The Troponin C G159D Mutation Blunts Myofilament Desensitization Induced by Troponin I Ser23/24 Phosphorylation

Brandon J. Biesiadecki, Tomoyoshi Kobayashi, John S. Walker, R. John Solaro, Pieter P. de Tombe

Abstract—Striated muscle contraction is regulated by the binding of Ca\(^{2+}\) to the N-terminal regulatory lobe of the cardiac troponin C (cTnC) subunit in the troponin complex. In the heart, β-adrenergic stimulation induces protein kinase A phosphorylation of cardiac troponin I (cTnI) at Ser23/24 to alter the interaction of cTnI with cTnC in the troponin complex and is critical to the regulation of cardiac contractility. We investigated the effect of the dilated cardiomyopathy linked cTnC Gly159 to Asp (cTnC-G159D) mutation on the development of Ca\(^{2+}\)-dependent tension and ATPase rate in whole troponin-exchanged skinned rat trabeculae. Even though this mutation is located in the C-terminal lobe of cTnC, the G159D mutation was demonstrated to depress ATPase activation and filament sliding in vitro. The effects of this mutation within the cardiac myofilament are unknown. Our results demonstrate that the cTnC-G159D mutation by itself does not alter the myofilament response to Ca\(^{2+}\) in the cardiac muscle lattice. However, in the presence of cTnI phosphorylated at Ser23/24, which reduced Ca\(^{2+}\) sensitivity and enhanced cross-bridge cycling in controls, cTnC-G159D specifically blunted the phosphorylation induced decrease in Ca\(^{2+}\)-sensitive tension development without altering cross-bridge cycling. Measurements in purified troponin confirmed that this cTnC-G159D blunting of myofilament desensitization results from altered Ca\(^{2+}\)-binding to cTnC. Our results provide novel evidence that modification of the cTnC-cTnI interaction has distinct effects on troponin Ca\(^{2+}\)-binding and cross-bridge kinetics to suggest a novel role for thin filament mutations in the modulation of myofilament function through β-adrenergic signaling as well as the development of cardiomyopathy. (Circ Res. 2007;100:1486-1493.)

Key Words: skinned muscle ■ rat ■ human ■ tension cost ■ cross-bridge cycling ■ cardiomyopathy

Although familial cardiomyopathies are genetically associated with mutations of a large number of amino acid residues in most of the sarcomeric proteins, only 2 mutations in the Ca\(^{2+}\) receptor responsible for activating cardiac sarcomeric contraction, cardiac troponin C (cTnC), have been recently demonstrated as linked to cardiomyopathy. In the current study, we report the functional effects of the dilated cardiomyopathy linked cTnC Gly159 to Asp (cTnC-G159D) mutation identified by Mogensen et al.1 The site of this mutation is of particular interest as it is located within the C-terminal lobe of cTnC generally considered to be structural, rather than regulatory.

cTnC is a bilobed metal-binding protein that functions as the Ca\(^{2+}\)-sensing subunit of the troponin (Tn) complex also containing cardiac troponin I (cTnI), the inhibitory protein, and cardiac troponin T (cTnT), the tropomyosin (Tm)-binding protein, to regulate the Tm inhibition of the actin–myosin interaction. Both the cTnC N- and C-lobes contain metal-binding sites, but it is the binding of Ca\(^{2+}\) to a single site located in the N-regulatory lobe that regulates contraction, whereas divalent cation binding to the C-lobe has been considered structural, reviewed in.2 In diastole and the absence of Ca\(^{2+}\), cTnI exists in a “closed” conformation and the cTnI C-terminal region binds actin tightly, contributing to inhibition of the actin–myosin interaction. In systole, Ca\(^{2+}\) binding to the cTnI N-regulatory lobe induces an “open” conformation that exposes a cTnI binding site removing cTnI inhibition.

The binding of Ca\(^{2+}\) to the cTnC N-regulatory lobe in cardiac myofilaments is modulated by β-adrenergic receptor stimulation via activation of protein kinase A (PKA) and the subsequent phosphorylation of cTnI at Ser23/24 through mechanisms unique to cardiac muscle. The phosphorylation of cTnI Ser23/24 in the N-terminal extension of the cardiac isoform has been well established to diminish the myofilament Ca\(^{2+}\) sensitivity of activation, tension, and ATPase rate by reducing the affinity of the cTnC N-regulatory site for Ca\(^{2+}\).3 Interestingly, unlike the binding of Ca\(^{2+}\) to the cTnC regulatory site, cTnI phosphorylation at Ser23/24 also induces structural alterations within the cTnC C-lobe, including the interaction of cTnI with cTnC residue Gly159.4 In view of the G159D location within the cTnC C-lobe and the effect of...
cTnI phosphorylation on cTnC residue Gly159, we sought to determine whether this mutation alters myofilament function in the presence of cTnI Ser23/24 phosphorylation.

