The heart-specific NH2-terminal extension regulates the molecular conformation and function of cardiac troponin I

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Akhter S, Zhang Z, Jin JP. The heart-specific NH2-terminal extension regulates the molecular conformation and function of cardiac troponin I. Am J Physiol Heart Circ Physiol 302: H923–H933, 2012. First published December 2, 2011; doi:10.1152/ajpheart.00637.2011.—In addition to the core structure conserved in all troponin I isoforms, cardiac troponin I (cTnI) has an ~30 amino acids NH2-terminal extension. This peptide segment is a heart-specific regulatory structure containing two Ser residues that are substrates of PKA. Under β-adrenergic regulation, phosphorylation of cTnI in the NH2-terminal extension increases the rate of myocardial relaxation. The NH2-terminal extension of cTnI is also removable by restrictive proteolysis to produce functional adaptation to hemodynamic stresses. The molecular mechanism for the NH2-terminal modifications to regulate the function of cTnI is not fully understood. In the present study, we tested a hypothesis that the NH2-terminal extension functions by modulating the conformation of other regions of cTnI. Monoclonal antibody epitope analysis and protein binding experiments demonstrated that deletion of the NH2-terminal segment altered epitopic conformation in the middle, but not COOH-terminal, region of cTnI. PKA phosphorylation produced similar effects. This targeted long-range conformational modulation corresponded to changes in the binding affinities of cTnI for tropinin T and for tropinin C in a Ca2+-dependent manner. The data suggest that the NH2-terminal extension of cTnI regulates cardiac muscle function through modulating molecular conformation and function of the core structure of cTnI.

The contraction of cardiac muscle is regulated by binding of cytosolic Ca2+ to troponin, which activates cross bridge cycling between sarcomeric myosin and actin filaments. The troponin complex consists of three protein subunits: the Ca2+-binding subunit tropinin C (TnC), the tropomyosin-binding subunit tropinin T (TnT), and the inhibitory subunit tropinin I (TnI) (19). The function of TnI is essential to cardiac muscle contraction (34).

Three homologous genes are present in vertebrate species encoding the fast skeletal muscle, slow skeletal muscle, and cardiac isoforms of TnI (20, 29). Cardiac TnI (cTnI) is the newest member evolved in the family of TnI isoform genes (7). In addition to the ~180 amino acids core structure that is highly conserved in the three TnI isoforms in all vertebrates, cTnI has a unique NH2-terminal extension of ~30 amino acids, which is not found in the two skeletal muscle TnI isoforms.

Showing functional conservation and exchangeability among the TnI isoforms, embryonic cardiac muscle utilizes solely slow skeletal muscle TnI. During development, it is completely replaced by cTnI in the adult heart (22, 36). Therefore, the NH2-terminal extension of cTnI is not an essential structure for the basic contractility of cardiac muscle but an added structure specific to the adult heart.

The NH2-terminal extension of cTnI does not contain binding sites for TnC, TnI, and other known myofilament proteins (34). However, it contains two Ser residues (i.e., Ser23/24 in mouse cTnI) that are substrates of PKA (37). Upon β-adrenergic stimulation, phosphorylation of Ser23/24 by PKA lowers myofilament Ca2+-affinity and facilitates cardiac muscle relaxation (30). Therefore, the NH2-terminal extension of cTnI is an adult heart-specific regulatory element.

A deletion of the NH2-terminal extension of cTnI by restrictive proteolysis occurs in cardiac adaptation to hemodynamic stress and β-adrenergic deficiency (14, 33, 45). This posttranslational modification selectively removes the NH2-terminal extension without destruction of the conserved core structure of cTnI. The NH2-terminally truncated cTnI (cTnI-ND) remains associated with the cardiac myofilament and functionally (45). Genetically modified mice with solely cTnI-ND in the postnatal heart are viable through adulthood (15). cTnI-ND increased the relaxation velocity of ventricular muscle, which in turn increased the tolerance of cardiac function to decreased preload (1) and sustained cardiac output via Frank-Starling mechanism (14). In contrast with the chronic and permanent switch from slow TnI to cTnI during postnatal heart development (22, 36), cTnI-ND produced by restrictive proteolysis will be replaced by newly synthesized intact cTnI in several days during the normal turnover of myofilament proteins. Therefore, similar to PKA phosphorylation of Ser23/24, this restrictive proteolysis is an acute and reversible mechanism to posttranslationaly regulate cardiac function.

It is important to note that the phosphorylation of Ser23/24 and the restrictive NH2-terminal truncation both lower myofilament Ca2+-affinity and increase the rate of cardiac muscle relaxation (1, 14, 30). The similar outcomes of the two different structural modifications indicate a common molecular mechanism for the NH2-terminal extension to regulate the function of cTnI.

