The C Terminus of Cardiac Troponin I Is Essential for Full Inhibitory Activity and Ca\(^{2+}\) Sensitivity of Rat Myofibrils*  

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Although the C terminus of troponin I is known to be important in myofilament Ca\(^{2+}\) regulation in skeletal muscle, the regulatory function of this region of cardiac troponin I (cTnI) has not been defined. To address this question, the following recombinant proteins were expressed in *Escherichia coli* and purified: mouse wild-type cTnI (WT cTnI; 211 residues), cTnI-(1–199) (missing 23 residues), and cTnI-(1–151) (missing 60 residues). The inhibitory activity of cTnI and the mutants was tested in myofibrils, from which cTnI binds to cTnC causing the Ca\(^{2+}\) sensitivity of the myofilaments to be lost. Addition of increasing amounts of exogenous WT cTnI or cTnI-(1–199) to cTnT-treated myofibrils at pCa 8 caused a concentration-dependent inhibition of the maximum ATPase activity. However, cTnI-(1–188) and cTnI-(1–151) inhibited this activity to about 75% and 50% of that of the WT cTnI, respectively. We also formed a complex of either WT cTnI or each of the mutants with cTnC, reconstituted the complex into the cTnT-treated myofibrils, and measured Mg\(^{2+}\)-ATPase activity as a function of pCa. We found that the cTnI-(1–188)-cTnC complex only partially restored Ca\(^{2+}\) sensitivity, whereas the cTnI-(1–151)-cTnC complex did not restore any Ca\(^{2+}\) sensitivity. Each cTnI C-terminal deletion mutant was able to bind to cTnC, as shown by urea-polyacrylamide gel-shift analysis and size exclusion chromatography. Each mutant also co-sedimented with actin. Our results indicate that residues 152–199 (C-terminal to the inhibitory region) of cTnI are essential for full inhibitory activity and Ca\(^{2+}\) sensitivity of myofibrillar ATPase activity in the heart.

The transition of heart muscle from diastole to systole involves a Ca\(^{2+}\)-dependent process in which an inhibitory protein, cTnI, plays a key role in switching on the reaction of myosin cross-bridges with actin. A current model for the switching mechanism has been derived largely from studies with fast skeletal TnI and is as follows. During diastole, cTnI interacts tightly with actin and contributes to the inhibition of the actin-cross-bridge reaction. During systole, with Ca\(^{2+}\) binding to cTnC, there is an increased affinity of cTnI for cTnC, which results in weakening of cTnI binding to actin. This leads to changes in Tm position, resulting in activation of contraction through promotion of the transition of cross-bridges from blocked or weak binding states to strong force-generating states (1).  

In the case of heart muscle, Ca\(^{2+}\) signaling appears different from that of fast skeletal muscle, and little is known about regions of cTnI that are essential for the Ca\(^{2+}\) switch. An anti-parallel interaction between cTnI and cTnC has been demonstrated (2). In the anti-parallel arrangement, an interaction of the N-terminal region of cTnI with cTnC acts as an anchor maintaining the two proteins in the correct spatial orientation. The C-terminal region of cTnI is thought to bind to the N terminus of cTnC, which contains the low affinity Ca\(^{2+}\)-specific site (site II) (2, 3). Ca\(^{2+}\) binding to site II on cTnC is essential for the regulation of cardiac contraction (4–6). Interestingly, it has been shown recently by heteronuclear, multidimensional NMR spectroscopy that upon transition from the apo- to the Ca\(^{2+}\)-saturated states of the N-domain of cTnC, fewer hydrophobic residues are exposed in cTnC than in fsTnC (7, 8). This hydrophobic region of cTnC is thought to bind to cTnI (9). The finding that the structure of the regulatory N-domain of cTnC is significantly more compact relative to fsTnC (7) may be related to differences in how cTnI and fsTnI interact with TnC and ultimately to differences in the regulation of cardiac versus skeletal muscle contraction (6, 10).  

