggest that the reduction in force and ATPase activation is possibly due to a thin filament conformation that promotes fewer accessible S-1-binding sites. In the absence of any family segregation data, the functional results presented herein support the concept that this is likely a dilated cardiomyopathy-causing mutation.

There are numerous findings that suggest a hallmark of mutations in the regulated thin filament (RTF) is to alter the Ca\(^{2+}\) regulation of cardiac muscle contraction (1, 2). In general, mutations in the RTF associated with dilated cardiomyopathy (DCM) tend to decrease the Ca\(^{2+}\) sensitivity of contraction, whereas mutations associated with hypertrophic and restrictive cardiomyopathies tend to increase the Ca\(^{2+}\) sensitivity. Some of these mutations, notably in cardiac troponin T (CTnT), can also alter the maximal force generating capabilities of muscle (1, 3, 4), suggesting that a subset of RTF mutants can also alter the number and/or efficiency of attached myosin heads at all Ca\(^{2+}\) concentrations. Nevertheless, it is still unclear how and why some mutants alter the maximal force generating properties of muscle, whereas others do not.

Cardiac contraction is regulated by the RTF, which contains cardiac troponin (CTn), tropomyosin (Tm), and F-actin. CTn consists of three interacting subunits: troponin C (CTnC) is the Ca\(^{2+}\)-binding subunit, tropomin I (CTnI) is the inhibitory subunit, and CTnT is the Tm-binding subunit. Cardiac contraction occurs upon Ca\(^{2+}\) binding to CTnC, which promotes CTn-Tm translocation. This allows for the interaction between actin and myosin and the subsequent generation of tension (5). CTnC contains three metal ion-binding sites: two identical C-terminal sites capable of binding Ca\(^{2+}\) and Mg\(^{2+}\) competitively (K\(_{Ca} = 1.4 \times 10^{-7} M^{-1}\), K\(_{Mg} = 1.0 \times 10^{-5} M^{-1}\)) and one N-terminal, Ca\(^{2+}\)-specific, regulatory site (K\(_{Ca} = 2.5 \times 10^{-5} M^{-1}\)) responsible for transmitting the Ca\(^{2+}\)-binding signal and switching on contraction (6).

Currently, six cardiomyopathy-linked mutations in CTnC exist that have been characterized in vitro (7–12). However, the first potential DCM mutation in CTnC was a double mutation at residues 59 and 75 (E59D/D75Y) in a patient with idiopathic DCM (13). No pedigree information is available to assess the segregation of this mutation with the disease; therefore, relevant functional studies are critical in determining the significance and molecular impact of this mutation.

In a recent report, the expression of the individual mutants in cardiomyocytes showed that the D75Y mutant was able to recapitulate the E59D/D75Y phenotype, which impaired the myofilament response to activation (12). In contrast, our studies found that the D75Y and E59D/D75Y mutants differentially affect the force generating and ATPase activation properties of skinned cardiac muscle.

Utilizing NMR techniques, one group reported that in the presence of Ca\(^{2+}\), the affinity of the N-domain of E59D/D75Y-CTnC for the CTnI switch peptide (147–163) was different in vitro compared with the WT-CTnC control (11). Currently, it is unknown how CTnI will influence the maximal ATPase activation properties of skinned cardiac muscle.
these mutations to affect the ATPase activities of skinned cardiac myofibrils (CMF) and reconstituted actomyosin (RAM).

The fluorescent probe 2-(4′-(iodoacetamido)anilino)naphtalene-6-sulfonic acid (IAANS) covalently bound to CTnC was used to correlate the effects of Ca\(^{2+}\) binding and myofilament protein interactions on the structure of CTnC (14). This enables the comparisons of apparent CTnC Ca\(^{2+}\) affinities measured by fluorescence in CTnC, CTn, and RTF with measurements of tension and ATPase activity in skinned muscle preparations because all are indirect measurements of Ca\(^{2+}\) binding to the regulatory site of CTnC. Our results indicate that the reduction in contractile force arising from the double mutant is accompanied by a decrease in CTnC Ca\(^{2+}\) affinity and a thin filament conformation that reduces the number and/or accessibility of myosin subfragment-1 (S-1)-binding sites.

**EXPERIMENTAL PROCEDURES**

**Mutation, Expression, and Purification of Proteins**—The CTnC sequence was derived from a human cardiac cDNA library and subcloned into the pET3-d vector. CTnC mutants were generated by following the Stratagene guidelines for the QuikChange site-directed mutagenesis kit. Monocysteine CTnC derivatives were engineered by mutating Cys\(^{35}\) to Ser. IAANS labeling of CTnC and subsequent purification followed previous methods and nomenclature, whereby IAANS labeled solely at Cys\(^{84}\) and both at Cys\(^{35}\) and Cys\(^{84}\) are, respectively, CTnC(C84)\(_{IA}\) and CTnC(C84)\(_{IA}\) (14). The expression and purification of human CTnC, CTnI, and CTnT followed previous methods (7). Porcine CMF, Tm, and myosin were prepared from left ventricles (7). Myosin subfragment-1 was prepared from rabbit skeletal myosin (15). F-actin was prepared from rabbit skeletal acetone powder (16).