We report here the results of experiments in which we investigated the effects of the cTnC-G159D mutation on Ca\(^{2+}\) regulation of myofilament tension and cross-bridge cycling rate in Tn-exchanged skinned rat trabeculae, both in the absence and presence of cTnI Ser23/24 phosphorylation. Our results demonstrate that the mutation by itself does not alter myofilament function. However, in the presence of cTnI phosphorylation the mutation virtually eliminates the characteristic cTnI Ser23/24 phosphorylation–induced myofilament desensitization in the absence of an effect on cross-bridge cycling. These data provide novel insights into the linkage of the TnC-G159D mutation to cardiomyopathy and the role of the cTnI-cTnC C-lobe interaction in β-adrenergic modulation of myofilament function.

**Materials and Methods**

**cDNA Constructs**

The human cardiac cTnC-G159D mutation and human cardiac TnI Ser23/24 pseudo-phosphorylation (cTnI-DD) cDNA were constructed by site-directed mutagenesis (Quick Change II kit; Stratagene). The cTnC-G159D construction was performed by mutagenesis of the human cTnC cDNA codon encoding Gly159 to Asp.\(^1\) The construction of the cTnI-DD charge mutation to mimic Ser23/24 phosphorylation was performed by mutagenesis of the human cTnI cDNA codons encoding Ser23/24 to Asp. The single Cys mutant human cardiac wild type and TnC-G159D were constructed as previously described.\(^5\)

**Tn Expression, Purification, and Complex Reconstitution**

The recombinant human wild-type cTnC, cTnC-G159D, and human cTnIs were expressed and purified as previously described.\(^6\) The expression and purification of the recombinant human cTnI containing an N-terminal myc tag was performed as previously described with slight modification of the purification protocol.\(^6\) Tn complex was reconstituted by sequential dialysis to remove urea and decrease salt of an equimolar amount of Tn components and purified using Resource-Q chromatography as previously described.\(^5\) Fractions containing pure Tn were dialyzed against exchange buffer (in mol/L): KCl 200, MgCl\(_2\) 5, EGTA 5, dithiothreitol (DTT) 1, MOPS 20 (pH 7.0); aliquots were stored at \(-80°C\) until use.

**Exchange of Recombinant Cardiac Tn Into Skinned Rat Trabeculae**

All animals were handled in accordance with the guidelines of the Animal Care and Use Committee at the University of Illinois, Chicago. Free-running, unbranched right ventricular trabeculae from male Lewis brown Norway–F1 rats (Harlan Laboratories; \(\sim 14\) weeks of age) were dissected, treated with Triton X-100 to extract membranes (skinned), and attached to aluminium T-clips as previously described.\(^7\) Endogenous Tn from trabeculae was exchanged over a range of free \([Ca^{2+}]\) to measure steady-state isometric tension and ATPase activity. Only muscles that maintained greater then 80% maximal tension were included for analysis. Following the mechanical experiment, each trabeculae was briefly dried and stored frozen for biochemical analysis.

**Quantification of Tn Exchange by Western Blot**

Recombinant cTnT in the present experiments included an N-terminal myc tag to allow for quantification of the amount of Tn exchange by Western blotting techniques.\(^6\) Previously we have demonstrated that the presence of this myc tag does not affect myofilament function.\(^8\)

**Tn Complex Ca\(^{2+}\)-Binding Affinity**

\([Ca^{2+}]\) binding was measured by fluorescence emission intensity of 2-(4′-idoacetamido)anilino-naphthalene-6-sulfonic acid (IAANS) attached to Cys35 in a single Cys mutant cTnC (C84S) of the Tn complex as described previously.\(^8\) The solutions contained (in mol/L): NaCl 100, MgCl\(_2\), 5, EGTA 1, DTT 1, MOPS 20 (pH 7.0); tiritations with \([Ca^{2+}]\) were performed at 25°C, and free \([Ca^{2+}]\) was calculated with the WEBMAXC program.\(^9\)

**Data Processing and Statistical Analysis**

Tension–\([Ca^{2+}]\) relationships were fit to a modified Hill equation; tension cost was determined by linear fit to the tension–ATPase data. Statistical analyses were performed either as multiple linear regression, 1-way or 2-way ANOVA as appropriate using the R statistical analyses packages. \(P<0.05\) was considered statistically significant; data are presented mean±SEM.