Moreover, it is known that TnI contains an inhibitory region (residues 139–150 in mouse cTnI) that binds to both TnC and actin, but not to both simultaneously (11, 12). This region is believed to be largely responsible for the ability of TnI to inhibit actomyosin ATPase activity and constitutes a key part of the molecular switch turning on the thin filament. Although structural and modulatory functions have been identified within the unique cardiac-specific N-terminal region of cTnI, the functional role of its C-terminal region has yet to be delineated.  

Our laboratory is using recombinant DNA technology to investigate structure-function relations of cardiac TnI. The C terminus of cTnI is highly conserved among the TnI isoforms and its binding to the N terminus of cTnC containing the low affinity Ca\(^{2+}\)-specific site indicates it may be important for Ca\(^{2+}\)-dependent regulation of cardiac muscle contraction. Although it has been shown that the C terminus of fsTnI appears to be important for Ca\(^{2+}\) sensitivity in myofilaments in skeletal muscle (13, 14), there is no evidence for such a role in cardiac muscle. In this study, we generated three deletion mutants of cardiac TnI to examine the function of the C-terminal domain of cTnI in the cardiac myofilament. Our results indicate that regions of cTnI C-terminal to the inhibitory region are essential

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for full inhibitory activity and Ca$^{2+}$ sensitivity of cardiac myofilaments.

**EXPERIMENTAL PROCEDURES**

**Purification of Native Myofilament Proteins**—Crude cardiac troponin was prepared from bovine left ventricular ether powder and cTnI and cTnC were subsequently purified by chromatography as described by Poter (15). Purified cTnT or cTnC was resuspended in 50 mM Trizma base, pH 8.0, 10 mM EDTA, 6 mM urea, 0.1 mM DTT for immediate use or dialyzed against deionized H$_2$O, lyophilized, and stored at $-80^\circ$C. F-actin was purified from bovine left ventricular ether powder essentially as described by Pardee and Spudich (16) and resuspended in 2 mM MOPS, pH 7.0, 50 mM KCl, 1 mM MgCl$_2$, 1 mM ATP, 0.2% NaN$_3$, 0.2 mM DTT. Tropomyosin was purified from the 42.5–60% (NH$_4$)$_2$SO$_4$ pellet containing WT cTnI, cTnI-(1–199), cTnI-(1–188), or cTnI-(1–151) mutants were eluted with a linear dialysis as described above. The cTnI-(1–199), cTnI-(1–188), or cTnI-(1–151) mutants were further purified by affinity chromatography on an Affi-Gel-15 (Bio-Rad) cTnC column equilibrated with 50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 6 mM urea, 0.1 mM DTT, 0.2% NaN$_3$, 0.1 mM DTT. The WT cTnI and mutant cTns were analyzed on SDS-polyacrylamide gels (15%) (19). The cTnI-(1–151) was 24,260, 22,925, 21,656, and 17,407, respectively.

**Expression and Purification of cTnI**—Mouse cardiac wild-type TnI was cloned by reverse transcriptase PCR as described previously (17). Construction of cTnI C-terminal deletion mutants (cTnI-(1–199), cTnI-(1–188), cTnI-(1–151), cTnI-(1–199), cTnI-(1–188), and cTnI-(1–151) mutants were expressed by PCR amplification using a common 5'-primer (5'-ctggagatacacgatgctgatigar-3') and 5 different 3'-primers (5'-TGGATCCTGGCATCTCAAGAGATCCTCACTC-3', 5'-TCTCAGTTTTCCTTCTCAATG-3', 5'-GCTGATGAAAGCAGC-3', 5'-GCTGATGAAAGCAGC-3', and 5'-GCTGATGAAAGCAGC-3'). The PCR products were cloned directly into a pCR™ cloning vector containing WT cTnI, cTnI-(1–199), cTnI-(1–188), or cTnI-(1–151) were prepared at a 1:1 molar ratio by sequential dialysis as described above. The cTnI-(1–199), cTnI-(1–188), or cTnI-(1–151) were 24,260, 22,925, 21,656, and 17,407, respectively.