**Multimodular Complex Formation**—Formation of fluorescent and nonfluorescent binary and ternary CTn complexes were prepared using standard methods (7, 14). CTnC(C84)\(_{IA}\)-labeled RTF (F-actin:Tm:CTn in a 7:1:1 ratio) were prepared as described previously (7).

**Determination of Apparent Ca\(^{2+}\) Affinities**—Ca\(^{2+}\) binding was measured by monitoring the peak emission intensity (448 nm) from the IAANS-labeled proteins in standard buffer as described previously (7). The data were fitted to a version of the Hill equation using SigmaPlot 11.0 software, and the Ca\(^{2+}\) affinities were reported within this manuscript as pCa\(_{50}\) values (−log[Ca\(^{2+}\)]\(_{free}\) at which 50% of the maximal response is observed). In cases of biphasic binding, the data were fit to Equation 1,

\[
Y = \frac{S_1[Ca^{2+}]^{n_1}}{(K_{d1}^{n_1} + [Ca^{2+}]^{n_1})} + \frac{S_2[Ca^{2+}]^{n_2}}{(K_{d2}^{n_2} + [Ca^{2+}]^{n_2})} \quad (\text{Eq. 1})
\]

where Y is the fluorescence intensity, \(S_1\) and \(S_2\) are the contributions of each class of site to the fluorescence, \(K_{d1}\) and \(K_{d2}\) are the dissociation constants for each class of site, and \(n_1\) and \(n_2\) are the respective Hill coefficients.

**Binding of Subfragment-1 with RTFs**—Labeled RTFs were prepared as above except that the RTF pellets were resuspended in buffer containing 150 mmol/liter KCl, 50 mmol/liter MOPS, 1.25 mmol/liter MgCl\(_2\), 0.5 mmol/liter EGTA, 1 mmol/liter nitritolactiic acid, 1 mmol/liter dithiothreitol, pH 7.0 (\(\mu = 183\)). The S-1 was dialyzed in the same buffer before use. S-1 was incrementally added to a cuvette containing 2 ml of CTnC(C84)\(_{IA}\)-labeled RTFs (50 nmol/liter) at 23 °C with constant mixing. Each addition ([S-1]\(_{final}\) = 500 nmol/liter) was followed by a 2-min pause before the solution was excited at 330 nm and the emission (445 nm) intensity was recorded. Each signal reading was averaged from 750 points recorded over 10 s. The dilution never exceeded 3.5% (at the final S-1 addition), and the averaged signals were corrected. The averaged baseline signal intensity (in the absence of S-1) was subtracted from the averaged signal intensities and fit to Equation 2, which is a solution for a quadratic equation (17),

\[
\Delta F_i/\Delta F_{max} = \left(\frac{nE_o + X_i + K_d - ((nE_o + X_i + K_d)^2 - 4nE_oX_i)^{1/2}}{2nE_o}\right) \quad (\text{Eq. 2})
\]

where \(E_o\) is the concentration of RTF, which is held constant; \(X_i\) is the concentration of S-1, which is varied; and \(\Delta F_i\) is the base line-subtracted fluorescence signal after the ith addition of S-1. \(n\) is the number of S-1-binding sites; \(K_d\) is the dissociation constant; and \(\Delta F_{max}\), the base line-subtracted maximum fluorescence signal, can all be obtained from the fit.

**Reconstituted Actomyosin ATPase Assays**—F-actin, Tm, and myosin were mixed together to prepare RAM, followed by the addition of preformed CTn or CTnC-CTnI binary complexes to assess the RAM Mg\(^{2+}\)-ATPase activity as described previously (7).

**Ca\(^{2+}\)-activated Cardiac Myofibrillar ATPase Assays**—Trans-1,2-Cyclohexane-N\(_2\),N\(_2\),N\(_2\),N\(_2\)-tetraacetic acid (CDTA) treatment of CMF, CTnC (WT and mutant) reconstitution, and subsequent determination of the Ca\(^{2+}\) sensitivity of CMF ATPase activation followed previous methods (7). The data were fitted to the Hill equation using the software of SigmaPlot 10.0.

**Skinned Fiber Preparations**—Skinned porcine papillary fibers were prepared using established protocols (7). Briefly, the skinned cardiac fibers were mounted using stainless steel clips to a force transducer and immersed in a relaxation solution (pCa 8.0) containing 10\(^{-4}\) mol/liter free [Ca\(^{2+}\)], 1.0 mmol/liter free [Mg\(^{2+}\)], 7 mmol/liter EGTA, 20 mmol/liter MOPS, 2.5 mmol/liter [Mg-ATP\(^2+\)], 15 mmol/liter creatine phosphate, 15 units/ml phosphocreatine kinase, pH 7.0, and between 33.6 and 47.9 mmol/liter KPr to achieve a constant ionic strength of 150 mmol/liter in all solutions. The fiber slack was removed until tension developed, and then the fiber was stretched 1.2× its length. This results in estimated sarcomere lengths between 2.2 and 2.3 μm. The various pCa solutions were prepared using the binding constants at 20 °C (18). The contraction solution (pCa 4.0) was the same composition as the pCa 8.0 solution, except that the free Ca\(^{2+}\) concentration was 10\(^{-4}\) mol/liter and was used to measure the initial force. Endogenous CTnC was extracted with 5 mmol/liter CDTA (pH 8.4) until the fibers developed −20% (1.5–2.0 h) of the initial tension (residual tension) in pCa 4.0 before reconstitution with CTnC (WT or mutant). The Ca\(^{2+}\) dependence of tension was tested in solutions containing intermediate concentrations of Ca\(^{2+}\). The data were analyzed as above.
Functional Effects of CTnC Mutations Associated with DCM