Previous studies suggested that the NH2-terminal extension of cTnI might participate in inter- or intramolecular interactions. One model is that in the absence of Ser23/24 phosphorylation, the NH2-terminal extension of cTnI weakly interacts with the N-lobe of TnC and stabilizes its Ca2+-bound conformation. Phosphorylation of Ser23/24 weakens this interaction and reduces the stabilizing effect, thus accelerating Ca2+ release (16, 17, 41). Cross-linking experiments using cTnI mutants with cysteines introduced at positions 5, 10, 18, and/or 26 indicated that the NH2-terminal extension was in contact with the N-lobe of TnC in the troponin complex in the presence of Ca2+ and to a lesser extent in the absence of Ca2+ (41, 42).
Consistently, NMR studies demonstrated that the NH2-terminal extension of cTnI in the absence of phosphorylation interacted with the N-domain of cardiac TnC via residues immediately downstream of the phosphorylation sites (40). Phosphorylation of Ser23/24 weakened this interaction (40, 41).

Another model proposed that phosphorylation of Ser23/24 increased the affinity of the switch peptide of cTnI to the N-domain of cardiac TnC (2). NMR showed that the phosphorylation of Ser23/24 extended and stabilized the local helix (residues 21–30) in the NH2-terminal extension of cTnI, which weakened the interaction of cTnI with the N-lobe of TnC and repositioned the NH2-terminal extension for interaction with the inhibitory region of cTnI (21).

These models are based on interactions of the NH2-terminal extension of cTnI with TnC or intramolecularly with another region of cTnI to elicit functional effects. In addition, studies using fluorescence-labeled cTnI suggested that the phosphorylation of Ser23/24 led to substantial changes in the conformation of the NH2-terminal extension of cTnI and a more compact hydrodynamic shape of the protein (10). Förster resonance energy transfer experiments showed that the mean distance between the NH2- and COOH-terminal portions of cTnI decreased by 9–12 Å upon phosphorylation of Ser23/24, indicating a change in the overall molecular conformation (9).

In the present study, we investigated the hypothesis that the NH2-terminal extension regulates the function of cTnI by modulating the conformation of other regions in the TnI core structure. The NH2-terminal extension of cTnI was not resolved in the crystal structure of cardiac troponin complex (38), precluding direct observation of the effect of its phosphorylation or proteolysis on the conformation of specific regions of TnI. Therefore, we employed monoclonal antibody (mAb) epitope analysis to demonstrate that the restrictive deletion of the NH2-terminal extension and PKA phosphorylation had similar effects on altering epitopic conformations in the middle region of cTnI. Protein-binding assays showed that the NH2-terminal extension-terminated conformational changes in the core structure of cTnI resulted in effects on the binding affinity for TnC and TnT, revealing a novel mechanism to modulate the Ca2+ regulation of cardiac muscle contraction.

MATERIALS AND METHODS

All protocols involving the use of experimental animals were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the Guiding Principles in the Care and Use of Animals, as approved by the Council of the American Physiological Society.

Cardiac TnI and engineered fragments. Bovine cTnI was purified from adult left ventricular muscle as described previously (4). Intact pET3d mouse cTnI was expressed from cloned cDNA in Escherichia coli culture. The construction of recombinant pAED4 expression plasmid, preparative scale protein expression and purification were done as described previously (5).

An NH2-terminal truncated mouse cTnI with a deletion of amino acids 1–28 (cTnI-ND) was engineered in pET3d expression plasmid and prepared by bacterial expression as described previously (5). An NH2-terminal and COOH-terminal truncated mouse cTnI (cTnI29–192) was engineered using the cTnI-ND expression plasmid as template by PCR to create a translational stop codon to replace codon R193. The PCR product with a 5’ Xhol in the vector sequence and a 3’ EcoRI site built in the PCR primer was digested with the restriction enzymes and cloned into pET3d vector. The cDNA insert was sequenced to verify the construction and authenticity.

cTnI29–192 protein was expressed by transformation of BL21(DE3)pLySS E. coli with the expression plasmid. Freshly transformed bacterial cells were cultured in 2× TY liquid media of (in g/l) 16 Tryptone, 10 yeast extract, 5 NaCl, and 1.32 NaH2PO4 (pH 7.3) containing 100 mg/ml ampicillin and 25 mg/l chloramphenicol at 37°C with vigorous shaking, and the expression of cTnI29–192 was induced with 0.4 mM isopropyl-1-thio-β-D-galactoside at early log phase of growth. After 3 h of culture under induction, the bacterial cells were harvested by centrifugation, suspended in 2.5 mM EDTA and 50 mM Tris-HCl (pH 8.0), and lysed by three passages through a French press.

Inclusion bodies that contain cTnI29–192 protein were washed with high salt buffer containing 50 mM Tris-HCl, 5 mM EDTA, and 1 M KCl (pH 8.0). The pellets were dissolved in 6 M urea, 0.1 mM EDTA, 6 mM β-mercaptoethanol, and 10 mM imidazole-HCl (pH 7.0), and clarified by centrifugation before loading to a CMS2 column for cation-exchange chromatography. The column was eluted by 0–500 mM linear KCl gradient, and the protein peaks were analyzed by SDS-PAGE. Fractions containing cTnI29–192 were dialyzed, lyophilized, and further purified by Sepharose G75 gel filtration chromatography in 6 M urea and (in mM) 500 KCl, 0.1 EDTA, 6 mM β-mercaptoethanol, and 10 imidazole-HCl (pH 7.0). Protein peaks were analyzed by SDS-PAGE and the fraction containing pure cTnI29–192 was dialyzed against 0.1% formic acid and lyophilized. The purification was carried out at 4°C.

