Selective Deletion of the NH_2-Terminal Variable Region of Cardiac Troponin T in Ischemia Reperfusion by Myofibril-Associated μ-Calpain Cleavage†

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ABSTRACT: The structure of the NH_2-terminal region of troponin T (TnT) is hypervariable among the muscle type-specific isoforms and is also regulated by alternative RNA splicing. This region does not contain binding sites for other thin filament proteins, but alteration of its structure affects the Ca^{2+} regulation of muscle contraction. Here we report a truncated cardiac TnT produced during myocardial ischemia reperfusion. Amino acid sequencing and protein fragment reconstruction determined that it is generated by a posttranslational modification selectively removing the NH_2-terminal variable region and preserving the conserved core structure of TnT. Triton X-100 extraction of cardiac muscle fibers promoted production of the NH_2-terminal truncated cardiac TnT (cTnT-ND), indicating a myofibril-associated proteolytic activity. μ-Calpain is a myofibril-associated protease and is known to degrade TnT. Supporting a role of μ-calpain in producing cTnT-ND in myocardial ischemia reperfusion, calpain inhibitors decreased the level of cTnT-ND in Triton-extracted myofibrils. μ-Calpain treatment of the cardiac myofibril and troponin complex specifically reproduced cTnT-ND. In contrast, μ-calpain treatment of isolated cardiac TnT resulted in nonspecific degradation, suggesting that this structural modification is relevant to physiological structures of the myofilament. Triton X-100 treatment of transgenic mouse cardiac myofibrils overexpressing fast skeletal muscle TnT produced similar NH_2-terminal truncations of the endogenous and exogenous TnT, despite different amino acid sequences at the cleavage site. With the functional consequences of removing the NH_2-terminal variable region of TnT, the μ-calpain-mediated proteolytic modification of TnT may act as an acute mechanism to adjust muscle contractility under stress conditions.

Cardiac and skeletal muscle contraction is activated by Ca^{2+} via troponin−tropomyosin in the actin thin filament regulatory system (*1*−*3*). Troponin T (TnT)[1] is the anchoring subunit of the troponin complex (*4*). Three muscle type-specific TnT isoform genes have evolved in higher vertebrates (*5*−*7*), and alternative RNA splicing further produces multiple protein isoforms (*8*−*10*). The various TnT isoforms mainly differ in their NH_2-terminal structures. The amino acid sequence of the NH_2-terminal region of TnT is hypervariable among the cardiac, slow, and fast skeletal muscle TnT isoforms and is regulated by alternative splicing during perinatal heart and muscle development (*8*, *9*). The clearly regulated developmental switches from embryonic to adult TnT isoforms suggest a functional significance of the NH_2-terminal structural variation of TnT.

Nonetheless, the NH_2-terminal region of TnT does not contain any known binding sites for other thin filament proteins (*11*−*13*). Deleting the NH_2-terminal variable region does not diminish the regulatory activity of troponin (*14*−*16*), suggesting that the NH_2-terminal variable region of TnT may function as a modulatory structure. Supporting the hypothesis that alterations in TnT NH_2-terminal structure affect the Ca^{2+} regulation of muscle contraction, a previous study demonstrated that NH_2-terminal alternatively spliced TnT isoforms convey significant changes in the activation of actomyosin ATPase (*17*). Aberrant splicing of cardiac TnT (cTnT) in the NH_2-terminal region is found in both hypertrophic and failing human hearts (*18*) and animal models with dilated cardiomyopathy (*19*, *20*). Consistent with the functional effects, studies showed that NH_2-terminal alterations in TnT affect the overall protein conformation (*21*, *22*) and binding to tropomyosin, TnI, and TnC (*21*, *23*).

Altered TnT isoform gene expression (*24*) and alternative RNA splicing (*18*) have been found during muscle adaptation to stress conditions. In contrast to the relatively slow response at the gene regulation and RNA splicing levels, posttranslational regulation provides a mechanism for rapid adaptation to acute stress. The mostly prominently studied posttranslational mechanism of myofilament protein regulation is phosphorylation (*25*−*27*). Proteolysis is usually considered a deteriorating mechanism in muscle under physiological or pathological stress conditions (*28*, *29*). However, restricted

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[1] Abbreviations: BSA, bovine serum albumin; cTnT, cardiac troponin T; cTnT-ND, NH_2-terminal truncated cTnT; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; p*I*, isoelectric point; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TnC, troponin C; TnI, troponin I; TnT, troponin T.

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proteolysis of cardiac troponin I (TnI) with a deletion of the NH_2-terminal phosphorylation sites has been found in rat cardiac muscle under simulated microgravity conditions (*30*). This specific structural modification of cardiac TnI has been demonstrated to enhance the relaxation of cardiac muscle as compensation to the decrease in cardiac preload in the microgravity model (*31*). It has been reported that NH_2-terminal truncated fast skeletal muscle TnT is produced during post-mortal proteolysis in rabbit (*32*) and porcine muscle (*33*). It is not known whether this modification occurs in vivo.

Calpain is a calcium-activated cysteine protease that has been found to play regulatory modification of proteins (*34*). Two major calpain isoforms have been identified in muscle cells. The μ-calpain (calpain 1) requires micromolar concentrations of calcium to activate, and the m-calpain (calpain 2) requires millimolar concentrations of calcium (*34*, *35*). μ-Calpain is a myofibril-associated enzyme (*34*). It has been observed that μ-calpain can degrade TnT and TnI in vitro (*36*).

In the present study, we found a truncated cTnT produced during myocardial ischemia reperfusion, a stress condition that results in cardiac muscle injuries (*37*). Amino acid sequencing and protein fragment reconstruction determined that it is generated by a posttranslational modification to selectively remove the NH_2-terminal variable region and preserve the conserved core structure of TnT. Triton X-100 extraction of cardiac muscle fibers promoted the production of the NH_2-terminal truncated cTnT (cTnT-ND), suggesting a myofibril-associated proteolytic activity. Supporting a role for μ-calpain in producing cTnT-ND, calpain inhibitors reduced cTnT-ND production in Triton-extracted myofibrils. μ-Calpain treatment of the cardiac myofibril and troponin complex reproduced cTnT-ND. μ-Calpain treatment of isolated cTnT resulted in nonspecific degradation, suggesting that this structural modification is relevant to physiological structures of the myofilament. Triton X-100 treatment of transgenic mouse cardiac myofibrils overexpressing fast skeletal muscle TnT produced similar NH_2-terminal truncations of the endogenous and exogenous TnTs, despite the different amino acid sequences at the cleavage site, indicating that it is the myofilament structure that determines the specific cleavage. With the functional consequences of removing the NH_2-terminal variable region of TnT, the μ-calpain-mediated proteolytic modification of TnT may act as an acute mechanism to adjust muscle contractility under stress conditions. In contrast to the commonly observed proteolytic destruction during ischemia-reperfusion injury (*28*), our finding demonstrates a novel specific modification of the troponin structure as a potentially functional adaptation.

MATERIALS AND METHODS

Cardiac Muscle Tissues. Fresh bovine cardiac muscle was obtained from the local slaughterhouse and kept on ice for ~1.5 h before being frozen at −80 °C prior to use. Fresh rodent cardiac muscles were obtained from Sprague-Dawley rats, Balb/c mice, and C57BL/6 transgenic mice that overexpress the embryonic cTnT and/or exon 7-deleted cTnT (*20*) or chicken fast skeletal muscle TnT (*38*) in the heart.

Double transgenic mice expressing both embryonic and exon 7-deleted cTnT in the adult heart were produced by crossing between homozygous single transgenic parents bearing each of the transgene alleles. The F1 offspring was verified for the double transgenic genotype by PCR analysis of genomic DNA extracted from tail tissue as described previously (*20*).

All animal procedures 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.

Anti-TnT Antibodies. A mouse monoclonal antibody (mAb) CT3 was previously developed by immunization with purified bovine cTnT (*23*). mAb CT3 cross-reacts with slow skeletal muscle TnT but not fast skeletal muscle TnT. The distinct mobility of cTnT and slow TnT in SDS−polyacrylamide gel electrophoresis (SDS−PAGE) allows an easy identification of cTnT in Western blots. The CT3 epitope has been mapped in the central region of TnT (*23*).

A polyclonal rabbit anti-TnT antiserum (RATnT) was previously generated by immunization using purified chicken breast muscle TnT as the antigen (*21*). The RATnT antiserum recognizing multiple epitopes on TnT strongly reacts with chicken fast skeletal muscle TnT and cross-reacts with avian and mammalian cardiac and slow skeletal muscle TnTs.

A mouse mAb 2C8 was previously developed by immunization with human cTnT (*39*). mAb 2C8 recognizes cardiac, slow, and fast TnTs almost equally in Western blots (*39*). The 2C8 mAb epitope is located in the central region of TnT.