**Results**

**Skinned Trabeculae Exchange With Recombinant Exogenous Tn**

We used the whole Tn-exchange technique as a means to investigate the functional effect of the cTnC-G159D mutation on sarcomere dynamics in the cardiac myofilament lattice. We generated and purified recombinant wild-type (WT-Tn) and mutated human Tn consisting of the cTnC-G159D mutation (G159D-Tn), Ser23/24 Asp pseudo-phosphorylated cTnI to mimic phosphorylation at these residues (WT-Tn-DD), or cTnC-G159D in the presence of the Ser23/24 Asp pseudo-phosphorylated cTnI (G159D-Tn-DD). Analysis of the various purified Tn complex preparations by SDS-PAGE revealed 3 protein bands without detectable evidence of protein degradation (Figure 1A). Recombinant cTnT migrates slower than the endogenous rat cTnT allowing the electro-phoretic separation of exogenous from endogenous cTnT for subsequent Tn exchange quantification of each trabeculae independently by Western blot using a cTnT-specific monoclonal antibody CT3 (Figure 1B). On average, trabeculae included in the analyses contained 68.4±2.7% recombinant human cTnT. Furthermore, the presence of the cTnC-G159D, the TnI-DD, or the combination of these did not significantly affect the extent of Tn exchange.

**cTnC-G159D Alone Does Not Alter Myofilament Function**

As illustrated in Figure 2A, exchange of human Tn containing the cTnC-G159D mutation into skinned rat cardiac trabeculae did not significantly alter myofilament Ca\(^{2+}\) sensitivity, cooperativity, or maximum tension development compared with trabeculae exchanged with human wild-type Tn. Furthermore, as shown in Figure 2B, there was a small, but insignificant, increase in ATP-hydrolysis rate at all levels of
tension, indicating that this mutation alone does not affect the
cycling kinetics of strongly bound, tension-developing cross-
bridges. There was also a lack of the mutant cTnC to affect
tension cost (Figure 2B, inset), a parameter reflecting the rate
of cross-bridge detachment.7 The average parameters of the
Hill fit to the tension data and tension cost obtained in each
individual muscle preparation support this notion (Table 1).

cTnC-G159D Blunts the Impact of cTnI Ser23/24
Pseudo-Phosphorylation on Myofilament Function

In light of the cTnC G159D’s location within the Tn complex
and its altered environmental change on cTnI Ser23/24
phosphorylation,4 we hypothesized that the cTnC-G159D
mutation may have different effects on cardiac contractile
function following β-adrenergic stimulation. To investigate
this hypothesis we used a recombinant cTnI with Ser residues
23 and 24 mutated to negatively charged Asp (cTnI-DD) to
mimic β-adrenergic–stimulated, PKA-mediated phosphoryla-
tion of cTnI at these residues. The cTnI-DD mutation has
previously been demonstrated to mimic the effects of cTnI
PKA phosphorylation at Ser23/24 both structurally4,10 and
functionally in cardiac trabeculae. 11,12 Figure 3A demon-
strates the well-known13 impact of cTnI Ser23/24 phosphor-
ylation to desensitize the myofilament to Ca2+
observed as a
significant right shift of the tension–Ca2+
relationship. Max-
imum myofilament tension–generating capacity was also
slightly reduced, albeit not significantly (Figure 3A, inset).
Moreover, myofilament ATP hydrolysis rate and the tension
cost parameter were significantly increased (Figure 3B),
indicating that Ser23/24 pseudo-phosphorylation of cTnI
induced an acceleration of cross-bridge cycling kinetics. We
next investigated the impact of cTnC-G159D on myofilament
function in the presence of the cTnI-DD. As is shown in
Figure 4A, the cTnC-G159D mutation almost completely
abolished the characteristic decrease in myofilament Ca2+
sensitivity following Ser23/24 phosphorylation. In contrast,
maximum tension generating capacity remained similar to that observed in the presence of the WT-Tn-DD (Figure 4A, inset). Moreover, myofilament ATP hydrolysis rate and the tension
cost parameter were significantly increased (Figure 3B),
indicating that Ser23/24 pseudo-phosphorylation of cTnI
induced an acceleration of cross-bridge cycling kinetics. We
next investigated the impact of cTnC-G159D on myofilament
function in the presence of the cTnI-DD. As is shown in
Figure 4A, the cTnC-G159D mutation almost completely
abolished the characteristic decrease in myofilament Ca2+
sensitivity that normally accompanies cTnI Ser23/24 phos-
phorylation. In contrast, maximum tension generating capacity
remained similar to that observed in the presence of the WT-Tn-DD (Figure 4A, inset). Furthermore, exchange of the
G159D-Tn-DD did not alter the rate of ATP hydrolysis or
tension cost compared to the WT-Tn-DD, both of which were
significantly increased from that observed in WT-Tn ex-
changed trabeculae (Figure 4B). The average fit parameters
are summarized in Table 1.