**Expression and Purification of cTnC**—Mouse cardiac wild-type TnC was expressed in an E. coli expression system as described by Johnson et al. (23) with slight modifications. Purified cTnI and cTnC (1:1 molar ratio) were mixed to give a final concentration of 8 mg/ml in 25 mM MOPS, pH 7.0, 6 mM urea, 1 mM KCl, 5 mM MgCl$_2$, 1 mM CaCl$_2$, 1 mM DTT and incubated on ice for 1 h. The mixture was sequentially dialyzed against (25) by first removing the urea and then reducing KCl from 1.0 to 0.75, 0.5, 0.3, and 0.1 mM. The cTnI-cTnC complex was finally washed in Buffer C (20 mM MOPS, pH 7.0, 100 mM KCl, 5 mM MgCl$_2$, 5 mM EDTA, 0.5 mM DTT, 0.1 mM glycine, 0.02% boehrnem blue with either 10 mM EGTA or 10 mM CaCl$_2$. Aliquots (20 µl) were analyzed on 8% polyacrylamide gels, pH 8.6, containing 6 mM urea essentially as described by Ornstein (24).

**Analysis of cTnI-cTnC Complexes by Size-exclusion Chromatography**—Complexes containing cTnI with WT cTnC, cTnI-(1–199), cTnI-(1–188), or cTnI-(1–151) were prepared at a 1:1 molar ratio by sequential dialysis as described above. The cTnI-cTnC complexes (20 µm) in 100 µl of 25 mM MOPS, pH 7.0, 100 mM KCl, 5 mM MgCl$_2$, 1 mM CaCl$_2$, 1 mM DTT were injected onto a Superdex-75 HR 10/30 HPLC gel filtration column (Pharmacia Biotech, Inc.) that was equilibrated with the same buffer. Proteins were eluted from the column with a flow rate of 0.5 ml/min and monitored by monitoring at $A_{280}$. Fractions (0.5 ml) were collected and analyzed by SDS-PAGE.

**Removal of Tropomyosin by Excess cTnI and Reconstitution with cTnI-cTnC Complexes**—This method removes endogenous cardiac troponin from the myofilibr with treatment with excess exogenous cTnT. The treated myofilibr contains the exogenous cTnT, but removal of cTnI and cTnC makes the myofilibr insensitive to Ca$^{2+}$. The procedure was originally suggested by Hatakenaka and Ohtsuki (25) and was performed as described by Rarick et al. (26). Purified bovine cTnI was dissolved in Buffer D (20 mM MOPS, pH 6.5, 250 mM KCl, 5 mM EGTA, 5 mM MgCl$_2$, 0.5 mM DTT, 0.1 µg/ml pepstatin A). The cTnI, at a concentration of 0.4 mg/ml was added to centrifuged rat myofilibr (4 mg/ml) in a 1:10 weight ratio of cTnI:myofilibr. The volume was adjusted to 2.5 ml with Buffer D, and the mixture was incubated at 25 °C for 60 min with gentle mixing every 2 min. The cTnI-treated myofilibr were centrifuged (2,000 × g; 10 min), and the pellet was resuspended in 2.5 ml of Buffer C containing 2 mg of WT cTnI, 2 mg of cTnI mutants, or 4 mg cTnI-cTnC complex. This mixture was incubated at 25 °C for 75 min with gentle mixing at 2-min intervals. The reconstituted myofilibr were centrifuged (2,000 × g; 10 min) and the pellets resuspended in 2 ml of Buffer A, recentlyrifuged, and suspended in a final volume of 1 ml of Buffer A. The protein concentration was determined by the Lowry method (22).

**ATPase Measurements**—ATPase activity was determined by measuring inorganic phosphate release using a modification of the methods of White (27) and Dobrowolski et al. (28). Assays were carried out in 96-well microtiter plates at 30 °C in an incubator. The assay conditions varied and are described in the figure legends. The total volume was 70 µl, and all reactions were initiated with the addition of cTnT or cTnI or cTnI mutants (0.286 nmol) were mixed (7:2:2 molar ratio of cTnT:cTnI:cTnC) and cTnC complex. This mixture was incubated at 25 °C for 75 min with gentle mixing at 2-min intervals. The reconstituted myofilibr were centrifuged (2,000 × g; 10 min) and the pellets resuspended in 2 ml of Buffer A, recentlyrifuged, and suspended in a final volume of 1 ml of Buffer A. The protein concentration was determined by the Lowry method (22).