SDS−PAGE and Western Blotting. Ventricular muscle tissues or myocytes were homogenized in Laemmli SDS−PAGE sample buffer containing 2% SDS, heated at 80 °C for 5 min, and clarified by centrifugation. Total protein extracts were resolved by 14% Laemmli gel with an acrylamide:bisacrylamide ratio of 180:1 (low cross-linker) or by 15% Laemmli gel with an acrylamide:bisacrylamide ratio of 29:1 (high cross-linker). The gels were stained with Coomassie Brilliant Blue R-250 to reveal the resolved protein bands. Duplicate gels were electrically blotted to nitrocellulose membranes, as described previously (*21*). After blocking in Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA), the membrane was incubated with anti-TnT mAb CT3 or 2C8 or polyclonal antibody RATnT. The membranes were then washed with high stringency using TBS containing 0.5% Triton X-100 and 0.05% SDS, incubated with alkaline phosphatase-conjugated anti-mouse IgG or anti-rabbit IgG second antibodies (Sigma), washed again, and developed in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate solution, as described previously (*21*).

Ex Vivo Ischemia Reperfusion of Working Rat Heart Preparations. The Langendorff−Neely working heart preparation was used to perfuse isolated rat hearts and apply ex vivo ischemia reperfusion. As described previously (*31*), rats were anesthetized with sodium pentobarbital (50 mg/kg body weight, intraperitoneally). The heart was removed and placed in chilled Krebs−Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 2.25 mM $CaCl_2$, 2.25 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.32 mM EGTA, 11 mM D-glucose, and 25 mM $NaHCO_3$) aerated with 95% O_2 and 5% CO_2 (pH 7.4 at 37 °C). The aorta was cannulated with a 16-gauge needle and the apex

of the heart placed in 37 °C Krebs−Henseleit buffer to maintain physiological temperature. The heart was then perfused with aerated and warmed Krebs−Henseleit buffer in the retrograde at a pressure of 70 mmHg for 15 min to stabilize the heart. During this period, the left atrium was cannulated with a 16-gauge atrial cannula set at a filling pressure of 15 mmHg. The heart was then converted to working mode by switching the tube delivering retrograde perfusion from 70 mmHg to an open column and initiating flow through the atrial cannula. The heart was stabilized in the working mode for 30 min at an afterload pressure of 55 mmHg. Following stabilization, low-flow ischemia was initiated by decreasing the afterload, and hence the coronary filling pressure, to 20 mmHg for 20 min. At a filling pressure of 20 mmHg coronary flow is insufficient to maintain adequate O_2 delivery to the myocardium, and the heart becomes ischemic. During ischemia the heart continued to beat weakly. Following the ischemic period coronary perfusion was resumed by returning the afterload to 55 mmHg for 40 min. At the end of the perfusion protocol the heart was removed from the cannula, flash frozen in liquid nitrogen, and stored at −80 °C for SDS gel and Western blot analysis. Samples from 105 min working hearts without ischemia reperfusion were used as controls.

As a control of simple myocardial ischemia, eight-week-old C57/BL6 mice were euthanized by cervical dislocation and the bodies placed at room temperature (22 °C) in a sealed plastic bag to prevent dehydration. The hearts were removed at 0, 2, 4, and 8 h post-mortem and homogenized in SDS−PAGE sample buffer for SDS gel and Western blot analysis to determine degradation of cTnT resulting from post-mortem ischemia.

Ischemia-Reperfusion Treatment of Mouse Cardiomyocytes. To induce ischemia-reperfusion damage in cardiac myocytes, mouse ventricular myocytes were isolated in low oxygen buffer followed by incubation in oxygenated buffer. Similar to that previously described (*20*), cardiomyocytes were isolated from transgenic mouse hearts overexpressing embryonic cTnT or exon 7-deleted cTnT by retrograde perfusion with Ca^{2+}-free Joklik solution containing 1% BSA and collagenase without oxygenation. Following isolation, the cardiac myocytes were incubated at room temperature without oxygenation for 30 min before initiating reoxygenation and returning Ca^{2+} to 1.25 mM stepwise over a 35 min period. Once Ca^{2+} had been restored, oxygenation was continued, and the myocytes were incubated at room temperature for an additional 30 min before the myocytes were collected by centrifugation, washed in TBS, and lysed in SDS−sample buffer for Western blot analysis.

Isolation of the cTnT Fragment for NH_2-Terminal Sequencing. To investigate the primary structure of the ischemia-reperfusion-produced cTnT fragment, mouse cardiac myocytes treated with ischemia reperfusion were homogenized in TBS and fractionated by ammonium sulfate precipitation. The 30−50% saturation fraction was dialyzed against three changes of 100 volumes of 0.1 mM EDTA at 4 °C. After dialysis, the precipitated material was collected by centrifugation at 25000*g*, 4 °C, for 30 min. The cTnT fragment in the low salt precipitate was further purified by a two-step preparative SDS−PAGE procedure. The sample was first resolved by electrophoresis on a high cross-linker gel (12% Laemmli gel with an acrylamide-to-bisacrylamide ratio of 29:1). The resulting gel was stained with Coomassie Brilliant Blue R-250, and the band containing the cTnT fragment as determined by parallel Western blot using the CT3 mAb was cut out. The protein contents were recovered from the gel slices by electrophoresis elution in SDS gel running buffer. After dialysis against 0.1% formic acid and concentration by lyophilization, the protein sample was redissolved in SDS gel sample buffer and further resolved by electrophoresis on a low cross-linker SDS−PAGE (14% Laemmli gel with an acrylamide-to-bisacrylamide ratio of 180:1). The resulting gel was electrically transferred to PVDF membrane and stained with Amido Black to visualize the protein bands. A parallel strip of the membrane was subjected to Western blot using the anti-cTnT mAb CT3 as described above. The CT3-positive band of the Western blot was aligned to the Amido Black stained membrane, and the corresponding band was excised for NH_2-terminal sequencing at the Biotechnology Resource Laboratory Protein Sequencing and Peptide Synthesis Facility, Medical University of South Carolina, Charleston, SC.

Expression of TnT and the Reconstructed TnT Fragment in Escherichia coli. Intact mouse cTnT (TnT4) was expressed in *E. coli* culture. The construction of the pAED4 expression plasmid from a cloned cDNA (*40*), large scale expression, and purification were done as described previously for the turkey cTnT (*19*).

A cDNA template encoding an NH_2-terminal-deleted mouse cTnT was engineered by polymerase chain reaction (PCR) mutagenesis to create a translational initiation codon prior to the cleavage site (Leu_{72}) as determined by NH_2-terminal sequencing. As shown in Figure 4, cloned adult mouse cTnT cDNA in pBluescript SK(−) plasmid (*40*) was used as template, and PCR was carried out using T7 primer and a custom-designed 5′-oligonucleotide primer that contained an *Nde*I restriction site (underlined), a translational initiation codon ATG, and the region corresponding to the coding sequence for amino acids 72−77 (McTnT-NDF: 5′-AGCCC<u>CATATG</u>CTCTTCATGCCCAACTT-3′). The PCR product was modified at the 5′ and 3′ ends by *Nde*I and *Xho*I cuts and cloned into the pAED4 expression plasmid (*41*). The cDNA insert was sequenced by the dideoxy chain termination method to verify the construction and sequence authenticity.

The truncated mouse cTnT cDNA was expressed by transformation of BL21(DE3)pLyseS *E. coli* cells with the expression plasmid. Freshly transformed bacterial cells were cultured in 2× TY-rich liquid media (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, 1.32 g/L Na_2HPO_4, pH 7.3) containing 100 mg/L ampicillin and 25 mg/L chloramphenicol at 37 °C with vigorous shaking and induced with 0.4 mM isopropyl 1-thio-β-D-galactoside at mid-log phase. After an additional 3 h of culture, the bacterial cells were harvested by centrifugation at 4 °C. The bacterial pellet was suspended in 2.5 mM EDTA and 50 mM Tris-HCl, pH 8.0, and lysed by three passes through a French press cell. The bacterial lysate was clarified by centrifugation and precipitated with ammonium sulfate to obtain the 0−35% saturation fraction. Following dialysis against 0.1 mM EDTA containing 6 mM β-mercaptoethanol, the 0−35% fraction was brought to 6 M urea, 0.1 mM EDTA, 6 mM β-mercaptoethanol, and 20 mM sodium acetate, pH 6.0, and fractionated by chromatography on a CM52 cation-exchange column equilibrated

in the same buffer. The column was eluted by a 0−500 mM linear KCl gradient, and the protein peaks were analyzed by SDS−PAGE. Fractions containing the NH_2-terminal truncated TnT were further purified by G75 gel filtration chromatography in 6 M urea, 500 mM KCl, 0.1 mM EDTA, 6 mM β-mercaptoethanol, and 10 mM imidazole hydrochloride, pH 7.0. Protein peaks were analyzed by SDS−PAGE, and the fractions containing pure NH_2-terminal truncated TnT were dialyzed against 0.1% formic acid and lyophilized. All purification steps were carried out at 4 °C.