cTnC-G159D Blunts the Impact of cTnI
Ser23/24 Pseudo-Phosphorylation on Tn
Ca2+-Binding Affinity

It is established that the decreased myofilament Ca2+
sensitivity following Ser23/24 phosphorylation of cTnI is associ-
ated with a decrease in Ca\textsuperscript{2+}-binding affinity to the Tn complex.\textsuperscript{3} The absence of the characteristic cTnI phosphorylation–induced myofilament Ca\textsuperscript{2+} desensitization in the presence of cTnC-G159D (Figure 4A) could, therefore, be attributable to a direct effect of this mutation to inhibit the modulation of Tn Ca\textsuperscript{2+}-binding affinity by cTnI phosphorylation. Consistent with the lack of an affect on the tension–Ca\textsuperscript{2+} relationship in skinned cardiac trabeculae (Figure 2A), the presence of cTnC-G159D alone did not affect Tn Ca\textsuperscript{2+}-binding affinity (Figure 5). Furthermore, cTnI-DD induced a decrease in Tn Ca\textsuperscript{2+}-binding affinity that was prevented in the presence of cTnC-G159D, consistent with the results obtained in Tn exchanged skinned cardiac trabeculae (Figures

<table>
<thead>
<tr>
<th>Tn Exchanged</th>
<th>pCα&lt;sub&gt;50&lt;/sub&gt;</th>
<th>ΔpCα&lt;sub&gt;50&lt;/sub&gt;</th>
<th>F&lt;sub&gt;max&lt;/sub&gt;</th>
<th>Hill</th>
<th>pCα&lt;sub&gt;50&lt;/sub&gt;</th>
<th>ΔpCα&lt;sub&gt;50&lt;/sub&gt;</th>
<th>F&lt;sub&gt;max&lt;/sub&gt;</th>
<th>Hill</th>
<th>TC</th>
<th>n</th>
</tr>
</thead>
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<tr>
<td>WT-Tn</td>
<td>5.63±0.11</td>
<td>N/A</td>
<td>38.6±2.8</td>
<td>2.62±0.17</td>
<td>5.69±0.11</td>
<td>N/A</td>
<td>194±12</td>
<td>3.01±0.19</td>
<td>4.04±0.36</td>
<td>9</td>
</tr>
<tr>
<td>G159D-Tn</td>
<td>5.64±0.14</td>
<td>+0.01</td>
<td>36.1±3.7</td>
<td>2.33±0.13</td>
<td>5.69±0.13</td>
<td>0.00</td>
<td>210±21</td>
<td>2.52±0.24</td>
<td>5.06±0.23</td>
<td>9</td>
</tr>
<tr>
<td>G159D-Tn-DD</td>
<td>5.60±0.17</td>
<td>−0.03</td>
<td>30.8±2.2</td>
<td>2.03±0.14</td>
<td>5.67±0.15</td>
<td>−0.02</td>
<td>204±12</td>
<td>2.30±0.19</td>
<td>6.44±0.50*</td>
<td>10</td>
</tr>
<tr>
<td>WT-Tn-DD</td>
<td>5.47±0.18*</td>
<td>−0.16</td>
<td>31.9±2.5</td>
<td>2.33±0.18</td>
<td>5.55±0.20*</td>
<td>−0.12</td>
<td>206±24</td>
<td>2.52±0.23</td>
<td>5.44±0.66*</td>
<td>8</td>
</tr>
</tbody>
</table>