Anti-cTnI mAbs. A mouse mAb TnI-1 was developed previously by immunization with purified chicken fast skeletal muscle TnI (28). TnI-1 cross-reacts with cardiac and skeletal muscle TnIs in all vertebrate species examined, and its epitope was located in the COOH terminal end segment of the TnI polypeptide chain (i.e., amino acids 193–211 of mouse cTnI).

Two other mouse mAbs, 4H6 (IgG2bκ) and 4B7 (IgG2aκ), were developed from immunization of a Balb/c mouse using purified mouse cTnI29–192. The fusion of mouse spleen cells with SP2/0 myeloma cells, hybridoma screening, and limiting dilution subcloning was performed as described previously (39).

SDS-PAGE and Western blotting. As described previously (5), cTnI preparations or total protein extracts from mouse hearts were bisacrylamide gel sample buffer containing 2% SDS. The samples were resolved by 14% SDS-PAGE with an acrylamide:bisacrylamide ratio of 29:1. The protein bands resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose membrane for Western blotting. Following blocking in 1% bovine serum albumin (BSA), the membrane was incubated with an anti-TnI mAb at 4°C overnight. The blots were washed and incubated with alkaline phosphatase-labeled anti-mouse IgG second antibody (Santa Cruz) followed by final washes before 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium substrate reaction.

Peptide mapping of mAb epitopes. Purified mouse cTnI29–211 (cTnI-ND) protein (2 mg/ml) was incubated with low or high concentration of chymotrypsin (1 μg/ml or 5 μg/ml, respectively) in 0.1 M ammonium bicarbonate buffer (pH 8.0) at room temperature to produce limited fragmentation for the mapping of 4H6 and 4B7 mAb epitopes. Samples were withdrawn from the reaction mixtures at 5, 10, 20, and 40 min, and the reaction was terminated by adding half volume of 3× SDS-PAGE sample buffer and heating at 90°C for 5 min. The serial digestions were examined on 15% SDS-PAGE with an acrylamide:bisacrylamide ratio of 29:1, and the protein bands were visualized with Coomassie Brilliant Blue R250 staining. Selected samples were examined in Western blot analysis using polyvinylidene difluoride membranes and mAbs TnI-1, 4H6, and 4B7 as described above.

Phosphorylation of recombinant mouse cTnI. Recombinant non-phosphorylated mouse cTnI produced in E. coli (0.16 mg/ml) was incubated with 0.1 mg/ml rabbit skeletal muscle cAMP-dependent PKA (Sigma Chemical) in a buffer containing 50 mM Tris-HCl, 0.1 M NaCl, 10 mM MgCl2, 1 mM DTT, 2 mM ATP, and 0.1 mM CaM at pH 7.5...
at 30°C for 2 h and 15 min. The reaction was terminated by freezing the mixture at −80°C. The PKA-catalyzed phosphorylation of cTnI was examined by staining SDS-PAGE gels with the Pro-Q Diamond reagent (Invitrogen). A parallel gel was stained with Coomassie Brilliant Blue R250 to determine the amount of total cTnI in the samples.

ELISA epitope analysis for conformational changes of cTnI induced by NH2-terminal truncation or phosphorylation. Antigen-antibody binding is a protein-protein interaction that depends on conformational fits between three-dimensional structures at the antigenic epitope and the antibody paratope. mAbs provide reagents for recognizing specific epitopes and can be used in monitoring protein conformation, structural dynamics, and folding (18). We have applied antibody probes against epitopes in allosteric proteins to detect conformational differences and changes using high throughput microtiter plate ELISA under native conditions (23, 24, 27, 39).

Using this highly efficient approach and mAb probes recognizing epitopes located in different regions of cTnI, we investigated the effects of NH2-terminal deletion or PKA phosphorylation of Ser23/Ser24 on the molecular conformation of cTnI. As described previously in detail for the conformational studies of TnT (39), intact mouse cTnI (McTnI), McTnI-ND, or PKA phosphorylated McTnI (1 μg/ml) in a buffer containing 100 mM KCl, 3 mM MgCl2, and 20 mM PIPES (pH 7.0; buffer A) was coated on microtiter plates (Immulon 2HB; Thermo Scientific) at 4°C overnight. Following washes with buffer A containing 0.05% Tween 20 (buffer T) to remove unbound protein and blocking the remaining plastic surface, the wells were incubated with serial dilutions of mAb TnI-1, 4H6, or 4B7 in buffer A containing 0.1% BSA for ELISA affinity titration. Washed with buffer T to remove unbound antibody, the wells were further incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG second antibody (Santa Cruz). After washes to remove the unbound second antibody, the plates were developed with H2O2-ABTS [H2O2-2′-azinobis-(3-ethylbenzthiazolinesulfonic acid)] substrate in 0.1 M citrate buffer (pH 4.0). A415nm for each assay well was monitored with an automated microplate reader (Bio-Rad Benchmark), and the titration curves were plotted to quantify the affinity between the anti-TnI mAbs and their specific epitopes. The experiments were carried out in triplicate wells and repeated.