According to the NH_2-terminal truncation site (between Thr45 and Ala46) reported in rabbit fast TnT (*32*), an expression vector encoding NH_2-terminal truncated mouse fast skeletal muscle TnT was constructed by similar procedures and the protein was expressed in *E. coli* as described above.

Triton X-100 Extraction of Ventricular Muscle Strips. Operated on ice, ventricular muscle was cut with a sharp razor blade into fine pieces of approximately the size of isolated trabeculae. The muscle strips were washed in relaxing solution containing 0.1 M KCl, 2 mM $MgCl_2$, 2 mM EGTA, 10 mM Tris, 0.5 mM DTT, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and 2 mM $Na_4P_2O_7$. After centrifugation at 2800*g* at 4 °C for 15 min, the pellet was skinned in relaxing solution plus 0.5% (w/w) Triton X-100 at 4 °C with rotation for 10 min. After centrifugation at 14000*g* at 4 °C for 20 min, the pellet was suspended in relaxing solution without Triton X-100 and incubated at 37 °C with rotation. Samples were collected at a series of time points for SDS−PAGE and Western blotting to examine the modifications of cTnT and other myofibrillar proteins.

Calpain Inhibition. To test whether endogenous calpain in the cardiac muscle contributes to TnT NH_2-terminal modification, several different calpain inhibitors were applied to the Triton X-100 extraction procedure. It has been reported that μ-calpain proteolytic activity is strongly inhibited by the application of an oxidant, e.g., 100 μM hydrogen peroxide (*42*). Triton treatment of mouse ventricular muscle strips was carried out as above in the presence or absence of 100 μM hydrogen peroxide, and the effect on cTnT modification was examined by Western blotting.

We also tested the effects of two cell membrane permissible nonpeptide calpain inhibitors, PD150606 and PD151746 (Calbiochem, San Diego, CA). PD150606 exhibits similar apparent inhibition constants against μ-calapin (K_i 0.21 ± 0.01 μM) and m-calpain (K_i 0.37 ± 0.03 μM), whereas PD151746 has a 20-fold selectivity for μ-calpain (K_i 0.26 ± 0.03 μM) over m-calpain (K_i 5.33 ± 0.77 μM) (*43*). PD150606 and PD151745 were separately added to the relaxing solution before Triton treatment and incubated with the minced ventricular muscle at 4 °C for 10 min to allow the inhibitor to penetrate the cell membrane and bind to calpain. After Triton X-100 extraction, samples were collected for SDS−PAGE and Western blotting to examine the effect on cTnT modification.

Muscle Protein Purifications. Bovine cTnT was purified from left ventricular muscle as previously described (*8*). Bovine cardiac TnI was purified from ventricular muscle as described previously (*19*). Rabbit α-tropomyosin was purified from ventricular muscle as described previously (*44*).

Preparation of Cardiac Myofibrils. Cardiac myofibrils were prepared from ventricular muscle according to the method described previously (*45*) with modifications. All steps were conducted at 4 °C. The ventricular muscle was pulverized in a food blender in 10 volumes (w/v) of the above relaxing buffer. The homogenization was passed through two layers of cheesecloth and centrifuged at 2000*g* for 15 min. After three washes using the relaxing buffer without $Na_4P_2O_7$, the pellet was suspended in the washing buffer containing 0.5% (w/w) Triton X-100 for 10 min with occasional stirring. Four more washes were performed to remove Triton X-100. The myofibrils were stored −20 °C in the washing buffer containing 50% glycerol until use.

Isolation of the Troponin Complex from Cardiac Muscle. The cardiac troponin complex was isolated by immunoaffinity chromatography using a mouse mAb TnI-1 against the COOH terminus of TnI (*46*). The TnI-1 epitope is exposed in the troponin complex and can be used as a handle to isolate the troponin complex from muscle homogenates (*30*). The TnI-1 mAb (IgG1) was purified from hybridoma ascites fluid using a Protein G−Sepharose (Amersham Pharmacia Biotech) affinity column and coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer's protocols. Bovine left ventricular muscle was minced into small pieces and extracted by 20 volumes (w/v) of Guba−Straub solution containing 300 mM KCl, 100 mM K_2HPO_4, 50 mM KH_2PO_4, 2.5 mM $MgCl_2$, 1 mM EGTA, and 0.1 mM PMSF, pH 6.5, on ice for 15 min. After centrifugation at 16000*g* at 4 °C for 20 min, the supernatant containing mainly myosin was removed. The pellet was extracted in 20 volumes (w/v) of 1 M KCl, 10 mM Tris-HCl, pH 8.0, and 0.1 mM PMSF by stirring on ice for 30 min. After centrifugation as above, the extract was diluted 5-fold in TBS and loaded on a TnI-1 mAb affinity column of 0.5 mL bed volume equilibrated in TBS. The column was washed with TBS, and the proteins bound to the TnI-1 affinity beads were eluted with 50 mM glycine hydrochloride, pH 2.7. Fractions of 0.3 mL were collected into tubes containing 0.05 mL of neutralizing buffer containing 1 M Tris-HCl, 1.5 M NaCl, and 1 mM EDTA, pH 8.0. The fractions were analyzed by SDS−PAGE and Western blotting as described above to identify the troponin peak. The fractions containing the three troponin subunits were examined on a Sepharose G75 column (Amersham Pharmacia Biotech) under nondenaturing conditions to verify their formation of the troponin complex.

μ-Calpain Treatment of cTnT. Purified bovine cTnT and the troponin complex were incubated at 37 °C in 50 mM sodium borate buffer, pH 7.5, containing 3 mM $MgCl_2$, 1.25 mM $CaCl_2$, and various concentrations (0.25−4 units/mL) of μ-calpain (Calbiochem, San Diego, CA). After 30 min incubation, the reaction was stopped by adding 3× SDS−PAGE sample buffer and heating at 80 °C for 5 min. The samples were analyzed by SDS−PAGE and Western blotting as described above.

The isolated bovine cardiac myofibrils were centrifuged at 3000*g* for 15 min to remove glycerol. The pellet was suspended in 50 mM sodium borate buffer, pH 7.5, containing 3 mM $MgCl_2$ and 1.25 mM $CaCl_2$. The calpain treatment conditions were the same as those for purified proteins except for the use of higher concentrations of μ-calpain (5−20 units/mL). The effects on myofilament proteins were examined by SDS−PAGE and mAb CT3 Western blotting as above.

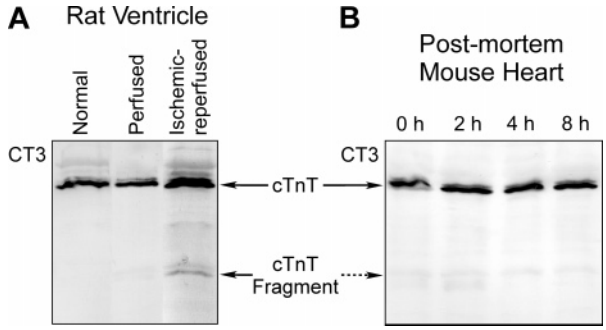

FIGURE 1: A cTnT fragment found in ischemia-reperfused cardiac muscle. (A) While there was no detectable degradation of cTnT in rat heart after 105 min ex vivo perfusion, a CT3 mAb Western blot showed that a specific cTnT fragment was produced in working rat heart preparations after ischemia-reperfusion treatment. (B) Cardiac muscle samples from adult C57BL/6 mice were prepared at 0, 2, 4, and 8 h post-mortem and analyzed by SDS−PAGE and CT3 mAb Western blotting. The results showed that there was no detectable degradation of cardiac TnT due to the post-mortem ischemia.

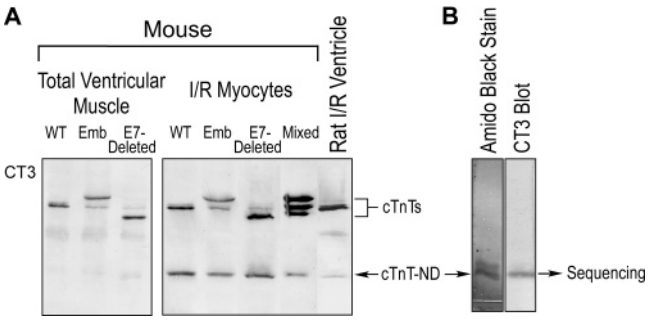

FIGURE 2: Isolation of the cTnT fragment from ischemia-reperfused mouse cardiomyocytes to determine the NH_2-terminal sequence. (A) The cTnT fragment was produced in isolated mouse cardiomyocytes that had undergone ischemia-reperfusion conditions. Samples from transgenic mouse hearts expressing embryonic cTnT and/or exon 7-deleted cTnT that differ from the endogenous adult cTnT in the NH_2-terminal region (Figure 5) showed a cTnT fragment identical in size to that produced in the wild-type adult mouse cardiomyocytes, indicating that a removal of the NH_2-terminal variable region produces the cTnT fragment. (B) The cTnT fragment band isolated from high cross-linker preparative SDS−PAGE gel (see Materials and Methods) was resolved on a low cross-linker SDS gel and transferred to PVDF membrane for NH_2-terminal sequencing. Amido Black staining revealed the yield of the cTnT fragment that is confirmed by a mAb CT3 Western blot.