The Ca\textsuperscript{2+}-activated tension and myofibrillar ATPase measurements from skinned rat trabeculae exchanged with recombinant Tn containing WT-Tn, G159D-Tn, G159D-Tn-DD, or WT-Tn-DD. Values are means±SEM. pCα<sub>50</sub> indicates inverse log of the [Ca\textsuperscript{2+}] in μmol/L required to develop 50% maximal tension; ΔpCα<sub>50</sub> change in pCα<sub>50</sub> from WT-Tn; F<sub>max</sub> maximally developed tension in mN/mm\textsuperscript{2}; Hill, slope of the tension–Ca\textsuperscript{2+} plot; TC, slope of the ATPase vs tension plot; n, no. of fibers in each group. *P<0.05 vs WT-Tn.
Ca\textsuperscript{2+} signaling by the quenching of IAANS-labeled cTnC fluorescence on Ca\textsuperscript{2+} binding to cTn. Ca\textsuperscript{2+} binding to purified phosophorylated cTn containing either WT-cTnC (WT-Tn), cTnC-G159D (G159D-Tn), WT-cTnC, and cTnI-DD (WT-Tn-DD) or cTnC-G159D and cTnI-DD (G159D-Tn-DD) was measured by the quenching of IAANS-labeled cTnC fluorescence on Ca\textsuperscript{2+} binding. Titration curves demonstrate that the binding of Ca\textsuperscript{2+} to the WT-Tn-DD exhibited the expected decrease in Ca\textsuperscript{2+}-binding affinity. However, the effect of cTn Ser23/24 phosphorylation on Ca\textsuperscript{2+} binding to cTnC was significantly blunted in the G159D-Tn-DD such that Ca\textsuperscript{2+} binding was not different from that of the WT-Tn.

3A and 4A. The average fit parameters of these data are summarized in Table 2.

**Discussion**

The results presented here represent the first investigation to test the effects of a cTnC cardiomyopathy-linked mutation in the cardiac myofilament lattice and provide new insights not only into the molecular pathology but also in the molecular mechanisms that underlie the modulation of myofilament function through \(\beta\)-adrenergic signaling. A previous report demonstrated that the cTnC-G159D mutation induced a decrease in ATPase activity of reconstituted myofilaments as well as filament sliding velocity in an in vitro motility assay.\(^{14}\) Another study demonstrated the exchange of the cTnC-G159D into fast skeletal fibers did not affect the development of steady-state tension but reduced the rate of tension development on rapid Ca\textsuperscript{2+} activation.\(^{15}\) Similar to the findings in skeletal muscle, we demonstrate here that the cTnC mutation exhibits no effect on the Ca\textsuperscript{2+}-regulated steady-state tension or ATP-hydrolysis rate when exchanged into cardiac myofilaments (Figure 2). In addition to this finding, our data further demonstrate that cardiac filaments regulated by cTnC-G159D lack the characteristic Ca\textsuperscript{2+} desensitization that normally results from the presence of cTnI phosphorylated by PKA (Figure 4). This inability of sarcomeres regulated by cTnC-G159D to respond to cTnI PKA-mediated phosphorylation is likely to alter myocardial functional responses, especially the \(\beta\)-adrenergic–induced reduction in cardiac cycle length. Studies\(^{16}\) using a transgenic mouse model have demonstrated that cTnI Ser23/24 phosphorylation, acting in conjunction with the phosphorylation of membrane proteins involved in Ca\textsuperscript{2+} homeostasis, is essential to the increased cardiac relaxation required to maintain adequate diastolic ventricular filling during the shortened contractile cycle induced by \(\beta\)-adrenergic stimulation. Thus our data predict an impairment of myocardial relaxation during elevated \(\beta\)-adrenergic activity, for example, as occurs during exercise, and this phenomenon may trigger maladaptive cardiac remodeling similar to the dilated cardiomyopathy that is observed in individuals who express the cTnC-G159D mutation.\(^{1}\)