Fig. 1. The heart-specific NH2-terminal extension of cardiac troponin I (cTnI) and peptide mapping of monoclonal antibody (mAb) epitopes. A: heart-specific NH2-terminal extension of cTnI is shown by aligning the 3 muscle type TnI isoforms. The cardiac troponin C (TnC) and cardiac troponin T (TnT) binding segments in the conserved regions are outlined. The PKA phosphorylation sites in the NH2-terminal extension of cTnI and the site for its selective removal by restrictive proteolysis to generate NH2-terminal truncated cTnI (cTnI-ND; McTnI29–211) are indicated. Three possible chymotrypsin fragments generated from cleavage at Y30, Y113, and Y134 are outlined, and the position of K118C mutation is indicated. The location of mAb TnI-1 epitope in the COOH-terminal end segment determined previously is also indicated. B: limited chymotrypsin digestion of cTnI-ND was carried out at high (5 μg/ml) and low (1 μg/ml) enzyme concentrations for a series of time periods. The protein fragments produced were detected in the SDS-PAGE gels. C: peptide specificity of mAbs 4H6 and 4B7 was mapped by Western blotting of cTnI fragments generated from 10 min and at 40 min digestions with low and high concentrations of chymotrypsin, respectively. With the use of mAb TnI-1 recognizing the COOH-terminal end of cTnI as an anchored reference to identify the chymotryptic fragment 135–211, the Western blots showed that mAb 4H6 recognized the large middle fragment of mouse cTnI-ND (fragment 31–134) but not the small fragment 114–134, localizing its epitope to the region between amino acids 31 and 113. In contrast, mAb 4B7 recognized both fragment 31–134 and fragment 114–134, placing its epitope in the region of amino acids 114–134. The arrowheads in B and C indicate chymotryptic products of cTnI-ND, which showed an inverted quantitative relationship to fragment 135–211 and weakly recognized by mAb TnI-1, implicating COOH-terminal truncations of fragment 135–211. The amino acid numbers describing the cTnI fragments reflect that in mouse cTnI. MW, molecular weight.
Locating mAb epitopes with TnC blocking experiments. ELISA blocking experiments were carried out to investigate the spatial relationship between the mAb 4H6 and 4B7 epitopes and the TnC-binding region of cTnI. Mouse cTnI was coated on a microtiter plate at 0.2 μg/ml, 100 μl/well at 4°C overnight. After excess cTnI was removed by washing with buffer T, the plate was incubated with or without 1 μM TnC in buffer B (buffer T containing 0.1% BSA) at pCa 4 at room temperature for 30 min. Serial dilutions of mAbs 4H6, 4B7, and TnI-1 in buffer B at pCa 4 were added, and the plate was shaken in a microplate reader for 3 × 10 s to mix the reaction solution. After further incubation at room temperature for 1 h, the binding of the mAbs to TnI in the presence or absence of TnC was determined with ELISA at pCa 4 as described above using HRP-conjugated anti-mouse IgG secondary antibody. The experiments were done in triplicate wells and repeated.

Locating mAb epitopes using a cTnI point mutation. Cardiac TnI was partially purified from hearts of wild-type and double transgenic mice expressing K118C mutant cTnI in the absence of endogenous cTnI gene (43). The cardiac tissue was homogenized in ice-cold buffer containing 10 mM Tris·HCl (pH 8.0), 0.1 mM PMSF, and 0.5 mM DTT. The homogenate was centrifuged at 4,300 g for 10 min, and the pellet was washed once with the same buffer before being extracted in 1 M KCl, 50 mM Tris·HCl (pH 8.0), 1 mM DTT, 1 mM EGTA, and 0.5 mM PMSF at 4°C for 2 h. Troponin subunits in the supernatant were then enriched by (NH4)2SO4 precipitation between 20% and 40% saturation, and the fraction was dialyzed against 10 mM Tris·HCl and 0.1 mM EGTA (pH 8.0) until the conductivity was below 2 mS/cm. Solid urea was added to 6 M, PMSF to 0.1 mM, DTT to 1 mM, and Tris·HCl to 10 mM, and the pH was adjusted to 8.0. The sample was then centrifuged at 17,000 g for 10 min to remove any precipitates and fractionated by CM52 anion exchange chromatography at 4°C (pH 7.0). The column was eluted with 0–500 mM KCl in 6 M urea, 10 mM Tris·HCl (pH 8.0), 0.1 mM EGTA, and 1 mM DTT were coated on microtiter plates, and ELISA titrations were carried out as above to examine the effect of K118C mutation on the affinity of mAb 4B7. The affinity of mAb TnI-1 was titrated as a control.