**Results**

**Ca\(^{2+}\) Sensitivity of Tension in Skinned Cardiac Fibers**—Skinned cardiac papillary muscle had its endogenous CTnC extracted by treatment with CDTA and then reconstituted back with recombiant CTnCs (WT or mutant). Fig. 1 (A and B) illustrates the effects of mutant CTnC reconstitution on the Ca\(^{2+}\) dependence of force development. Incorporation of E95D/D75Y-CTnC into CTnC-depleted fibers significantly decreased the Ca\(^{2+}\) sensitivity of tension 0.1 pCa units when compared with the WT control (Table 1). Additionally, Fig. 1C shows that the reconstitution of E95D/D75Y-CTnC into fibers only restored 55% of the “initial” (before CDTA treatment) maximal tension in comparison with the 75% that is restored by the WT-CTnC. After CDTA treatment, the residual tension in maximal tension observed in fibers reconstituted with WT and E95D/D75Y-CTnC was 46.78 ± 5.77 KN/m\(^2\) and normalized to represent 100% maximal tension.

**TABLE 1**

Summary of pCa-tension relationships of cardiac fibers reconstituted with mutant CTnCs

<table>
<thead>
<tr>
<th>Reconstituted fiber</th>
<th>pCa(_{50})</th>
<th>ΔpCa(_{50})</th>
<th>n(_{st})</th>
<th>Residual tension</th>
<th>Restored tension</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>5.69 ± 0.05</td>
<td>4.07 ± 0.68</td>
<td>21.4 ± 3.5</td>
<td>74.9 ± 8.2</td>
<td></td>
</tr>
<tr>
<td>E95D/D75Y</td>
<td>5.59 ± 0.05</td>
<td>3.73 ± 0.18</td>
<td>18.4 ± 5.3</td>
<td>55.3 ± 6.3</td>
<td></td>
</tr>
<tr>
<td>E95D</td>
<td>5.71 ± 0.02</td>
<td>3.44 ± 0.26</td>
<td>19.4 ± 6.6</td>
<td>66.0 ± 10.3</td>
<td></td>
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<tr>
<td>D75Y</td>
<td>5.61 ± 0.06</td>
<td>3.71 ± 0.77</td>
<td>18.5 ± 3.4</td>
<td>67.5 ± 7.3</td>
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*p < 0.001 versus WT.

*p < 0.05 versus WT.

Muscle Fiber CTnC Quantification—Five muscle fibers resulting in ~20% residual force after CDTA treatment had their proteins separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Anti-human cardiac tropinin C, clone 7b9 (Research Diagnostics, Inc., Concord, MA), and anti-human myosin light chain I (essential light chain) (Accurate Chemical, Westbury, NY) were diluted 1:500 and 1:40,000, respectively, for primary detection. Secondary detection for quantitative Western blots was performed with goat anti-mouse Alexa-680 (1:4000) from Rockland Immunologicals (Gilbertsville, PA). Visualization and quantification of gel bands was performed with the Odyssey infrared imaging system (LI-Cor Biosystems, Homburg, Germany). Normalization of gel bands was accomplished by ratiometric analysis using essential light chain as a loading control. The changes in CTnC content from the CDTA-treated fiber were estimated by comparing the ratios of CTnC/essential light chain between the experimental and control fibers.

Preparation of Buffered Calcium Solutions—Methods for solving the free and bound metal ion equilibria in our solutions were provided for by the computer program pCa Calculator (18).

Statistical Analysis—The data are expressed as the averages of four to six experiments ± S.D. (unless otherwise stated). Significant differences were determined using a one-way analysis of variance (SigmaPlot version 11.0), with significance defined as p < 0.05.

**RESULTS**

**Ca\(^{2+}\) Sensitivity of Tension in Skinned Cardiac Fibers**—Skinned cardiac papillary muscle had its endogenous CTnC extracted by treatment with CDTA and then reconstituted back with recombiant CTnCs (WT or mutant). Fig. 1 (A and B) illustrates the effects of mutant CTnC reconstitution on the Ca\(^{2+}\) dependence of force development. Incorporation of E95D/D75Y-CTnC into CTnC-depleted fibers significantly decreased the Ca\(^{2+}\) sensitivity of tension 0.1 pCa units when compared with the WT control (Table 1). Additionally, Fig. 1C shows that the reconstitution of E95D/D75Y-CTnC into fibers only restored 55% of the “initial” (before CDTA treatment) maximal tension in comparison with the 75% that is restored by the WT-CTnC. After CDTA treatment, the residual tension in maximal tension observed in fibers reconstituted with WT and E95D/D75Y-CTnC was 46.78 ± 5.77 KN/m\(^2\) and normalized to represent 100% maximal tension.