Protein Binding Assays. Enzyme-linked immunosorbent assay (ELISA) solid-phase protein binding experiments (*21*) were performed to investigate the interactions of the NH_2-terminal truncated cTnT with TnI and tropomyosin. Purified intact and NH_2-terminal truncated mouse cTnT or BSA control was dissolved at 5 μg/mL in buffer A (0.1 M KCl, 3 mM $MgCl_2$, 20 mM PIPES, pH 7.6) and coated onto 96-well microtitering plates by incubation at 4 °C overnight. After washes with buffer T (buffer A containing 0.05% Tween-20) at pH 7.6 to remove the unbound protein, the plates were blocked with buffer T at pH 7.2 or 6.3 containing 1% BSA. The immobilized cTnT was incubated with serial dilutions of bovine cardiac TnI or rabbit α-tropomyosin in buffer T (at the blocking pH) containing 0.1% BSA at room temperature for 2 h. After one wash with buffer T of the blocking pH and two washes with buffer T at pH 7.2, the bound TnI or tropomyosin was quantified via incubation at pH 7.2 and room temperature for 1 h with the anti-TnI mAb TnI-1 (*46*) or an anti-tropomyosin mAb CH1 (*47*), respectively. The plates were then processed by a standard ELISA procedure, including pH 7.2 buffer T washes, horseradish peroxidase-conjugated anti-mouse immunoglobulin second antibody (Sigma) incubation, and H_2O_2/2,2′-azinobis(3-ethylbenzothiazolinesulfonic acid) substrate reaction (*21*). A_{405nm} of each assay well was recorded at a series of time points by an automated microtiter plate reader (Bio-Rad Benchmark). The A_{405nm} values in the linear course of the color development were used to plot the protein binding affinity curves. All experiments were done in triplicate.

Data Analysis. The DNA and protein sequence analyses were done using computer programs from DNAStar. Statistical analysis of the SDS gel and Western blot densitometry and the protein binding data was done by Student's *t* test. All values are presented as the mean ± SD.

RESULTS

A cTnT Fragment Produced in Myocardial Ischemia Reperfusion. Western blots using mAb CT3 recognizing cardiac and slow TnT (*23*) detected a significant amount of an additional protein band in ischemia-reperfused rat heart (Figure 1A). This band has a significantly lower apparent molecular weight than that of the slow skeletal muscle TnT or any known alternatively spliced cTnT isoforms. This band is also recognized by several other anti-cTnT mAbs as well as the anti-TnT rabbit polyclonal antibody RATnT (data not shown), indicating that it is a modified TnT protein. Considering the facts that slow and fast skeletal muscle TnT are not expressed in postnatal cardiac muscles (*48*), this low M_r TnT is likely a proteolytic fragment of cTnT.

The cTnT fragment is present in normal cardiac muscle although at very low levels (Figure 6B), suggesting a physiological relevance. Simple post-mortal ischemia for up

		A.A.	Mr
Mouse cTnT	...EGPVEDTKPKPSRLFMPNLVPPKIPDGERVDFDDIHRK...	291	34,546
Mouse cTnT-ND$_{72-291}$	**(M) LFMPNLVPPKIPDGERVDFDDIHRK...**	**221**	**26,859**
Rat cTnT$_{77-298}$	(M)VPPKIPEGEKVDFDDIHRK...	215	26,046
Rabbit 26 kDa fsTnT	APKIPEGEKVDFDDIQKK...	214	25,400
Mouse 25 kDa cTnT	DIHRK...	200	24,447

FIGURE 3: NH_2-terminal truncations of cTnT. NH_2-terminal amino acid sequencing of the cTnT fragment revealed a single truncation site between residues Arg_{71} and Leu_{72}. The Met in parentheses indicates the addition of an initiation codon for expressing cTnT-ND_{72-291} in *E. coli* (Figure 4). Amino acid sequence alignment demonstrated that the cTnT NH_2-terminal truncation removes the entire variable region (Figure 5), similar to the NH_2-terminal truncated fast TnT previously isolated from rabbit skeletal muscle (rabbit 26 kDa fsTnT; *32*) and a model protein previously studied (rat $cTnT_{77-298}$; *16*). In contrast, a caspase cleavage-produced cTnT fragment (mouse 25 kDa cTnT; *29*) involves the deletion of a part of the conserved region. The number of amino acids (A.A.) and calculated molecular weights of these proteins are indicated after the sequences.

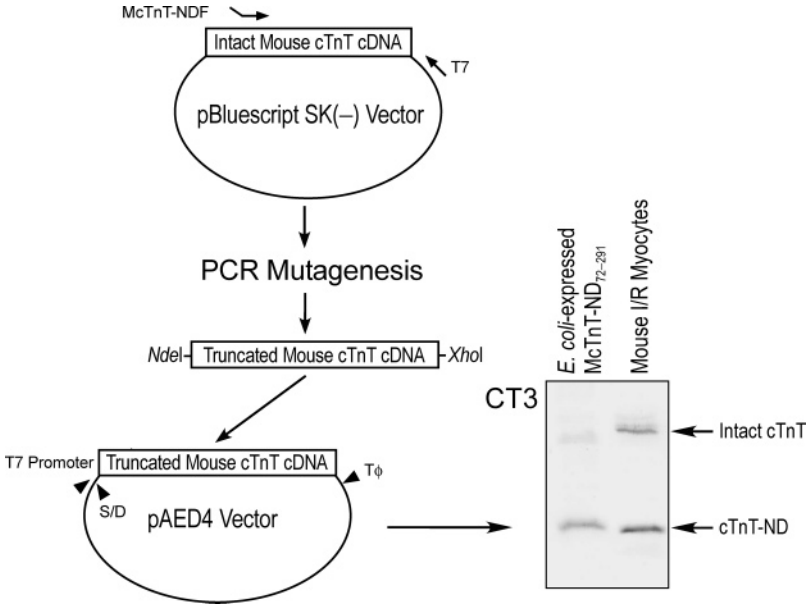

FIGURE 4: Bacterial expression of the reconstructed cTnT fragment. 5′-Truncated mouse cTnT cDNA was constructed according to the NH_2-terminal truncation site for protein expression in *E. coli* (see Materials and Methods). S/D and Tϕ in the pAED4 expression vector indicate the Shine−Dalgarno ribosomal binding site and the transcription termination sequence, respectively. The cTnT fragment expressed from the truncated cDNA shows a size identical to that of the cTnT fragment produced in ischemia-reperfused cardiac muscle (the slightly slower gel mobility seen in the blot may be due to the addition of an NH_2-terminal Met in the expression construct), indicating that the NH_2-terminal truncation is the only primary structure modification.

to 8 h did not increase the cTnT fragmentation (Figure 1B). There was no detectable change of the cTnT fragment in the rat heart after 105 min ex vivo perfusion, demonstrating a correlation to the ischemia-reperfusion stress conditions.

The cTnT Fragment Is Produced by a Restricted NH_2-Terminal Truncation. Suggesting an NH_2-terminal deletion that produces the cTnT fragment, ischemia-reperfusion treatment of transgenic mouse cardiomyocytes expressing embryonic or exon 7-deleted cTnT that differ from the wild-type cTnT only in the length of the NH_2-terminal variable region produced cTnT fragments with identical size (Figure 2A).

We successfully isolated the low molecular weight cTnT fragment from ischemia-reperfused mouse cardiomyocytes for NH_2-terminal sequencing. Figure 2B shows the enrichment of the cTnT fragment by preparative SDS−PAGE. The NH_2-terminal sequencing result revealed that this low molecular weight TnT protein is indeed a cTnT fragment with a deletion of the NH_2-terminal amino acids 1−71 (Figure 3).

To investigate the integrity of the COOH terminus in the cTnT fragment, we reconstructed the NH_2-terminal truncation in mouse cTnT by generating a 5′-truncated mouse cTnT cDNA (Figure 4). Expression of the truncated cDNA in *E. coli* produced a cTnT protein with a size identical to that of the cTnT fragment produced in ischemia-reperfused cardiac muscle. The results demonstrate that there was no COOH-terminal deletion in this cTnT fragment.

The NH_2-terminal truncation site (Arg_{71}-Leu_{72}) is not at an exon boundary, and therefore, the cTnT-ND_{72-291} fragment is not generated by alternative RNA splicing but by proteolytic cleavage. Sequence alignment (Figure 3) demonstrates that the cleavage site is different from the previously reported caspase cleavage site in cTnT (the mouse 25 kDa cTnT) under ischemia-reperfusion conditions (*29*).