Solid phase protein binding experiments. ELISA-based protein binding experiments (5, 39) were utilized to determine the effect of conformational changes in TnI on its affinity for TnT and TnC. Bovine cardiac TnT was purified from adult ventricular muscle as described previously (25). Chicken fast TnC was expressed from cloned cDNA and purified as described previously (24). Microtiter plates were coated as above except that the TnI proteins were at a concentration of 2.5 μg/ml and the coating buffer A contained 1 mM DTT. After removal of excess protein by wash with buffer T containing 1 mM DTT, the wells were blocked with buffer T containing 1% BSA and 1 mM DTT at room temperature for 1 h. After rinse with buffer T containing 1 mM DTT, the wells were incubated with serial dilutions of bovine cardiac TnT in Buffer B and 1 mM DTT at room temperature for 2 h. The plates were then washed with buffer T to remove unbound TnT and reducing agent, incubated with an anti-TnT mAb CT3 at room temperature for 1 h, and washed again. The plates were then incubated with HRP-conjugated anti-mouse immunoglobulin secondary antibody at room temperature for 1 h. After washes to remove excess secondary antibody, the substrate reaction and A415nm

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Fig. 2. Effect of deleting the NH2-terminal extension on the core conformation of cTnI. ELISA epitope analysis was carried out for intact cTnI and cTnI-ND to investigate the conformational effect of removing the NH2-terminal extension. Three mAbs recognizing epitopes in the middle and COOH-terminal regions of cTnI were used as structural probes (Fig. 1). A: results showed that the binding affinity of mAb TnI-1 did not change after deletion of the NH2-terminal extension, indicating no detectable conformational effect on the very COOH-terminal region recognized by TnI-1. B: deletion of NH2-terminal extension also did not alter the conformation of the epitope recognized by mAb 4H6 in the region of amino acids 31–113 of mouse cTnI as shown by the similar affinity titration curves against intact cTnI and cTnI-ND. C: however, deletion of the NH2-terminal extension decreased the binding affinity of mAb 4B7 that recognizes an epitope in the region of amino acids 114–134 of mouse cTnI, indicating a targeted long-range conformational effect.
recording were performed as above. Data within the linear course of the color development were used to plot the cTnI-TnT binding curves. cTnI-TnC binding experiments were carried out similarly with the following modifications. After washing with buffer T containing 1 mM DTT, the wells were incubated with TnC in buffer B containing 5 mM EGTA, 5 mM CaCl$_2$ (pCa4), and 1 mM DTT or buffer B containing 5 mM EGTA and 11 μM CaCl$_2$ (pCa 9) and 1 mM DTT. The wells were then washed with buffer T at pCa 4 or pCa 9. The binding of TnC to immobilized TnI was detected using an anti-TnC mAb 2C3 (24), and the ELISA steps were the same as above, except that the washing and antibody dilution buffers were maintained at either pCa 4 or pCa 9.

Data analysis. Protein sequence analysis was done using DNAStar software (Lasergene). Statistic analysis was performed using Student’s t-test.

RESULTS

The mAb 4H6 and 4B7 epitopes in the middle region of cTnI. Figure 1A is a linear illustration of the structure of cTnI. Mouse cTnI-ND (amino acids 29–211) was used in the peptide mapping of 4H6 and 4B7 epitopes. Corresponding to predicted cleavages at Y$_{30}$, Y$_{113}$, and Y$_{134}$, the SDS-PAGE gel in Fig. 1B identified three fragments generated from limited chymotrypsin digestion of McTnI-ND. The Western blots in Fig. 1C showed their reactivity with the three anti-cTnI mAbs used in the present study. It is known that the mAb TnI-1 epitope is at the COOH-terminal end of TnI (28; Fig. 1A) and the chymotryptic band recognized by TnI-1 was identified as the COOH-terminal fragment 135–211 (predicted Mr = 8,951) of McTnI-ND. Both mAb 4H6 and mAb 4B7 recognized the larger chymotryptic band predicted to be fragment 31–134 (Mr = 12,122). 4B7 but not 4H6 recognized the small chymotryptic band predicted to be fragment 114–134 (Mr = 2,393). Altogether, the peptide mapping results located the 4H6 and 4B7 epitopes to the segments of amino acids 31–113 and 114–134, respectively, in mouse cTnI.

Fig. 3. Effect of NH$_2$-terminal phosphorylation on the core conformation of cTnI. A: pro-Q Diamond (Pro-Q) and Coomassie Blue staining of bacterially expressed mouse cTnI in SDS-gel showed effective phosphorylation after PKA treatment. In vivo phosphorylated bovine cTnI was used as a positive control. B: mAb epitope analysis for the effect of PKA-catalyzed phosphorylation of Ser$_{23/24}$ in the NH$_2$-terminal extension on the molecular conformation of cTnI. The results showed that the NH$_2$-terminal phosphorylation did not alter the COOH-terminal conformation of cTnI since the binding affinity of mAb TnI-1 was identical before and after PKA treatment. C: similarly, the NH$_2$-terminal phosphorylation did not alter the conformation of the 4H6 epitope in the middle region of cTnI as shown by the unchanged affinity titration curve. D: however, NH$_2$-terminal phosphorylation resulted in a conformational change in the epitope recognized by mAb 4B7 in the region of amino acids 114–134 of mouse cTnI as detected by the decreased binding affinity of mAb 4B7 after PKA treatment.
Selective deletion of the NH2-terminal extension of cTnI altered the molecular conformation in the middle region. ELISA epitope analysis using the three anti-cTnI mAbs against epitopes outside of the NH2-terminal extension as conformational probes found that mAb TnI-1 exhibited identical affinity to intact and NH2-terminal truncated mouse cTnI (Fig. 2A), indicating that the deletion of the NH2-terminal extension did not cause detectable conformational change in the COOH-terminal end segment recognized by TnI-1 (Fig. 1A). Similarly, the results in Fig. 2B showed that the deletion of NH2-terminal extension of cTnI did not alter the conformation of the epitope structure recognized by mAb 4H6 in the region of amino acids 31–113.