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When E59D or D75Y-CTnC is reconstituted into the CDTA-treated fibers, the maximal restored tensions are not statistically different from the WT control (Fig. 1C). However, in Fig. 1B, only D75Y-CTnC has the ability to significantly decrease the Ca$^{2+}$/H11001$^{+}$ sensitivity of tension ($p_{Ca50}$). The $p_{Ca50}$ and $n_{H}$ values of fibers reconstituted with D75Y-CTnC are statistically insignificant in comparison with the values derived from the double mutant reconstituted fibers (summarized in Table 1).

**Effect of CTnC Reconstitution on CMF and RAM ATPase Activity**—Fig. 2 (A and B) shows the Ca$^{2+}$ sensitivities of ATPase activation from CDTA-treated CMF that have been reconstituted with WT and E59D/D75Y-CTnC (A) and E59D and D75Y-CTnC (B). C, the effect of CTn prepared with mutant CTnCs on the inhibition ($\text{Ca}^{2+}$) and activation ($\text{Ca}^{2+}$) of reconstituted actomyosin Mg$^{2+}$-ATPase activity. D, the effect of mutant CTnC-CTnI binary complexes on the inhibition ($\text{Ca}^{2+}$) and neutralization ($\text{Ca}^{2+}$) of reconstituted actomyosin Mg$^{2+}$-ATPase activity. All of the CMF concentrations were 0.5 mg/ml, and the specific activities were normalized in A and B (insets) for illustrative purposes. The concentrations of F-actin, myosin, and Tm were 3.5, 0.6, and 1 μmol/liter, respectively; and the averages of three experiments are shown in D.

When E59D or D75Y-CTnC is reconstituted into the CDTA-treated fibers, the maximal restored tensions are not statistically different from the WT control (Fig. 1C). However, in Fig. 1B, only D75Y-CTnC has the ability to significantly decrease the Ca$^{2+}$/H11001$^{+}$ sensitivity of tension ($p_{Ca50}$). The $p_{Ca50}$ and $n_{H}$ values of fibers reconstituted with D75Y-CTnC are statistically insignificant in comparison with the values derived from the double mutant reconstituted fibers (summarized in Table 1).

**Effect of CTnC Reconstitution on CMF and RAM ATPase Activity**—Fig. 2 (A and B) shows the Ca$^{2+}$ sensitivities of ATPase activation from CDTA-treated CMF that have been reconstituted with WT or mutant CTnCs. The CDTA-treated myofibrils are essentially inactivated at all Ca$^{2+}$/H11001$^{+}$ concentrations (data not shown). After CTnC (WT or mutant) reconstitution, all myofibrillar ATPase activities are similarly inhibited in pCa 8.0 (Fig. 2, A and B). However, Fig. 2A shows that the maximal ATPase levels (in pCa 4.5) are not fully restored in the presence of E59D/D75-CTnC. A 3-fold ATPase activation is observed in comparison with the 4-fold activation seen in the WT-CTnC reconstituted myofibrils (Table 2). The data obtained from this figure are normalized (Fig. 2, A and B, insets) to better illustrate the changes in the apparent Ca$^{2+}$ affinity caused by the mutants (summarized in Table 2).

When E59D or D75Y-CTnC is reconstituted into the CDTA-treated CMF, the maximal restored ATPase activities are not statistically different from the WT-CTnC reconstituted CMF (Fig. 2B and Table 2). However, significant differences in the $p_{Ca50}$ of ATPase activation occur for the E59D and D75Y reconstituted CMF in opposite directions ($p_{Ca50}$ = +0.16 and −0.19, respectively). Although the $p_{Ca50}$ of ATPase activation from CMF reconstituted with D75Y and E59D/D75Y-CTnC are statistically different from each other, they both show significant reductions in their Ca$^{2+}$ sensitivities when compared with the WT control.

F-actin, tropomyosin, and myosin were mixed together to prepare reconstituted actomyosin. Then preformed CTn complexes prepared with WT or mutant CTnCs were added to
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determine their abilities to affect the inhibition and activation of RAM Mg$^{2+}$-ATPase activity. The specific ATPase activity of “unregulated” actin-Tm-myosin (i.e. in the absence of CTn) is considered 100% in our calculations (Table 2). Fig. 2C shows that RAM prepared with WT and mutant CTn are not statistically different in their abilities to inhibit (−Ca$^{2+}$) the ATPase activity at all tested CTn concentrations (i.e. from 0.0 to 2.0 μmol/liter CTn). With respect to the ability to activate (+Ca$^{2+}$) the ATPase activity, RAM prepared with E59D/D75Y-CTn is not statistically different from the unregulated ATPase levels when all CTn concentrations are tested. In the presence of 2.0 μmol/liter CTn, the specific ATPase activity of RAM prepared with E59D/D75Y-CTn increases 3.75-fold (101.2%) upon changing the [Ca$^{2+}$] from low to high, as opposed to the 6.1-fold (162.5%) activation seen in the RAM prepared with WT-CTn. Incorporation of D75Y-CTn into RAM significantly reduces the ATPase activities as well, yielding a 4.2-fold (133.1%) activation in the presence of Ca$^{2+}$. Although the ATPase activities of RAM prepared with E59D/D75Y and D75Y-CTn are statistically different from each other in the presence of Ca$^{2+}$, they both significantly reduced the ATPase activation (summarized in Table 2).