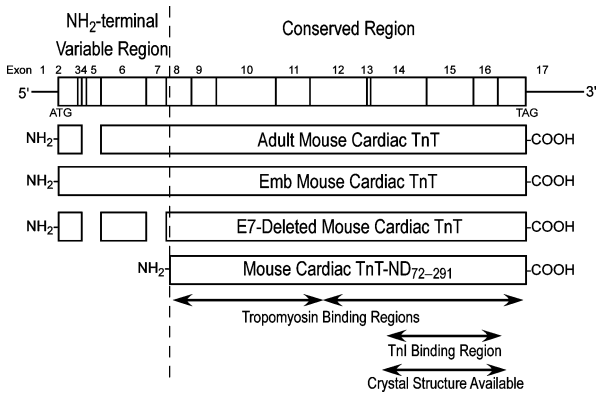

FIGURE 5: Structural comparison of the NH_2-terminal truncated cTnT with cTnT splicing variants. A cTnT mRNA map noted with exon boundaries is shown on the top. Protein primary structural maps of the cTnT variants studied are aligned with the coding region of the mRNA map. The NH_2-terminal and adjacent regions of intact wild-type adult mouse cTnT and two alternatively spliced variants are compared with the NH_2-terminal truncated cTnT. The NH_2-terminal truncation specifically removes the entire variable region that is alternatively spliced to produce the adult mouse cardiac TnT (Adult cTnT-4, exclusion of exons 4 and 5), embryonic isoform (Emb cTnT-1, contains all of the exons), and E7-deleted mouse cardiac TnT (E7-deleted TnT, exclusion of exons 4, 5, and 7). The central and COOH-terminal conserved regions of TnT contain the core functional structure that binds other thin filament regulatory proteins, TnI, TnC, and tropomyosin (Tm). These protein binding sites and the portion of TnT with the X-ray crystallography structure available are outlined. The NH_2-terminal truncated cTnT (McTnT-ND_{72-291}) retains the integrity of the conserved core structure, implying a functional role in myocardial ischemia reperfusion.

The Restricted NH_2-Terminal Truncation of cTnT Preserves the Core Functional Structure of TnT. Figure 5 compares the NH_2-terminal truncated cTnT with several intact cTnT variants and demonstrates that the NH_2-terminal cleavage of the cTnT polypeptide chain specifically removes

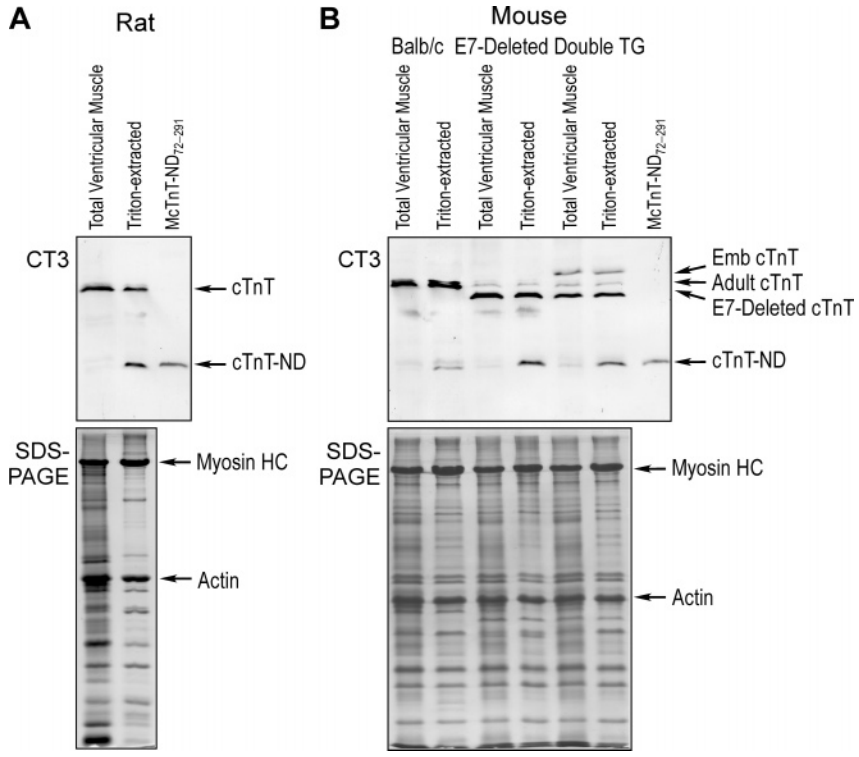

FIGURE 6: Triton X-100 extraction promotes the production of the NH_2-terminal truncated cTnT. Rat (A) and mouse (B) ventricular muscle strips before and after Triton X-100 extraction were examined by SDS−PAGE and Western blot using anti-TnT mAb CT3. The results revealed that Triton X-100 extraction induced the production of a specific cTnT fragment in both rat and mouse cardiac muscle samples. In the mouse model, adult, exon 7 (E7) deleted, and embryonic (Emb) cTnTs with different NH_2-terminal structures produced a single fragment that has the same size as the reconstructed McTnT-ND_{72-291} protein, consistent with a selective NH_2-terminal truncation. The production of NH_2-terminal truncated cTnT (cTnT-ND) by Triton X-100 extraction suggests the role of activation of a myofibril-associated protease. The accompanying SDS−PAGE showed comparable amounts of protein loading normalized to that of actin and that other major myofibril proteins were not affected by the Triton treatment. HC, heavy chain; TG, transgenic.

entirely the hypervariable region encoded by exons 2−7. The central and COOH-terminal conserved regions that contain binding sites for other thin filament regulatory proteins, TnI, TnC, and tropomyosin, are preserved in this proteolytic modification. The integrity of the conserved core structure of TnT in the NH_2-terminal truncated cTnT (cTnT-ND_{72-291}) implies a functional effect in myocardial ischemia reperfusion. This notion is consistent with the fact that cTnT-ND_{72-291} was retained in the isolated cardiac myofibrils with a proportion identical to that in the total muscle extract, indicating its full ability to incorporate into the myofilament (Figure 6A).

Triton X-100 Extraction of Cardiac Muscle Activates an Endogenous Proteolytic Activity That Produces the NH_2-Terminal Truncated Cardiac TnT. Western blotting using mAb CT3 demonstrated that Triton X-100 treatment of rat and mouse cardiac muscle strips reproduced the specific cTnT-ND fragment (Figure 6). In the mouse model, Triton X-100 extraction of transgenic cardiac muscle containing wild-type adult, embryonic, and exon 7-deleted cTnTs that are different in the NH_2-terminal region produced a single fragment with the same size as that of McTnT-ND_{72-291}. This was most clearly shown in the double transgenic mouse heart that simultaneously expresses all of the three cTnT variants (Figure 6B). This result indicates that the cTnT fragment produced by Triton extraction is the same as the NH_2-terminal truncated cTnT-ND_{72-291} identified in ischemia reperfusion. Triton X-100 treatment is known to remove lipid contents from the muscle fiber without disruption of the myofibril structure. SDS−PAGE in Figure 6 showed that other major myofibril proteins were not affected by Triton X-100 treatment. The Triton treatment may have activated a myofibril-associated protease that is responsible for the specific production of cTnT-ND_{72-291} under stress conditions.

The Production of NH_2-Terminal Truncated cTnT by Myofibril-Associated Proteolytic Activity Is Suppressed by Calpain Inhibitors. Calpain has been reported to cleave cTnT (*36*). Therefore, we tested the effects of calpain inhibitors on the myofilament-associated endogenous proteolytic activity. Western blot examination showed that the presence of 100 μM hydrogen peroxide resulted in a ~35% decrease in the Triton X-100-induced production of cTnT-ND_{72-291} in mouse ventricular muscle strips (Figure 7A). Hydrogen peroxide is known to inhibit the proteolytic activity of μ-calpain (*42*). Therefore, this result suggests that cardiac myofilament-associated μ-calapin may be responsible for the production of the cTnT-ND_{72-291} fragment during ischemia reperfusion.

Consistently, calpain-specific inhibitors, PD150606 and PD151746, also decreased the productions of cTnT-ND_{72-291} in Triton X-100-extracted mouse cardiac muscle strips (Figure 7B). PD150606 and PD151746 resulted in ~37% and ~50% decreases in cTnT-ND_{72-291} production, respectively, further supporting the role of myofibril-associated μ-calpain.