In contrast, ELISA affinity titration detected that the deletion of NH2-terminal extension resulted in decreased binding affinity of mAb 4B7 that recognizes the segment of amino acids 114–134 in the middle region (Fig. 2C), demonstrating a long-range conformational effect on a specifically targeted region of the cTnI molecule. These data concur with the observation that restrictive removal of the NH2-terminal extension of cTnI in hemodynamic adaptation and heart failure (14, 45) does not cause global destruction of cTnI but functions as a specific regulatory mechanism.

PKA phosphorylation of Ser23/24 in the NH2-terminal extension produced conformational change in cTnI similar to that of NH2-terminal truncation. Pro-Q Diamond staining of SDS-PAGE gel demonstrated that the recombinant mouse cTnI expressed in E. coli was, as expected, nonphosphorylated. Treatment with PKA that is known to phosphorylate cTnI at residues Ser23 and Ser24 (37) resulted in significant phosphorylation of the bacterially made mouse cTnI (Fig. 3A).

Epitope conformational analysis using mAbs TnI-1, 4H6, and 4B7 showed that the phosphorylation of Ser23/24 in the NH2-terminal extension of cTnI did not have detectable effect on the conformations of the mAb TnI-1 epitope in the COOH-terminal region (Fig. 3B) or the mAb 4H6 epitope in the segment of amino acids 31–113 (Fig. 3C), but produced a detectable conformational change in the mAb 4B7 epitope corresponding to the segment of amino acids 114–134 as shown by the decreased binding affinity (Fig. 3D). This pattern was similar to that resulted from restrictive deletion of the NH2-terminal extension (Fig. 2).

Fig. 4. Localization of mAb 4H6 epitope to a troponin C (TnC)-binding site of cTnI. The mAb titration curves from ELISA blocking experiments plotted with both absolute absorbance values (A415nm), and percentage of maximum binding showed that the association of TnC did not affect the binding of mAb TnI-1, consistent with the nonoverlapping spatial relationship between the TnC binding sites and the COOH-terminal end segment of TnI (A) (38); the binding of mAb 4H6 was significantly blocked by the presence of TnC, indicating a close spatial relationship between the 4H6 epitope and the TnC binding site of cTnI (B); and the association of TnC to cTnI did not decrease but detectably increased the affinity of mAb 4B7, indicating a nonblockage relationship between the binding of TnC and the conformation of cTnI at the 4B7 epitope (C).
Spatial relationship between mAb 4H6 epitope and the TnC-binding site 1 of cTnl. ELISA experiments at pCa 4 detected that binding of mAb 4H6 (Fig. 4B), but not of TnI-1 (Fig. 4A), was significantly blocked by the presence of TnC, indicating a close spatial relationship between the 4H6 epitope and TnC binding sites of cTnl. Based on the crystallographic structure of cardiac troponin, the blockage of the 4H6-epitope in the amino acids 31–113 region of cTnl (Fig. 1) by the binding of TnC indicated that the 4H6-epitope is directly related to the amphiphilic portion of α-helix 1 (TnC-binding site 1, residues 43–65 in human cTnl) that is involved in tight binding to the C-lobe of TnC (38).

The binding of TnC did not have any blocking effect on mAb 4B7 but, instead, increased the affinity of 4B7 (Fig. 4C). This finding suggested that the binding of TnC at pCa 4 resulted in an altered exposure and/or flexibility of the 4B7 epitope of cTnl. This result further indicated that although the 4B7 epitope in the region of amino acids 114–134 is not spatially overlapping with the TnC binding sites of cTnl, it is a dynamic structure that responds to allosteric signals from TnC.

Verification of 4B7 epitope in the TnT-binding helix of cTnl. With the use of partially purified wild-type and K118C mutant mouse cTnl (Fig. 5A), ELISA titrations showed that mAb TnI-1 against a COOH-terminal epitope bound wild-type cTnl and cTnl-K118C with similar affinities (Fig. 5B). In parallel titrations, the affinity of mAb 4B7 for cTnl-K118C was increased compared with that for wild-type cTnl (Fig. 5C). This conformational relationship between the K118C single amino acid substitution and the 4B7 epitope supports the peptide mapping data that located the 4B7 epitope to the segment including amino acids 114–134.

Whereas our experimental condition had a limitation to the quantification of affinity changes by not reaching the saturated binding of 4B7 thus the percent maximum binding was calculated against the highest point in the data set, our goal here was to qualitatively localize the mAb epitope, and, therefore, the relative affinity change caused by the K118C mutation was sufficient for supporting the localization of 4B7 epitope to the region of amino acids 114–134.