Fig. 2D shows the effects of preformed CTnC-CTn binary complexes prepared with WT or E59D/D75Y-CTnC on their abilities to affect CTn inhibition (−Ca$^{2+}$) and neutralization (+Ca$^{2+}$) of RAM Mg$^{2+}$-ATPase activity. The specific ATPase activity of unregulated actin-Tm-myosin is considered 100% in our calculations. RAM prepared with binary complexes containing WT and E59D/D75Y-CTnC are not statistically different from each other in their abilities to inhibit (−Ca$^{2+}$) the ATPase activity or neutralize (+Ca$^{2+}$) the CTnC-based inhibition.

**Determining the Apparent Ca$^{2+}$ Affinity from IAANS-labeled Proteins**—Fig. 3A represents the Ca$^{2+}$-dependent changes in fluorescence arising from WT and mutant CTnC-labeled proteins. Because the curves in Fig. 3A are biphasic, the data are fit to a modified Hill equation (see “Experimental Procedures”) to account for two classes of binding sites with the pCa$\text{Ca}_{50}$ values and Hill coefficients summarized in Table 3. As the free Ca$^{2+}$ increases from pCa 8.0 to 6.6, the WT-CTnCIA$	ext{Mg}^{2+}$ fluorescence decrease with a pCa$\text{Ca}_{50}$ of 7.12, indicating Ca$^{2+}$ binding to the Ca$^{2+}$-$\text{Mg}^{2+}$ sites. However, when Ca$^{2+}$ binds to the Ca$^{2+}$-$\text{Mg}^{2+}$ sites of E59D/D75Y-CTnCIA in the same pCa range, there are larger decreases in fluorescence with a concomitant decrease in the pCa$\text{Ca}_{50}$ (ΔpCa$\text{Ca}_{50} = -0.18$). Increasing the concentration of free Ca$^{2+}$ from pCa 6.6 to 3.8 results in an increase in fluorescence for the WT-CTnCIA protein, indicating Ca$^{2+}$ binding to the Ca$^{2+}$-specific site with a pCa$\text{Ca}_{50}$ of 4.94. On Ca$^{2+}$ binding to the Ca$^{2+}$-specific site of E59D/D75Y-CTnCIA, a marked reduction in the pCa$\text{Ca}_{50}$ is observed (ΔpCa$\text{Ca}_{50} = -0.18$).

When D75Y-CTnCIA is titrated with Ca$^{2+}$ (Fig. 3A) from pCa 8.0 to 6.4, the fluorescence decreases with a concomitant reduction in the pCa$\text{Ca}_{50}$ for the Ca$^{2+}$-$\text{Mg}^{2+}$ sites (ΔpCa$\text{Ca}_{50} = -0.15$). Increasing the concentration of free Ca$^{2+}$ from pCa 6.4 to 3.8 results in an increase in fluorescence for D75Y-CTnCIA, indicating Ca$^{2+}$ binding to the Ca$^{2+}$-specific site with a marked reduction in the pCa$\text{Ca}_{50}$ (ΔpCa$\text{Ca}_{50} = -0.24$). These results show that the Ca$^{2+}$ affinities and structural changes of D75Y and E59D/D75Y-CTnCIA, as measured by fluorescence, are not statistically different from each other in the isolated state.

Fig. 3B shows the Ca$^{2+}$-dependent changes in fluorescence from CTnC-CTn binary complexes prepared with WT, E59D/D75Y, and D75Y-CTnC(C84)IA. The WT binary complex increases its fluorescence when the free Ca$^{2+}$ rises from pCa 8.0 to 5.0 with a pCa$\text{Ca}_{50}$ of 6.24. In comparison, the Ca$^{2+}$ affinities of the E59D/D75Y and D75Y binary complexes are significantly reduced (ΔpCa$\text{Ca}_{50} = -0.65$ and −0.74, respectively). The pCa$\text{Ca}_{50}$ values of E59D/D75Y and D75Y binary complexes are statistically different from each other.

Fig. 3C shows the Ca$^{2+}$-dependent changes in fluorescence from labeled CTn (CTnIA) prepared with WT, E59D/D75Y, E59D, and D75Y-CTnCIA. In comparison with WT-CTnIA, the Ca$^{2+}$ affinities of E59D/D75Y and D75Y-CTnCIA are significantly reduced (ΔpCa$\text{Ca}_{50} = -0.9$ and −0.85, respectively). Moreover, significant changes in the Hill coefficient that accompany these large shifts in pCa$\text{Ca}_{50}$ are indicative of irregularities in the cooperative events that regulate CTnC Ca$^{2+}$ binding in the ternary state. The Ca$^{2+}$ affinity of E59D-CTnCIA is significantly decreased, but to a much lesser extent than E59D/D75Y-CTnCIA.