The lusitropic effects of cTnI phosphorylation at Ser23/24 appear to result from 2 separate functional mechanisms (Figure 6B): (1) a decrease in the Ca\textsuperscript{2+}-binding affinity of the cTnC N-terminal regulatory domain to decrease the Ca\textsuperscript{2+}-sensitive activation of the thin filament\(^{17,18}\) and (2) an increase in cross-bridge cycling rate.\(^{17,19}\) Increased cross-bridge cycling attributed to cTnI PKA phosphorylation at Ser23/24 has been observed in some,\(^{17,20}\) but not all,\(^{10}\) studies using a variety of techniques such as velocity of shortening,\(^{21}\) rate of tension development, and frequency of minimum stiffness.\(^{19}\) The inconsistency of these findings may result from PKA treatment–mediated phosphorylation of other cardiac contractile proteins, notably myosin binding protein C or titin, to mask the effect of cTn Ser23/24 phosphorylation on cross-bridge cycling. Here we overcame this confounding factor by exchanging endogenous Tn in skinned cardiac trabeculae with recombinant human Tn containing cTn Ser23/24 mutated to the negatively charged Asp residue (cTn-DD). The exchange of cTn-DD allows for investigation of the sole effects of cTn Ser23/24 phosphorylation in the absence of other potentially confounding PKA-mediated cytoskeletal protein phosphorylation. The mutation cTn Ser23/24 residues to Asp functions both structurally\(^{4,10}\) and functionally,\(^{10,11}\) similar to PKA-mediated phosphorylation of cTnC in cardiac muscle. Indeed, in the present study, we observed both the characteristic decrease in Ca\textsuperscript{2+} sensitivity of steady-state tension (Figure 3) and a decrease in

**TABLE 2. Ca\textsuperscript{2+} Binding to the Purified Tn Complex**

<table>
<thead>
<tr>
<th>Tn Exchanged</th>
<th>pCa50 (\Delta pCa50)</th>
<th>Ka (10^4)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-Tn</td>
<td>6.51±0.009</td>
<td>0.10</td>
<td>3.21±10(^{-4})</td>
</tr>
<tr>
<td>G159D-Tn</td>
<td>6.52±0.004</td>
<td>0.01</td>
<td>3.28±10(^{-5})</td>
</tr>
<tr>
<td>G159D-Tn-DD</td>
<td>6.41±0.007*</td>
<td>-0.10</td>
<td>2.59±10(^{-4})</td>
</tr>
<tr>
<td>WT-Tn-DD</td>
<td>6.09±0.009*</td>
<td>-0.42</td>
<td>1.24±10(^{-4})</td>
</tr>
</tbody>
</table>

The \(\Delta pCa50\) indicates inverse log of the [Ca\textsuperscript{2+}] in \(\mu\)mol/L required to saturate 50% of TnC. \(\Delta pCa50\) change in pCa50 from WT-Tn; \(K_a\) binding constant of Ca\textsuperscript{2+} to Tn; n, no. of experiments in each group. *\(P<0.05\) vs WT-Tn.
binding affinity of Ca$^{2+}$ to Tn containing the recombinant cTnI-DD (Figure 5). Furthermore, we demonstrate an increase in tension cost (a measurement of cross-bridge detachment rate) in the presence of cTnI pseudo-phosphorylated at Ser23/24 (Figure 3) that, unlike Ca$^{2+}$-desensitization, was not affected by the presence or absence of the cTnC-G159D mutation (Figure 4). This increase in cross-bridge detachment rate, as measured by tension cost, is supported by our previous findings demonstrating that cTnI PKA phosphorylation increases the rate of force relaxation and minimum dynamic stiffness, as well as work from other investigators including an increase in the velocity of shortening following PKA treatment. The separate functional effects of cTnI PKA phosphorylation are also consistent with our recent observation that exchange of Tn containing slow skeletal Tn induced an alteration in myofilament Ca$^{2+}$ sensitivity without affecting activation/relaxation dynamics. Together these findings support the notion that the impact of cTnI Ser23/24 phosphorylation on myofilament Ca$^{2+}$ sensitivity and cross-bridge cycling rate function independently through separate and distinct mechanisms (Figure 6B).