**Actin Co-sedimentation Assay**—F-actin (1 nmol), Tn (0.286 nmol), and cTnI or cTnI mutants (0.286 nmol) were mixed (7:2:2 molar ratio of actin:Tn:cTnI) in 180 µl of 2 mM imidazole, pH 7.0, 100 mM NaCl, 5 mM MgCl$_2$, 0.1 mM EGTA, 1 mM DTT, and centrifuged for 30 min at 150,000 × g in a Beckman Airfuge. After centrifugation, the supernatant fraction was removed, and the pellets were resuspended in 6 mM urea. Both pellets and supernatant fractions were analyzed by SDS-PAGE (15%).

**Statistics**—Repeated measurements were at least $n = 3$, and are presented as mean ± S.E. Measured relationships between $p$ and ATPase activity were fit to the Hill equation using nonlinear least-squares regression with least square to the Hill equation using nonlinear least-squares regression to derive the $pC_{50}$ and Hill coefficient ($n$) (Inplot curve fitting software, GraphPAD Software, Inc., San Diego, CA). Statistical differences of $pC_{50}$ values were analyzed by a paired Student's t test with significance set at $p < 0.05$. 

C-terminal Deletion Mutants of cTnI
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RESULTS

To localize protein domains in the C terminus of cTnI that are important for Ca\(^{2+}\) regulation of cardiac muscle contraction, we generated three deletion mutants of mouse cardiac cTnI, which were compared with the WT cTnI in functional assays. The amino acid sequence of the mouse WT cTnI consists of 211 residues and is shown in Fig. 1 (upper panel). The WT cTnI and the cTnI C-terminal deletion mutants are shown schematically in Fig. 1 (lower panel). The cTnI-(1–199), cTnI-(1–188), and cTnI-(1–151) mutants have 12, 23, and 60 residues deleted from the C terminus, respectively. The WT cTnI (1–151) mutant terminates 1 amino acid residue past the inhibitory region of cTnI.

The mouse WT cTnI and each cTnI C-terminal deletion mutant were expressed in Escherichia coli and purified as described under “Experimental Procedures” to >99% purity (Fig. 2). The gel in Fig. 2 shows that the recombinant mouse WT cTnI (lane 3) migrated slightly slower than native cardiac TnI (lane 2) purified from bovine tissue, possibly because of the lack of acetylation of the N-terminal methionine.

We examined the binding of each cTnI C-terminal deletion mutant to cTnC by visualization of cTnI-cTnC complex formation on urea polyacrylamide gels and size exclusion chromatography. As shown in Fig. 3, WT cTnI and each C-terminal deletion mutant of cTnI formed a binary complex with cTnC on urea polyacrylamide gels in the presence of Ca\(^{2+}\), but not in the presence of EGTA. We could not detect any differences between WT cTnI and each cTnI mutant in their ability to bind to cTnC in the denaturing conditions used for the urea PAGE analysis. This suggests that the structural interaction between the N terminus of cTnI and the C terminus of cTnC is the major contributor to formation of the cTnI-cTnC complex. We also tested whether WT cTnI and each cTnI C-terminal deletion mutant formed a complex with cTnC under non-denaturing conditions by size exclusion chromatography. At physiological salt concentrations, after passage through a HPLC gel filtration column, WT cTnI and each cTnI C-terminal deletion mutant formed a stable binary complex with cTnC (data not shown).

We also examined the ability of WT cTnI and the C-terminal deletion mutants of cTnI to bind to F-actin. F-actin, cTnI, or cTnI deletion mutants, and Tm were combined, and the binding of cTnI or the cTnI deletion mutants to F-actin was assayed by centrifugation in an Airfuge. The supernatant fractions and pellets were analyzed by SDS-polyacrylamide electrophoresis.
alkaline urea polyacrylamide gel electrophoresis to show the removal and restoration of cTnC under these conditions.