μ-Calpain Treatment of Myofibrils Reproduced the NH_2-Terminal Truncated cTnT Fragment. The results in Figure

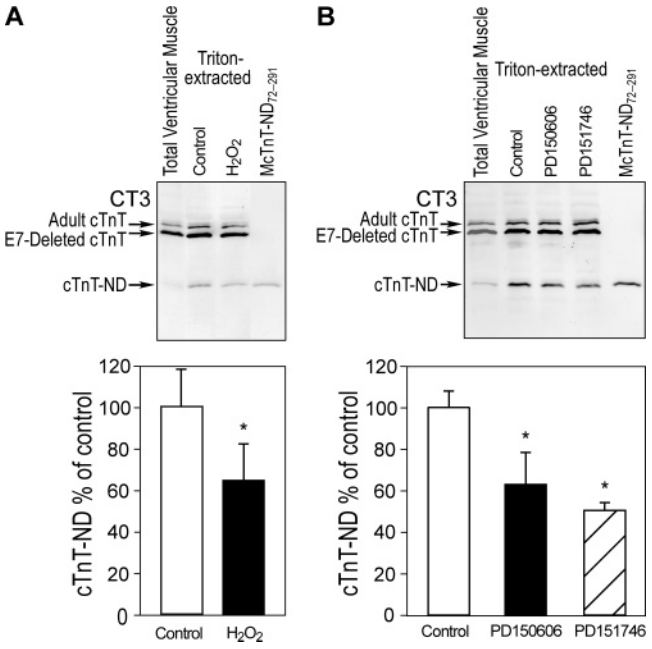

FIGURE 7: Calpain inhibitors reduce the production of NH_2-terminal truncated cTnT by myofibril-associated proteolytic activity. (A) Western blots were used to examine Triton X-100-extracted exon 7 (E7) deleted transgenic mouse ventricular muscle strips in the absence or presence of 100 μM hydrogen peroxide. Densitometric analysis showed that cTnT-ND production was reduced to 65% in the presence of hydrogen peroxide (*, $P < 0.01$), indicating an effect of inhibiting the activity of μ-calpain (*42*). (B) Calpain-specific inhibitors, PD150606 and PD151746, also decreased the production of cTnT-ND in Triton X-100-extracted mouse cardiac muscle strips (to 63% and 50%, respectively, *, $P < 0.01$), further supporting the role of myofibril-associated, μ-calpain in the production of cTnT-ND. Data are shown as the mean ± SD. The results were summarized from three repeated experiments.

8A demonstrate that μ-calpain treatment of purified bovine cTnT effectively decreased the level of intact cTnT in a concentration-dependent manner, consistent with that observed in a previous study (*36*). However, no specific cTnT fragment was produced at a significant amount from μ-calpain treatment of isolated cTnT, demonstrating a nonspecific degradation effect.

On the other hand, μ-calpain treatment of bovine cardiac myofibril effectively reproduced the cTnT-ND_{72-291} fragment (Figure 8B). SDS−PAGE and the gel densitometry plots (Figure 8B) further showed that other major myofibrillar proteins, including myosin, actin, and tropomyosin, were not significantly affected by the μ-calpain treatment. This result supports the observation that the production of cTnT-ND_{72-291} during myocardial ischemia reperfusion is by endogenous μ-calpain cleavage. In contrast to the nonspecific degradation of purified cTnT by μ-calpain, the specific reproduction of cTnT-ND_{72-291} by μ-calpain treatment of cardiac myofibrils demonstrates that this posttranslational modification of the cTnT structure is dependent on the physiological structure of the myofilament, consistent with a physiological relevance.

Quantitative densitometry analysis of the Western blots shows that the μ-calpain modification of cTnT in bovine cardiac myofibril had a nonlinear (reverse exponential) concentration relationship (Figure 9). The sensitive responses to the initial increasing concentrations of μ-calpain imply a

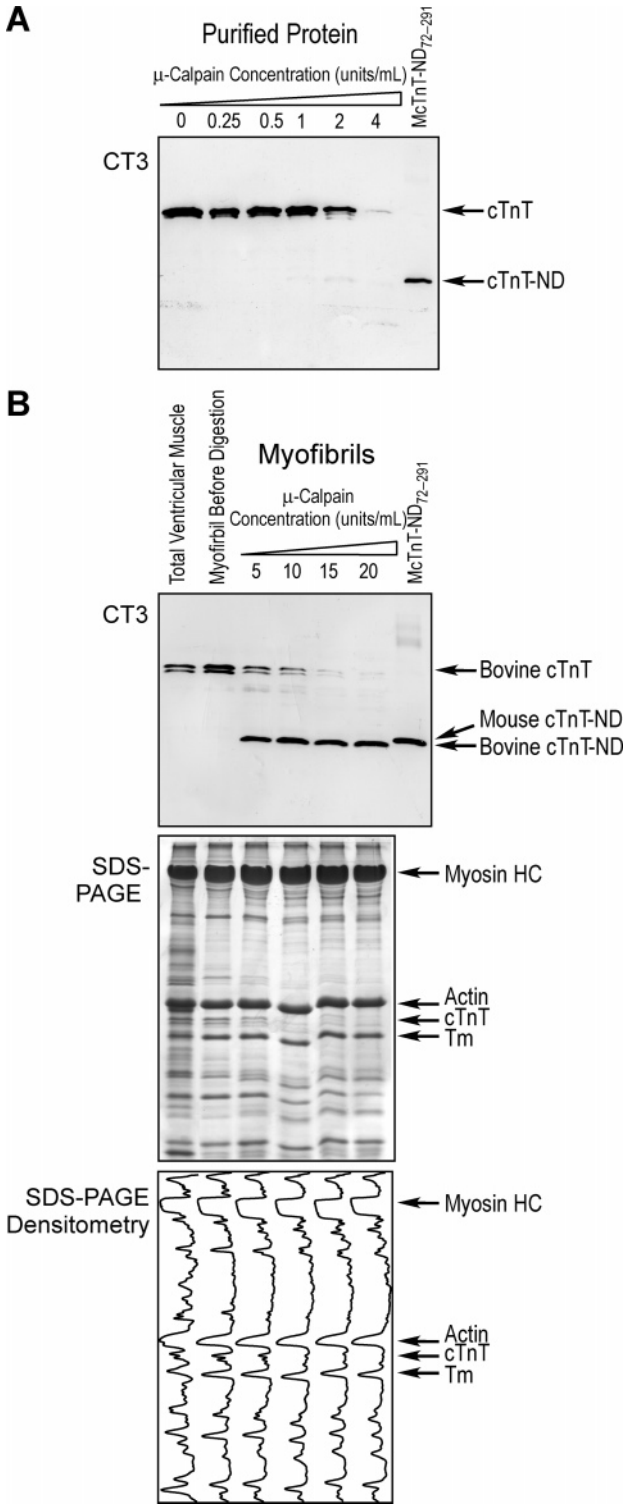

FIGURE 8: μ-Calpain treatment of cardiac myofibrils reproduced the NH_2-terminal truncated cTnT fragment. (A) A Western blot using anti-cTnT mAb CT3 showed that μ-calpain treatment degraded purified bovine cTnT as that reported previously (*36*) but did not produce a specific fragment. (B) In contrast, μ-calpain treatment of bovine cardiac myofibrils effectively reproduced the cTnT-ND fragment. The results demonstrate that the cTnT-ND modification by μ-calpain is dependent on the myofibril structure. Densitometry traces of the accompanying SDS−PAGE gel showed no apparent degradation of other major myofibrillar proteins, including myosin, actin, and Tm, under the μ-calpain treatment conditions. HC, heavy chain.

preferred selective cleavage of cTnT in the myofibrils. On the other hand, the reaching of a plateau at the higher

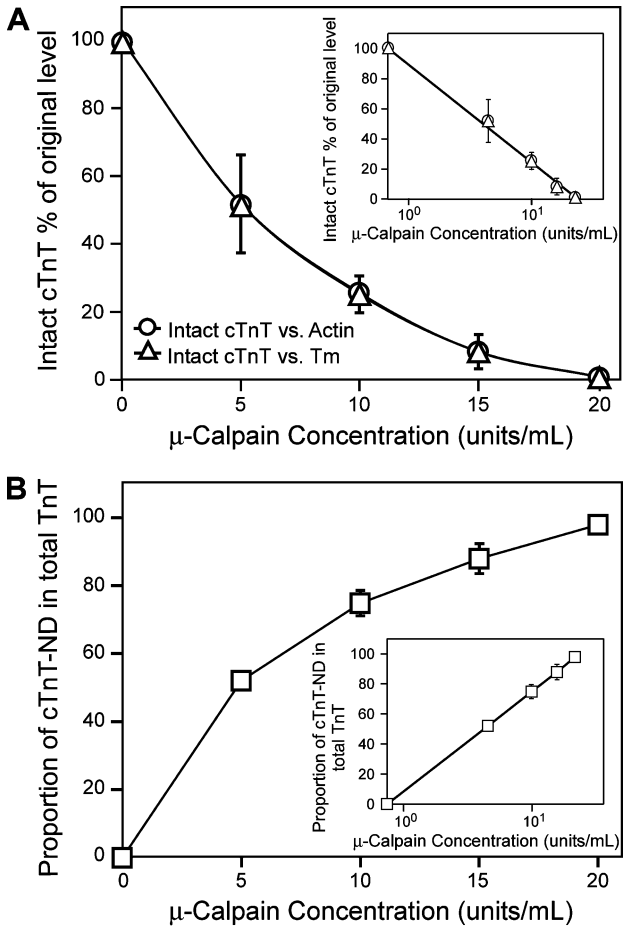

FIGURE 9: μ-Calpain production of NH_2-terminal truncated cTnT suggests a restricted proteolytic modification. Densitometry analysis of multiple copies of mAb CT3 Western blots (Figure 8) determined the relative amounts of intact and NH_2-terminal truncated cTnT in μ-calpain-treated bovine cardiac myofibrils. The results show that intact cTnT decreased from 100% to 1.5% when the concentration of μ-calpain increased from 0 to 20 units/mL (A), while the amount of the NH_2-terminal truncated cardiac TnT, cTnT-ND, increased from 0% to 98% of the total cTnT (truncated plus intact) (B). The reverse exponential concentration responses of the cleavage of intact cTnT as shown by the semilogarithmic plot inserts and the specific production of the NH_2-terminal truncated cTnT by μ-calpain treatment suggest a selective and restricted proteolytic modification.

concentration of μ-calpain suggests a restricted proteolytic modification.