The molecular mechanism of how the cTnC-G159D mutation blunts the decrease in myofilament Ca$^{2+}$ sensitivity on cTnI Ser23/24 phosphorylation remains to be identified. Our results indicate that cTnC-G159D blunts the cTnI Ser23/24 N-terminal phosphorylation–induced decrease of myofilament Ca$^{2+}$ sensitivity by altering the binding affinity of Ca$^{2+}$ to the cTnC N-terminal regulatory lobe (Figure 5 and Table 2), suggesting a direct effect of cTnC C-lobe residue 159 on Ca$^{2+}$ binding to the cTnC N-regulatory lobe in a phospho-specific manner. Consistent with this finding, biochemical studies investigating the function of various TnI fragments support the notion of a role for the TnI N terminus as responsible for regulating Ca$^{2+}$ sensitivity, whereas the C-terminal region may function to regulate cross-bridge cycling kinetics. In the absence of Ser23/24 phosphorylation, the cTnI N-terminal extension binds to the cTnC N-regulatory lobe to stabilize the open conformation, $^{4,24-26}$ After cTnI Ser23/24 phosphorylation, incorporation of the negatively charged phosphates repels the cTnI N-terminal extension, causing it to bend and rotate away from the main axial, $^{27,28}$ (Figure 6A). This conformational change in the cTnI N-terminal extension is believed to weaken its interaction with the cTnC N-lobe, $^{4,24-26}$ causing a shift of the regulatory domain open/closed conformational distribution toward the closed conformation to decrease Ca$^{2+}$-binding affinity. The phosphorylation of cTnI Ser23/24 also induces a shift in the NMR chemical signal that results from the interaction of the cTnI N terminus with the cTnC C-lobe, including the cTnI interaction with cTnC residue Gly159. $^{4,10}$ The cTnC-G159D mutation has previously been demonstrated to increase the binding affinity of cTnC to cTnI. This increase in binding affinity may strengthen the interaction between the cTnI N terminus and the cTnC C-lobe to inhibit the Ser23/24 phosphorylation weakening of the cTnI N-terminal extension/cTnC N-lobe interaction and prevent the shift in the open/closed conformation of the cTnC regulatory lobe responsible for decreased Ca$^{2+}$-binding affinity (Figure 6A). The detailed mechanisms by which the cTnC-G159D mutation alters cardiac contraction and...
the binding of Ca²⁺ to cTnC solely in the presence of the cTnl Ser23/24 phosphorylation is of great interest and deserves further investigation.

The cTnC Gly159 residue resides in a highly conserved region of the C-lobe that directly and tightly interacts with the cTnl N terminus and is generally not considered to be involved in myofilament regulation. We report here that the cTnC-G159D mutation inhibits cTnl PKA-mediated phosphorylation induced Ca²⁺ desensitization (Figures 4 and 5). This finding extends our earlier studies demonstrating the importance of the cTnC C-lobe in myofilament regulation. One indication of the importance of this cTnC-cTnI region in the regulation of the myofilament in response to Ca²⁺ is found in the binding of the Ca²⁺-sensitizing agent EMD 57003 to the cTnC C-lobe. We have previously demonstrated that cTnl phosphorylation at Ser43/45 (a region that also interacts with the TnC C-lobe) is sufficient to modify the impact of EMD 57033 on myofilament Ca²⁺ sensitivity.30 Furthermore, we have reported that the cTnl N-terminal region surrounding Ala66, a region that strongly interacts with the cTnC C-lobe, is a significant locus of signaling in the activation of the thin filament by strongly bound cross-bridges.31 Together with these previous findings, our current results provide further evidence indicating a unique role for the cTnC C-lobe interaction with the cTnl N terminus in myofilament contractile regulation.

A significant finding from our experiments is the linkage of increased myofilament Ca²⁺ sensitivity with dilated cardiomyopathy. Mutations in sarcomeric proteins associated with dilated cardiomyopathy typically demonstrate decreased myofilament Ca²⁺ sensitivity, yet the effect of the cTnl-G159D to inhibit cTnl phosphorylation-induced myofilament Ca²⁺ desensitization functions to effectively increase Ca²⁺ sensitivity. Alterations of cTnl phosphorylation levels are suggested to be important in the development of human cardiomyopathy. Increased myofilament Ca²⁺ sensitivity has been reported by some investigators in end-stage heart failure and is most likely associated with the reduced basal PKA-mediated phosphorylation of cTnl resulting from decreased β-adrenergic signaling through cAMP.32,33 Although it has been difficult to ascertain whether the increased myofilament Ca²⁺ sensitivity observed in end-stage human heart failure is related to the development of this condition, our data indicate that the altered myofilament response to Ca²⁺ may be an important factor in the transition of the failing heart to a dilated phenotype.

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Disclosures
None.

References


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