We analyzed the effect of C-terminal deletion of cTnI on its ability to inhibit the Mg$^{2+}$-ATPase activity of myofibrils lacking cTnI-cTnC. Fig. 5 shows the effect of increasing concentrations of the various forms of cTnI on the unregulated ATPase activity of the myofibrils. In the absence of added cTnI, as expected, the ATPase rate was independent of Ca$^2+$ and similar to that obtained in native myofibrils at maximal activity (pCa 4.9). WT cTnI reduced this activity to 25% of maximum with the inhibition reaching a plateau at 1.0 $\mu$M WT cTnI. The cTnI-(1–199) mutant had an inhibitory effect similar to WT cTnI. However, the inhibitory capacity of the cTnI-(1–188) mutant was reduced to 75% of that of WT cTnI and reached a plateau at a concentration of 1 $\mu$M. Inhibition by the cTnI-(1–151) mutant was about 50% of that of WT cTnI and attained a plateau at 2 $\mu$M. Even a concentration of 12 $\mu$M could not increase the inhibitory capacity of this mutant beyond 45% of that of WT cTnI (data not shown). Thus, loss of 23 residues at the C terminus of cTnI results in some loss of inhibitory activity that is further decreased after removal of an additional 37 residues.

We next tested the ability of cTnC to reverse the inhibitory effect of both WT cTnI and the cTnI C-terminal deletion mutants. The molar ratios of cTnC to cTnI tested varied from 0.5 to 4, and measurements were made in the absence and presence of Ca$^2+$. As Fig. 6 illustrates, addition of cTnC at a 1:1 molar ratio to cTnI in the presence of Ca$^{2+}$ reversed the inhibition caused by both WT cTnI and the cTnI-(1–199) mutant. The inhibition caused by cTnI-(1–188) was released by cTnC in the presence of Ca$^{2+}$ with restoration to within 20% of that for WT cTnI. However, the inhibition of maximum ATPase activity caused by cTnI-(1–151) was not released by the addition of cTnC (in the presence of Ca$^{2+}$) even at a molar ratio of 4:1 (cTnC:cTnI).

We also formed a complex of WT cTnI or cTnI C-terminal deletion mutants with cTnC before reconstitution into cTnT-extracted myofibrils, and measured the Mg$^{2+}$-ATPase activity as a function of pCa. Fig. 7 shows that under these experimental conditions, the Ca$^{2+}$ sensitivity was restored to that observed for control myofibrils with WT cTnI-cTnC and cTnI-(1–199)cTnC. On the other hand, reconstitution with the cTnI-(1–188)cTnC complex only partially restored Ca$^{2+}$ sensitivity (data not shown), and we observed a significant leftward shift in the pCa50 (Fig. 7). Data for the cTnI-(1–151)cTnC complex is not shown in Fig. 7 because after reconstitution with this complex, no Ca$^{2+}$ sensitivity was observed. These results indicate that in cardiac TnI, residues 152–188 (C-terminal to the inhibitory region) are essential for Ca$^{2+}$ sensitivity of the myofibrils.

**DISCUSSION**

Our results provide the first evidence that the C-terminal region of cardiac TnI, downstream from the inhibitory region, is essential for the Ca$^{2+}$-dependent regulation of cardiac myofilament activation. The functional significance of the C-terminal domain of TnI fits with evidence of an anti-parallel alignment between cTnI and cTnC in which the C-terminal region of cTnI binds to the N terminus of cTnC. This insight makes an important contribution to our objective to determine unique aspects of the detailed mechanism by which Ca$^{2+}$ switches on cardiac myofilaments.