Fig. 3D shows the Ca$^{2+}$-dependent changes in fluorescence from labeled RTFs prepared with WT, E59D/D75Y, and E59D-

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**TABLE 2**

Summary of cardiac myofibrillar and reconstituted actomyosin ATPase activity data from Fig. 2

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>E59D/D75Y</th>
<th>E59D</th>
<th>D75Y</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cardiac myofibrils</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCa$\text{Ca}_{50}$</td>
<td>5.95 ± 0.03</td>
<td>5.63 ± 0.03*</td>
<td>6.11 ± 0.04*</td>
<td>5.76 ± 0.04ab</td>
</tr>
<tr>
<td>n$_{1}$</td>
<td>1.44 ± 0.09</td>
<td>1.32 ± 0.03*</td>
<td>1.32 ± 0.07</td>
<td>1.29 ± 0.05*</td>
</tr>
<tr>
<td>Min. activity (nmol of PO$_4$-mg$^{-1}$-min$^{-1}$)</td>
<td>39.5 ± 4.6</td>
<td>40.8 ± 2.7</td>
<td>38.6 ± 4.4</td>
<td>41.2 ± 4.3</td>
</tr>
<tr>
<td>Max. activity (nmol of PO$_4$-mg$^{-1}$-min$^{-1}$)</td>
<td>157.3 ± 11.3</td>
<td>125.6 ± 10.0*</td>
<td>161.1 ± 9.9</td>
<td>165.3 ± 13.5</td>
</tr>
<tr>
<td>Fold activation</td>
<td>4.02 ± 0.43</td>
<td>3.08 ± 0.16*</td>
<td>4.19 ± 0.27</td>
<td>4.02 ± 0.26</td>
</tr>
</tbody>
</table>

| **Reconstituted actomyosin** |       |           |      |      |
| Min. activity (mol of PO$_4$-mol myosin$^{-1}$-min$^{-1}$) | 0.088 ± 0.011 | 0.090 ± 0.011 | 0.099 ± 0.013 | 0.105 ± 0.014 |
| Max. activity (mol of PO$_4$-mol myosin$^{-1}$-min$^{-1}$) | 0.335 ± 0.021 | 0.333 ± 0.03a | 0.552 ± 0.011 | 0.438 ± 0.015ab |
| % Activation (−Ca$^{2+}$) | 46.1 ± 3.3 | 27.4 ± 3.6 | 30.1 ± 4.0 | 31.8 ± 4.2 |
| % Activation (+Ca$^{2+}$) | 162.5 ± 6.3 | 101.2 ± 10.1 | 167.8 ± 3.2 | 133.1 ± 3.6 |

* $p < 0.001$ versus WT.
* $p < 0.001$ versus E59D/D75Y.
* $p < 0.05$ versus WT.
CTnC(C84)IA. The WT and E59D-RTF fluorescence intensities increase 1.65-fold when the free Ca\(^{2+}\) is raised from \(pCa\) 7.8 to 4.4 with \(pCa_{50}\) values of 6.12 and 6.17, respectively. In the same \(pCa\) range, the E59D/D75Y-RTF fluorescence increases 1.25-fold with a marked reduction in Ca\(^{2+}\) affinity (\(\Delta pCa_{50} = -0.36\)). The data from Fig. 3 are summarized in Table 3.

Binding of S-1 to CTnC(C84)IA-labeled Filaments—Fig. 4 shows the increase in fluorescence intensity arising from CTnC(C84)IA-labeled RTFs in response to the attachment of S-1 cross-bridges in the absence of Ca\(^{2+}\) and nucleotide (\(\mu = 183\) mmol/liter). At saturating S-1 concentrations, the fluorescence intensity increases 1.31- and 1.26-fold for the labeled WT and E59D/D75Y-RTFs, respectively. Further addition of S-1 does not change the fluorescence intensity, indicating that the effect of rigor S-1 attachment on the detectable conformational changes of CTnC(C84)IA is saturated. When the number of binding sites (\(n\)) and \(K_d\) are calculated from the labeled WT-RTF data, the overall means ± S.D. are 4.81 ± 0.72 and 11.86 ± 2.10 mmol/liter, respectively. However, the number of S-1-binding sites obtained from labeled E59D/D75Y-RTFs is significantly reduced 23% (\(n = 3.72 ± 0.39, p < 0.01\)) without significantly altering the \(K_d\) (19.50 ± 8.74 mmol/liter). S-1 detachment was observed as the fluorescence intensities of the labeled RTFs decreased when 2 mmol/liter Mg-ATP\(^{2-}\) was added at the end of the titration (data not shown).

**DISCUSSION**

Our results indicate that the E59D/D75Y mutation directly affects the Ca\(^{2+}\) binding properties of CTnC and the transmission of force production. Although both mutations are located in the N terminus of CTnC, a miscommunication between the two domains exists because we observe reductions in the Ca\(^{2+}\) affinities for the Ca\(^{2+}\)-Mg\(^{2+}\)- and Ca\(^{2+}\)-specific sites measured by fluorescence in the isolated CTnC (Table 3).