Residing in an α-helical structure of cTnl in the TnI-TnT interface (5), the K118C substitution would not result in overall destruction of the coiled coil structure at the TnI-TnT interface as suggested by molecular modeling (5). However, cTnl-K118C is a dominantly negative mutation in transgenic mouse hearts (43) and resulted in a changed overall conformation and/or decreased flexibility, which was detectable even in SDS-PAGE by the decreased gel mobility (5, 43; Fig. 5A). Together with the altered conformation of the 4B7 epitope upon the binding of TnC to cTnl (Fig. 4C), the results demonstrated that the mAb 4B7 epitope is an allosteric structure in cTnl at the TnI-TnT interface and its conformational change upon the removal of, or phosphorylation in, the NH2-terminal extension (Figs. 2 and 3) indicated a novel molecular mechanism in the regulation of tropinin function.

Restrictive NH2-terminal deletion altered the binding affinity of cTnl for TnT. Intact and NH2-terminally truncated mouse cTnl were examined in ELISA solid-phase protein binding experiments for bovine cardiac TnT. The results in Fig. 6A showed that the conformational change in cTnl induced by restrictive deletion of the NH2-terminal extension caused a small but statistically significant decrease in the binding affinity for TnT. Although the difference between the TnI-TnT binding curves was relatively small and the presaturation pattern did not allow the determination of absolute affinity, the difference was statistically significant to support our hypothesis for the NH2-terminal extension to modulate the molecular conformation of cTnl with a long-range functional effect.

Ca2+-dependent effect of the NH2-terminal truncation on the binding affinity of cTnl for TnC. Intact and NH2-terminal truncated mouse cTnl were further examined in ELISA solid-phase protein binding experiments for TnC in the absence or presence of Ca2+. The results in Fig. 6B showed that at pCa 9, the binding affinity of cTnl for TnC was low and the restrictive deletion of the NH2-terminal extension had no detectable
effect. Similar to what was shown previously in classic protein binding experiments (34), the binding affinity of cTnI to TnC was significantly higher at pCa 4 in the solid phase ELISA protein binding experiments. The deletion of the NH2-terminal extension of cTnI further increased the binding affinity for TnC at pCa 4 in a significant manner (Fig. 6C). Together with its effect on the binding of cTnI to TnT, the Ca2+ -dependent effect on the interaction of cTnI with TnC demonstrated a role of the NH2-terminal extension-based conformational modulation in tuning the Ca2+ regulation of cardiac muscle contraction.

**DISCUSSION**

To understand the mechanism for the NH2-terminal extension of cTnI to regulate cardiac muscle function, we investigated in the present study the effects of NH2-terminal modifications on the molecular conformation and function of cTnI. Summarized in Fig. 7, the results suggested new insights into the structure-function relationship of cTnI.

**Long-range conformational modulation in tropinin subunits.**

It is well known that the NH2-terminal segment of TnT, another subunit of troponin, is a variable region with structural differences among muscle type-specific isoforms and regulated by alternative RNA splicing during development and in pathophysiological adaptations (29, 44). Similar to the NH2-terminal extension of cTnI, the NH2-terminal variable region of TnT does not bind any known myofilament protein and can be removed by restrictive proteolysis under stress conditions (13, 46). Extensive studies have shown that the NH2-terminal structural variation had long-range effects on the molecular conformation and function of other regions of TnT (3, 26, 39). Our present study demonstrated that this mechanism also applies to the function of the NH2-terminal extension of cTnI. Extended from previous NMR studies suggesting long-range interactions within cTnI (2, 21), deletion of the NH2-terminal extension or PKA catalyzed NH2-terminal phosphorylation produced conformational changes in the middle region of cTnI corresponding to functional effects on the binding affinity for TnT and TnC. This common feature of the two troponin subunits is consistent with their nature as allosteric regulatory proteins functioning in the Ca2+ signal transduction in muscle thin filament.

The NH2-terminal extension functions by a targeted modulation of the molecular conformation of cTnI. Our results showed that the NH2-terminal deletion selectively altered molecular conformation in the TnT-binding helix of cTnI as indicated by the changes in the 4B7 epitope (Figs. 2C and 7). PKA-catalyzed phosphorylation in the NH2-terminal region...
had a similar effect (Fig. 3D). On the other hand, no such NH₂-terminal-dependent conformational change was detected in the COOH-terminal region (indicated by the mAb TnI-1 epitope; Figs. 2A and 3B) or in the TnC-binding site 1 (indicated by the mAb 4H6 epitope; Figs. 2B and 3C) of cTnI. Functionally, the NH₂-terminal deletion-originated conformational effect targeted on the TnT-binding segment affected the binding affinity of cTnI for TnT (Fig. 6A). These findings suggest that the TnI-TnT interface (the coiled coil I-T arm) (38) in cardiac troponin complex is a target for the NH₂-terminal extension-based regulation of cTnI function. Our recent finding that cTnI-K118C mutation in the TnI-TnT interface blunted the inotropic effect of 8-adrenergic stimulation in transgenic mouse hearts (43) supports functional significance of this targeted regulation.