Our findings suggest a need to rethink the relative contribution of the central inhibitory region of cTnI to the regulation of force. In previous studies, the primary focus has been the
inhibitory region of cTnI. An 11-amino acid peptide corresponding to the sequence of the inhibitory region of cTnI has the capacity to inhibit ATPase activity in vitro (12, 30, 31), although not to the same extent as full-length cTnI. Surprisingly, cardiac fiber bundles reconstituted with the cTnI inhibitory peptide are able to undergo sequential contraction-relaxation cycles (32). The importance of the inhibitory peptide in skeletal muscle is also supported by the inability of mutant fsTnI, missing the inhibitory region, to inhibit ATPase activity in vitro (33). However, our data indicate that in addition to the inhibitory region, two additional sites in cTnI, located C-terminal to the inhibitory region, are essential for the expression of maximum inhibition.

From our analysis of the effect of C-terminal deletions on the ability of cTnI to inhibit ATPase activity (Fig. 5), we deduce that the inhibitory region inhibits only about 50% of the ATPase activity. Two additional sites, between residues 152–188 and residues 189–199, contribute approximately 25% each to the inhibition of ATPase activity, as shown by reconstitution with mutants cTnI-(1–188) and cTnI-(1–199), respectively (Fig. 5). One interpretation of these data is that there are two additional actin-Tm binding sites located in the C-terminal domain of cTnI. Farah et al. (13) and Triplet et al. (34) also postulate the presence of an additional actin-Tm binding site located on the C-terminal side of the inhibitory region in fsTnI (residues 136–148). The second putative actin-Tm binding site in cTnI, comprising 10 amino acids (cTnI residues 189–199; fsTnI residues 157–167), does not appear to function in skeletal muscle. On the other hand, based on evidence that there is complete absence of inhibitory activity with removal of the inhibitory region (residues 104–120) in fsTnI (13, 33), we also cannot exclude the possibility that binding of the inhibitory region to actin-Tm induces conformational changes in actin-Tm that expose sites, which can then bind to actin-Tm binding sites located in the C-terminus of cTnI.

In a model of control of thin filament activation, Lehrer (35) suggested that cTnI may be involved in stabilizing a "blocked state" of the thin filament by sterically blocking the actin-myosin interaction, and that Ca\(^{2+}\) induces movement of cTnI away from actin. TnI would need to be in an elongated configuration and span at least several actin monomers to be involved in a blocking function. In a model of Ca\(^{2+}\)-saturated fsTnI-fsTnC complex derived from small-angle x-ray scattering data, fsTnI adopts an extended conformation in the presence of fsTnC and Ca\(^{2+}\) that is about 115 Å long (36). An actin monomer is estimated to be about 40 Å in diameter (37); thus, TnI may span 2–3 monomers of actin. Residues C-terminal to the inhibitory region may be critical for extension of TnI to stabilize a blocked state of the thin filament, especially in the case of cTnI, which has an additional 32 amino acids at the N terminus.

In addition to the importance of residues C-terminal to the inhibitory region of cTnI in expression of maximal inhibition of ATPase activity, this C-terminal region of cTnI appears to be essential for Ca\(^{2+}\)-dependent regulation of cardiac myofilament contraction. We observed that the the cTnI-(1–188) mutant had impaired ability to regulate Ca\(^{2+}\) sensitivity in myofibrils and the cTnI-(1–151) mutant had lost the ability to regulate Ca\(^{2+}\) sensitivity (Fig. 6). This loss of Ca\(^{2+}\)-dependent control of the myofilament after deletion of portions of the C terminus of cTnI implies an alteration in the interaction of cTnI and cTnC, most likely through removal of a binding site for cTnC in the C-terminal region of cTnI. Evidence, derived largely from studies on fast skeletal myofilaments, indicates multiple sites of interaction between fsTnI and fsTnC (13, 38, 39). Experiments have revealed that fsTnC may bind C-terminal to the inhibitory region of fsTnI. A fsTnI peptide, fsTnI\(_{96–148}\), which extends about 30 residues C-terminal to the inhibitory region (96–114), had tighter binding to fsTnC than the inhibitory peptide, fsTnI\(_{96–114}\) (40). Kobayashi et al. (41) found that residues 132–141 of fsTnI (corresponding to cTnI\(_{166–174}\)) cross-linked to Cys-12 in the N terminus of fsTnC. Moreover, an N-terminal part of fsTnC (cTnC\(_{46–78}\)) not only cross-linked to the inhibitory region, fsTnI\(_{96–114}\), but to residues 122–152 of fsTnI (cTnI\(_{156–186}\)). It has also been demonstrated that in the presence of Ca\(^{2+}\), Cys-133 of fsTnI (cTnI\(_{167}\)) moves 0.7 nm toward Cys-98 in the central helix of fsTnC (42, 43) and 1.5 nm away from actin (44). This latter finding underscores the importance of the C-terminal part of TnI in the Ca\(^{2+}\)-switch mechanism. In our experiments, evidence for a TnC binding site C-terminal to the inhibitory region comes from the finding that cTnC was not able to overcome the inhibition of maximal ATPase activity by the cTnI-(1–151) mutant in the presence of Ca\(^{2+}\) (Fig. 6).