Measuring Ca\(^{2+}\) binding to E59D/D75Y-CTnC interacting in complex and in RTFs continues to show significant reduc-
TABLE 3

Apparent Ca^{2+} affinities and Hill coefficients of IAANS-labeled CTnC mutants in isolation and in complex

The experiments were conducted in the presence of 1.25 mM MgCl_{2}. In the RTF and isolated CTnC level, D75Y-CTnC(C84)_{IA} showed no Ca^{2+}-dependent changes in fluorescence.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Low affinity binding</th>
<th>High affinity binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pCa_{50}</td>
<td>n_{H}</td>
</tr>
<tr>
<td>Isolated CTnC_{IA}</td>
<td></td>
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<tr>
<td>WT</td>
<td>4.94 ± 0.01</td>
<td>0.85</td>
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<tr>
<td>E59D/D75Y</td>
<td>4.76 ± 0.01a</td>
<td>0.94</td>
</tr>
<tr>
<td>D75Y</td>
<td>4.70 ± 0.05a</td>
<td>0.95</td>
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<tr>
<td>Isolated CTnC(C84)_{IA}</td>
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<td></td>
</tr>
<tr>
<td>WT</td>
<td>4.84 ± 0.01</td>
<td>0.84</td>
</tr>
<tr>
<td>E59D/D75Y</td>
<td>4.73 ± 0.03a</td>
<td>0.85</td>
</tr>
<tr>
<td>E59D</td>
<td>4.93 ± 0.09a</td>
<td>0.80</td>
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<tr>
<td>Binary CTnC(C84)_{IA}</td>
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<tr>
<td>WT</td>
<td>6.24 ± 0.02</td>
<td>1.11</td>
</tr>
<tr>
<td>E59D/D75Y</td>
<td>5.59 ± 0.04a</td>
<td>0.95b</td>
</tr>
<tr>
<td>D75Y</td>
<td>5.50 ± 0.04a</td>
<td>0.87c</td>
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<tr>
<td>Ternary CTnC_{IA}</td>
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<tr>
<td>WT</td>
<td>6.68 ± 0.02</td>
<td>0.94</td>
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<tr>
<td>E59D/D75Y</td>
<td>5.78 ± 0.05a</td>
<td>0.70b</td>
</tr>
<tr>
<td>D75Y</td>
<td>5.83 ± 0.05b</td>
<td>0.71b</td>
</tr>
<tr>
<td>E59D</td>
<td>6.60 ± 0.01b</td>
<td>1.03</td>
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<tr>
<td>CTnC(C84)_{IA} RTF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>6.12 ± 0.03</td>
<td>1.22</td>
</tr>
<tr>
<td>E59D/D75Y</td>
<td>5.76 ± 0.06b</td>
<td>1.14</td>
</tr>
<tr>
<td>E59D</td>
<td>6.17 ± 0.04b</td>
<td>1.27</td>
</tr>
</tbody>
</table>

* p < 0.001 versus WT.
* p < 0.05 versus WT.
* p < 0.05 versus E59D/D75Y.

FIGURE 4. Changes in the fluorescence intensity associated with the binding of S-1 to actin in IAANS-labeled cardiac thin filaments. WT and E59D/D75Y-CTnC(C84)_{IA} labeled regulated thin filaments were mixed with S-1 (from 0–500 nmol/liter), and the fluorescence emission intensity at 445 nm was measured. The x axis represents the total [S-1] added to the cuvette containing 50 nmol/liter labeled RTFs (or 350 nmol/liter actin). The fluorescence is plotted along the y axis as ΔF/ΔF_{max}, where ΔF is the fluorescence increase caused by the S-1 addition that is base line-subtracted. The solid and dashed lines are the calculated mean fits for thin filaments prepared with WT and E59D/D75Y-CTnC(C84)_{IA}, respectively. The mean values ± S.D. for the number of S-1-binding sites (n) and K_{D} are 4.81 ± 0.72 and 11.86 ± 2.10 nmol/liter, respectively for the WT-RTFs. The values for n and K_{D} obtained using E59D/D75Y-RTFs are 3.72 ± 0.39 and 19.50 ± 8.74 nmol/liter, respectively. The conditions and measurements were as described under "Experimental Procedures."

Functional Effects of CTnC Mutations Associated with DCM

Changes in the fluorescence intensity associated with the binding of S-1 to actin in IAANS-labeled cardiac thin filaments. WT and E59D/D75Y-CTnC(C84)_{IA} labeled regulated thin filaments were mixed with S-1 (from 0–500 nmol/liter), and the fluorescence emission intensity at 445 nm was measured. The x axis represents the total [S-1] added to the cuvette containing 50 nmol/liter labeled RTFs (or 350 nmol/liter actin). The fluorescence is plotted along the y axis as ΔF/ΔF_{max}, where ΔF is the fluorescence increase caused by the S-1 addition that is base line-subtracted. The solid and dashed lines are the calculated mean fits for thin filaments prepared with WT and E59D/D75Y-CTnC(C84)_{IA}, respectively. The mean values ± S.D. for the number of S-1-binding sites (n) and K_{D} are 4.81 ± 0.72 and 11.86 ± 2.10 nmol/liter, respectively for the WT-RTFs. The values for n and K_{D} obtained using E59D/D75Y-RTFs are 3.72 ± 0.39 and 19.50 ± 8.74 nmol/liter, respectively. The conditions and measurements were as described under "Experimental Procedures."
exceeds the levels of actomyosin and Tm alone in the presence of Ca\textsuperscript{2+} (Fig. 2C), suggesting that a diminished TnC-TnT interaction may exist that could be largely responsible for the Mg\textsuperscript{2+}-ATPase inactivation.