μ-Calpain treatment of isolated bovine cardiac troponin complex also showed a selective cleavage of the NH_2-terminal domain of TnT (Figure 10). The difference between free TnT and the troponin complex in μ-calpain proteolysis is consistent with the determining role of the substrate structural conformation. Nonetheless, the higher sensitivity and less effective preservation of the TnT core structure seen in the μ-calpain treatment of the isolated troponin complex than that of intact myofibrils (Figure 8B) suggest that this selective structural modification of TnT is most effective under physiological conditions.

Similar NH_2-Terminal Truncation of Cardiac and Fast Skeletal Muscle TnT by Calpain Proteolysis. After Triton X-100 extraction of transgenic mouse cardiac muscle strips containing both cTnT and chicken fast skeletal muscle TnT (*38*), Western blot using polyclonal antibody RATnT raised against chicken fast TnT and mAb 2C8 recognizing both cardiac and fast TnTs detected the production of both cardiac and fast skeletal TnT-ND fragments (Figure 11). Mouse cTnT-ND and chicken fast TnT-ND have different molecular weights due to their amino acid composition in the core structure. While the blot using a low cross-linker SDS gel (Figure 11A) showed only one TnT-ND band, the blot using a high cross-linker SDS gel resolved two TnT fragments with distinct immunoreactivities to RATnT and 2C8 antibodies indicating their cTnT and fast TnT origins. Although the amino acid sequences of the NH_2-terminal regions of mouse cardiac and chicken fast skeletal muscle TnTs flanking the truncation sites are very different, similar modification of both endogenous cTnT and transgenic-expressed fast TnT upon the Triton extraction-activated μ-calpain cleavage suggests a dependence on myofibril structure rather than the amino acid sequences at the cutting sites.

Selective Removal of the NH_2-Terminal Variable Region of cTnT Preserves the Binding of cTnT to TnI and Tropomyosin with Altered Affinities. To examine the effect of the NH_2-terminal truncation on cTnT's interactions within the thin filament regulatory system, we compared the binding of intact and the NH_2-terminal truncated cTnT to TnI and tropomyosin. The results of ELISA solid-phase protein binding experiments in Figure 12A demonstrate that the NH_2-terminal truncated cTnT has an increased binding affinity for TnI compared to that of intact cTnT. This is shown by the lower concentration of TnI required for reaching 50% of maximum binding (8.73 ± 1.15 nM for cTnT-ND versus 15.33 ± 1.36 nM for intact cTnT, $P < 0.005$), reflecting a higher K_a in the equilibrium binding step. No significant difference in the maximum binding was observed (Figure 12A insert), indicating no effect on the TnT−TnI coupling strength in the subsequent washing separation steps after the complex formation. The higher K_a in cTnT-ND-TnI binding may facilitate incorporation of the mutant cTnT into the troponin complex and the thin filament as well as affect the allosteric feature of the Ca^{2+}-regulatory system.

The binding of cTnT-ND to tropomyosin also exhibited a higher affinity than that of intact cTnT (Figure 12B). The concentrations of tropomyosin for 50% maximum binding of cTnT-ND and intact cTnT were 9.73 ± 0.185 and 13.50 ± 1.38 nM, respectively ($P < 0.01$). The level of maximum binding was not significantly changed (Figure 12B insert). Although having no effect on the anchoring strength of TnT on tropomyosin, the altered binding affinity of NH_2-terminal truncated cTnT for tropomyosin may affect the allosteric feature of the thin filament regulatory system, contributing to myocardial function during ischemia reperfusion.

Intracellular acidosis occurs in myocardial ischemic injury with a correlation to the function of troponin (*49*). Therefore, we compared the response of cTnT-ND to lowered pH with that of intact cTnT. Decrease of pH from 7.2 to 6.3 did not result in significant change in the binary binding affinity of both intact and NH_2-terminal truncated cTnT to TnI or tropomyosin (Figure 12). The results suggest that although environmental pH and the NH_2-terminal negative charge of TnT are known to affect the bindings of acidic and basic fast skeletal muscle TnT isoforms to skeletal muscle TnI and tropomyosin (*50*), the cardiac protein isoforms may have a higher resistance to acidosis. The removal of the entire NH_2-terminal variable region corresponding to a large amount of negative charges from cTnT did not convey the effect on

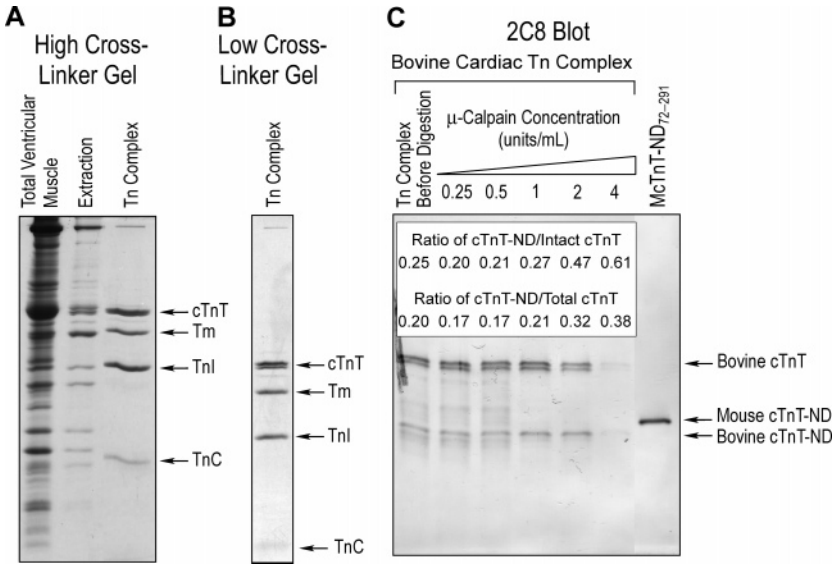

FIGURE 10: Isolation and μ-calpain treatment of the bovine cardiac troponin complex. (A) SDS−PAGE (15% high cross-linker gel) showed the effective isolation of the troponin (Tn) complex from bovine ventricular muscle homogenate by immunoaffinity chromatography. The TnI-1 mAb affinity column fraction contained a 1:1:1 ratio of TnT, TnI, and TnC. Some tropomyosin (Tm) was co-isolated with the troponin complex, reflecting the native binding between troponin and tropomyosin in the thin filament. (B) The 14% low cross-linker SDS−PAGE gel shows that the isolated bovine troponin complex contains both of the two adult cTnT isoforms (*8*), indicating a native state. (C) Western blot using anti-cTnT mAb 2C8 showed that μ-calpain treatment of isolated bovine cardiac troponin reproduced a single cTnT-ND fragment from the two NH_2-terminal alternatively spliced cTnT isoforms. The insert table shows the relative amounts of cTnT-ND produced. It is worth noting that while the troponin structure preserved the TnT core structure against calpain digestion in contrast to that in free cTnT, the protection was less effective than that in intact myofibrils (Figure 8B). Altogether, the results are consistent with the role of cTnT conformation in determining this selective structural modification under physiological conditions.

the core structure and function seen in the fast TnT isoforms in response to the environmental pH. This observation presents an analogy to a feature of cardiac TnI in which proteolytic removal of the entire NH_2-terminal region had a functional effect similar to that of protein kinase A phosphorylation in the NH_2-terminal region (*31*).

DISCUSSION

We report in the present study a restricted proteolytic NH_2-terminal truncation of cTnT in myocardial ischemia reperfusion. This structural modification selectively removes the NH_2-terminal variable region and preserves the TnT core structure with functional implications. From characterizing its production by μ-calpain cleavage, the following observations suggest the significance of this study.

Regulatory Role of the NH_2-Terminal Variable Region of TnT. TnT is known as a protein with extended conformation in which the NH_2-terminal variable region is a part of the "tail" domain of troponin. The presence of TnI and TnC binding sites in the TnT COOH-terminal domain is confirmed by the X-ray crystallographic three-dimensional structure of partial cardiac (*51*) and skeletal muscle (*52*) troponins. The NH_2-terminal amino acid sequence is hypervariable among TnT isoforms and is regulated by alternative RNA splicing during heart and skeletal muscle development. This region does not contain binding sites for other thin filament proteins, but its structural alteration shows fine-tuning effects on the Ca^{2+} regulation of muscle contraction. It has been proposed that the NH_2-terminal variable region has its functional effects by modulating the molecular conformation and activity of other regions of TnT (*21*−*23*).