The loss of inhibitory function after deletion of either 23 (cTnI-(1–188)) or 60 (cTnI-(1–151)) residues (Fig. 5) suggests a weakened interaction of cTnI with actin-Tm, which would promote the availability of actin-Tm for reaction with myosin. A weakened cTnI-actin-Tm interaction could also explain the apparent increase in Ca\(^{2+}\) sensitivity seen with the cTnI-(1–188) mutant (Fig. 7). The evidence for a proposed single cTnC binding domain downstream from the inhibitory region is based on the data presented in Fig. 6. Although inhibition of Mg\(^{2+}\)-ATPase activity was reduced in myofibrils reconstituted with the cTnI-(1–188) mutant, addition of cTnC and Ca\(^{2+}\) almost completely restored the Mg\(^{2+}\)-ATPase activity. However, addition of cTnC and Ca\(^{2+}\) to myofibrils reconstituted with the cTnI-(1–151) mutant could not reverse the inhibition of the Mg\(^{2+}\)-ATPase activity (Fig. 6). These results indicate the presence of a Ca\(^{2+}\)-dependent cTnC binding domain within residues 152–188. Thus, this region contains both actin-Tm and cTnC binding domains. The existence of these additional actin-Tm and cTnC binding sites C-terminal to the inhibitory region add to the complexity of thin filament protein interactions and may also be an important component of the Ca\(^{2+}\) regulation of thin filament activation.
Although our study has focused on the role of the C-terminal domain of cTnI in Ca\textsuperscript{2+} regulation of cardiac myofilaments, it is also important to emphasize that cTnI contains an unique N-terminal extension that undergoes covalent modification by protein kinase A, which decreases the pCa\textsubscript{50} for activation of the myofilaments. The mechanism involves phosphorylation of cTnI at Ser 23,24 and an increase in the rate of dissociation of Ca\textsuperscript{2+} from the N terminus of cTnC (45). This effect appears to involve global changes in the C-terminal domain of cTnI with cTnC and/or actin-Tm. Dong et al. (46) have demonstrated this global change by using fluorescence resonance energy transfer to determine a decrease in mean distance between N- and C-terminal regions of cTnI induced by protein kinase A phosphorylation. Moreover, Chandra et al. (47) have demonstrated that phosphorylation of the N terminus of cTnI by protein kinase A is able to depress Ca\textsuperscript{2+} binding to an N-terminal fragment of cTnC (cTnC-(1–89). Thus, covalent modifications at the N terminus of cTnI are sensed by the C terminus of cTnI, which appears able to modulate the interaction of the N-terminal domain of cTnC with Ca\textsuperscript{2+}.

In conclusion, our results contribute new information to the detailed understanding of the mechanism by which cTnI participates in the Ca\textsuperscript{2+} switch of the heart, and are important in the context of both cardiac physiology and pathophysiology. There is now evidence that mutations in the C-terminal region of the cTnI molecule are causal in familial hypertrophic cardiomyopathy in the Japanese population (48). Our results suggest that such mutations in cTnI could have severe effects on the control of cardiac myofilaments by Ca\textsuperscript{2+}. Moreover, the region of cTnI missing in the cTnI-(1–151) mutant, which interacts with the N terminus of cTnC, could be important with regard to rational design of pharmacological agents useful in heart failure.

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