The current results indicate that a failure to activate the ATPase activity beyond unregulated levels may be responsible for the lower force observed at all Ca\textsuperscript{2+} concentrations. We rationalized that because the double mutant is capable of neutralizing the CTnI-based inhibition (Fig. 2D), other mechanisms independent of CTnI could exist that decrease the number of attached myosin motors. Therefore, we measured the total number of accessible S-1 actin-binding sites in the absence of Ca\textsuperscript{2+} and ATP at physiological ionic strength. We show that the S-1 binding stoichiometry within cardiac RTFs has similar numbers of binding sites obtained when heavy meromyosin binds to skeletal thin filaments (20) and myofibrils (21). Fig. 4 shows that the S-1 affinity does not change significantly in the presence of E59D/D75Y-CTnC(C84)\textsubscript{15I} rather the combined fits indicate that fewer binding sites are available when RTFs are prepared with the double mutant (3.7 S-1/filament) in comparison with the WT control (4.8 S-1/filament). It may be a striking coincidence, but ~23% fewer S-1 sites are available, and ~25% of the maximal tension and CMF ATPase activation is lost. Although rigor cross-bridges (A-M) make up a small fraction of the various strongly attached cross-bridge states (22), it is possible that other strongly attached states may have similar properties. Moreover, because cross-bridge attachment is known to enhance TnC Ca\textsuperscript{2+} affinity (23, 24), fewer S-1-binding sites along the thin filament could decrease this enhancement.

Having shown that the inhibitory properties of CTnI are capable of being neutralized when the double mutant is present, it is appropriate to consider another molecular mechanism involved in reducing the contractile force. McKillop and Geeves (25) have proposed a three-state model that possibly that other strongly attached states may have similar properties. Moreover, because cross-bridge attachment is known to enhance TnC Ca\textsuperscript{2+} affinity (23, 24), fewer S-1-binding sites along the thin filament could decrease this enhancement.

Many investigators have found that Ca\textsuperscript{2+} desensitization of cardiac muscle contraction is a common effect caused by DCM-linked mutations in the sarcomere. Notably, cardiac fibers from 13 idiopathic DCM patients also required higher Ca\textsuperscript{2+} concentrations to generate their maximal ATPase activities and were unable to achieve the maximal ATPase response seen in nonfailing heart fibers (26). Similar studies in conjunction with our findings suggest that the following mechanisms may contribute to the systolic dysfunction and larger end diastolic dimensions associated with DCM. The decrease (direct or indirect) in CTnC calcium affinity and fewer available S-1 actin-binding sites are predicted to synergistically: 1) lower the Ca\textsuperscript{2+}-activated ATPase and tension, consequently reducing the ejection fraction in vivo; and 2) facilitate diastolic filling as a result of having fewer cross-bridges attached during the previous systole. These findings are consistent with the pathophysiology of DCM in man and may contribute to the compensatory dilation that occurs over time with this disease.

The diagnosis of family members affected with another CTnC mutation (G159D) associated with DCM ranged from 20 to 62 years of age with the pathogenicity ranging from moderate to severe (27, 28). Although no segregation and penetrance data are available for the E59D/D75Y mutation, the variable penetrance associated with G159D suggests that CTnC mutations, like other sarcomeric mutations, are governed by multifactorial processes (i.e. modifier genes and epigenetic factors) (29).

Although assigning pathogenicity of a mutation is often hindered by a lack of family studies showing segregation with disease, such information would be valuable; however, it is very difficult to obtain. Therefore, Hershberger et al. (30) have reasoned that relevant functional studies are extremely valuable in interpreting the molecular results of a mutation. Based upon this rationale, Hershberger et al. were able to assign three of five mutations in the gene encoding CTnT as likely causative agents of DCM in the absence of any family segregation data. Therefore, ongoing research that integrates molecular, clinical, and functional data are critical determinants of the significance of mutations. In conclusion, although studies showing segregation of the E59D/D75Y mutation with disease is not available, our data show that this mutation can recapitulate the DCM functional phenotype in vitro and provide support that this is likely a DCM-causing mutation.

Acknowledgment—We thank Dr. Danuta Szczesna-Cordary for many insightful discussions.

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A Dilated Cardiomyopathy Troponin C Mutation Lowers Contractile Force by Reducing Strong Myosin-Actin Binding
David Dweck, Daniel P. Reynaldo, Jose R. Pinto and James D. Potter

doi: 10.1074/jbc.M109.064105 originally published online April 6, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M109.064105

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