Binding of TnC at pCa 4 did not block but increased the affinity of mAb 4B7 (Fig. 4C). This observation suggests that the Ca²⁺ signal from TnC may be transmitted to alter the conformation and/or accessibility of the 4B7 epitope. Considering the relationship between the segment of 4B7 epitope and the TnC-binding site 2 of cTnI (Figs. 1A and 7), this region may also directly interact with TnC to respond to Ca²⁺ signaling.

Altogether, the data suggest that the NH₂-terminal extension-based conformational modulation of cTnI targeted to the region of the 4B7 epitope in the I-T interface and possibly involving the TnC-binding site 2 provides a molecular mechanism to regulate cardiac muscle contractility. Detailed mechanistic basis of this regulation requires further investigation. Several studies have demonstrated that conformational changes in TnI are different in isolated protein from that in binary or tertiary troponin complex and in reconstituted thin filaments (8, 11, 12). Further study of the conformational modulation of cTnI in higher order complexes will help to fully understand the functional mechanism of the NH₂-terminal extension.

Phosphorylation of Ser²³/²⁴ and restrictive deletion of the NH₂-terminal extension produced similar functional effects. Previous investigations have extensively studied the function of the NH₂-terminal extension of cTnI as a phosphorylation switch. Downstream of 8-adrenergic regulation of cardiac muscle contractility (37), phosphorylation of Ser²³/²⁴ either perturbs the interaction with the N-lobe of TnC and/or allows new interactions to take place with the other domains of cTnI to elicit functional effects (2, 9, 10, 21, 42).

Supporting functionality of the NH₂-terminal extension, a recent study showed that overexpression of cTnI with deletion of the NH₂-terminal 10 amino acids (cTnI⁰⁻¹⁰) resulted in decreased contractility in transgenic mouse cardiac muscle (35). Functional studies by several laboratories including ours have shown that the NH₂-terminal truncation and PKA phosphorylation of cTnI had similar effects on decreasing myofilament Ca²⁺ sensitivity and increasing the rate of myocardial relaxation (1, 6, 14, 31, 33, 45). The functional effects of NH₂-terminal phosphorylation and truncation were nonadditive (1), suggesting that they converge to the same downstream molecular mechanism. Supporting this hypothesis, our present study further found that the NH₂-terminal phosphorylation and restrictive deletion exhibited the same patterns of effects on cTnI molecular conformation.
Deletion of the NH2-terminal extension of cTnI by restrictive proteolysis as a regulatory mechanism complementary to \(\beta\)-adrenergic stimulation. The restrictive proteolysis of the NH2-terminal extension played a compensatory role to improve diastolic cardiac function when \(\beta\)-adrenergic stimuli were suppressed such as in the cases of \(G_{\alpha}\) deficiency (14) and disruption of the PKA targeting by A-kinase anchoring proteins (33). The observation that the two different NH2-terminal modifications conveyed similar physiological function demonstrated that in addition to the main \(\beta\)-adrenergic signaling pathway, cardiac muscle possesses an additional mechanism to enhance diastolic function.

Because \(\beta\)-adrenergic agonist has complex downstream effects including significant increases in Ca\(^{2+}\) activation of contraction, its effect on increasing the rate of cardiac muscle relaxation has little practical application in clinical treatment of diastolic heart failure. In contrast, removal of the NH2-terminal extension by restrictive proteolysis selectively mimics one of the \(\beta\)-adrenergic downstream mechanisms to increase relaxation rate of the cardiac muscle, providing an attractive target for the development of new treatment for diastolic heart failure. Plausible studies have shown that transgenic overexpression of cTnI-ND improved cardiac function in aged mice (6) and was able to partially rescue the diastolic dysfunction and heart failure in a restrictive cardiomyopathy mouse model (31).

The cellular mechanism controlling the production of cTnI-ND by restrictive proteolysis is not yet understood. Disruption of PKA interaction with A-kinase anchoring proteins in rat hearts in vivo demonstrated that the extent of cTnI-ND production was inversely correlated with the level of PKA phosphorylation (33). A significantly lower level of cTnI-ND was found in cardiomyocytes isolated from rats under \(\beta\)-adrenergic stimulation compared with controls. Therefore, PKA-dependent phosphorylation of cTnI may contribute to the determination of the production of cTnI-ND by restrictive proteolysis. Under conditions of decreased PKA-dependent cTnI phosphorylation, such as that in congestive heart failure, NH2-terminal proteolysis of cTnI may be upregulated as a mechanism to compensate for the suppressed \(\beta\)-adrenergic function (33).

Low levels of cTnI-ND are found in normal heart of a wide range of vertebrates including humans (45), suggesting that this posttranslational modification is an active mechanism in myocardial adaptation other than a destructive degradation. It is also worth noting that cTnI has a short half-life in normal cardiac muscle in vivo (several days as shown in adult dog hearts) (32). Therefore, the proteolytic modification of cTnI could play a transient function and be replaced with newly synthesized intact cTnI after the stress response, making this mechanism a reversible acute regulation like the PKA phosphorylation of Ser\(_{23/24}\). The findings in our study laid a foundation for further investigations.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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