The fact that the NH_2-terminal region of TnT does not contain binding sites for other thin filament proteins allows for its high sequence variability and forms the foundation for a wide range of modulating effects. The NH_2-terminal truncated cTnT produced during myocardial ischemia reperfusion selectively removes the entire NH_2-terminal variable region while retaining the conserved core structure of TnT. This mechanism represents the most extreme modification of TnT in comparison to the developmental (*8*) and pathological (*20*) alternative splicing variants. The molecular evolution of the TnT NH_2-terminal variable region demonstrates an increase in length and complexity (*53*). Therefore, the removal of the entire NH_2-terminal variable region in cTnT may be a mechanism to resume a fundamental functional state of troponin as a response to stress conditions.

Predicted Functional Consequences of Selective Deletion of the NH_2-Terminal Variable Region of cTnT. Several previous studies investigating the structure−function relationship of TnT have characterized cTnT model molecules with an NH_2-terminal deletion similar to cTnT-ND_{72-291} (Figure 3). For example, a naturally occurring NH_2-terminal truncation of the rabbit fast skeletal muscle TnT fragment (the TnT 26K fragment; *32*) is equivalent to the cTnT-ND_{72-291}. This fragment is able to form a functional troponin complex that exhibits a higher binding strength to tropomyosin compared with that of troponin containing the intact TnT. The reconstituted troponin complex containing the NH_2-terminal truncated fast TnT also conferred a decrease in the maximum activation of actomyosin−S1 MgATPase (*14*). Our results showed that cTnT-ND_{72-291} also has an increased affinity to tropomyosin (Figure 12B), indicating similar functional effects. Further studies using a similar NH_2-terminal-deleted cTnT in reconstituted myofilaments demonstrated that the removal of the NH_2-terminal domain resulted in decreased myofibril force development (*16*). These data suggest a hypothesis that the NH_2-terminal truncation of TnT is not a simple destruction but may

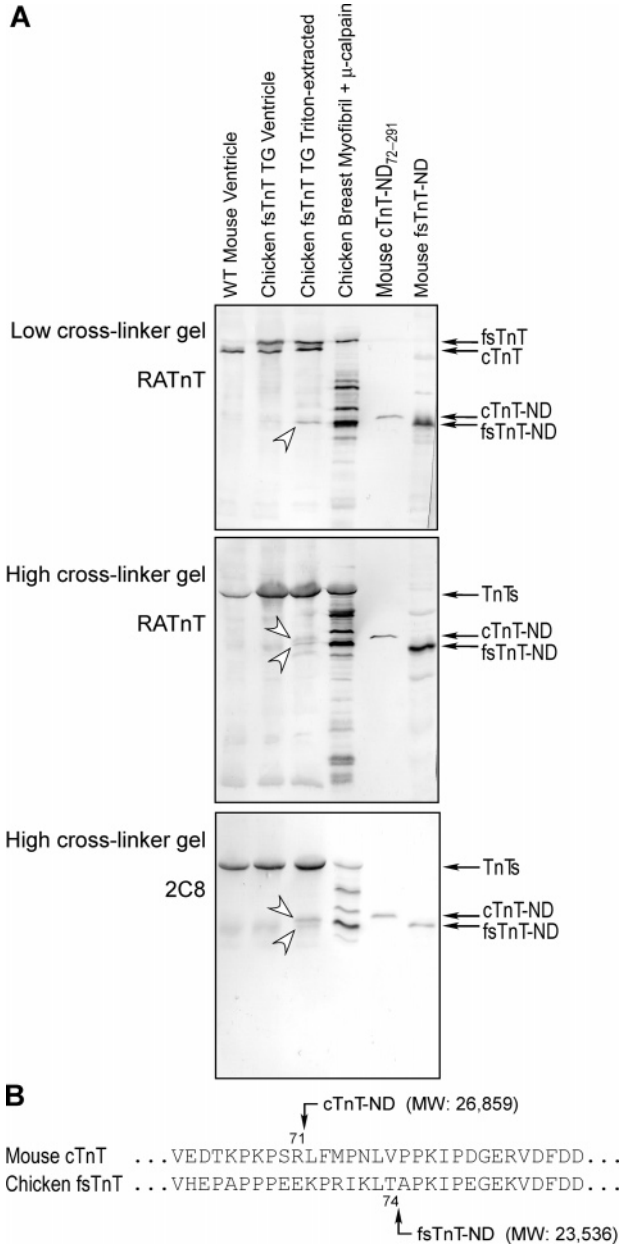

FIGURE 11: Similar NH_2-terminal truncation of cardiac and fast skeletal muscle TnT by μ-calpain modification. (A) A Western blot using polyclonal antibody RATnT raised against chicken fast TnT and mAb 2C8 recognizing both cardiac and fast TnTs detected TnT-ND fragments (indicated by the arrowheads) in Triton X-100-treated transgenic mouse cardiac muscle strips containing endogenous cTnT and transgenic-expressed chicken fast skeletal muscle TnT (fsTnT) (*38*). While the blot using the low cross-linker SDS gel (upper panel) showed only one TnT fragment band, the blot using the high cross-linker SDS gel resolved two TnT fragments with distinct immunoreactivities to RATnT and 2C8 antibodies, indicating their cTnT and fast TnT origins. μ-Calpain-treated chicken breast muscle myofibrils, reconstructed mouse cTnT-ND, and reconstructed mouse fast TnT-ND were used as controls. (B) Aligned amino acid sequences of the NH_2-terminal regions of mouse cardiac and chicken fast skeletal muscle TnTs flanking the truncation sites are shown. The predicted molecular weights of the NH_2-terminal truncated cTnT and fast TnT proteins are in agreement with the SDS gel mobility of the protein fragments detected in (A). The observation that cTnT and fast skeletal TnT were modified similarly by μ-calpain cleavage suggests a dependence on myofibril structure rather than the amino acid sequences at the cutting sites. TG, transgenic.

function as a regulatory mechanism in both cardiac and skeletal muscles, which may have a role in modulating

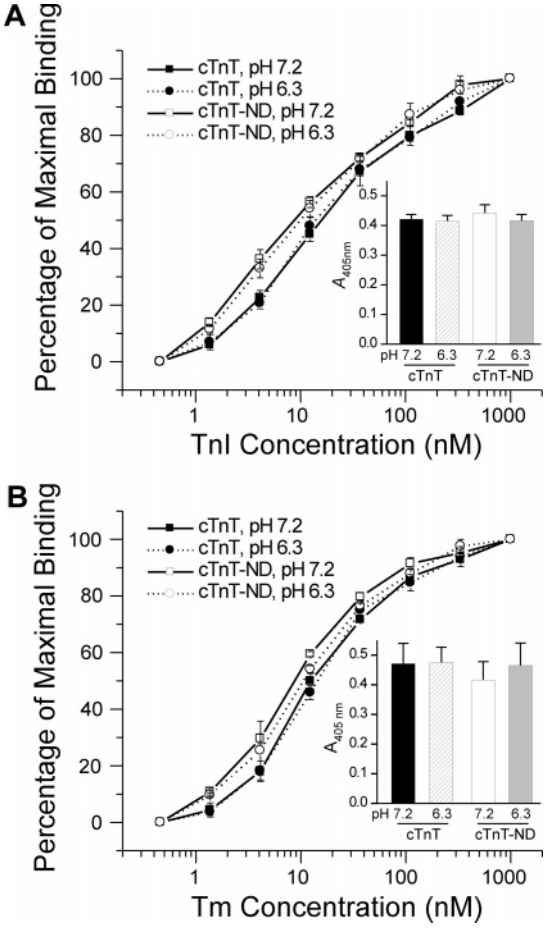

FIGURE 12: Removal of the NH_2-terminal variable region preserves cTnT's binding to TnI and tropomyosin (Tm) with altered affinities. ELISA protein binding curves demonstrate that the NH_2-terminal truncated cTnT has an increased binding affinity for TnI as compared to that of intact cTnT (A). The concentrations of TnI required to reach 50% of maximum binding were 8.73 ± 1.15 nM for cTnT-ND and 15.33 ± 1.36 nM for intact cTnT, $P < 0.005$. No significant difference was seen between the maximum bindings of cTnT-ND and intact cTnT to TnI (panel A insert). The binding of cTnT-ND to α-tropomyosin also exhibits a higher affinity than that of intact cTnT (B). The concentrations of the α-tropomyosin dimer for 50% maximum binding of cTnT-ND and intact cTnT were 9.73 ± 0.185 and 13.50 ± 1.38 nM, respectively ($P < 0.01$). The level of maximum binding was not significantly changed (panel B insert). The decrease of pH from 7.2 to 6.3 did not result in a significant change in the binding of both intact and NH_2-terminal truncated cTnT to TnI or α-tropomyosin.

contractility under physiological and pathological stress conditions.

$cTnT\text{-}ND_{72-291}$ is retained in the myofibrils of ischemia-reperfused cardiac muscle and, therefore, is anticipated to participate in the thin filament regulatory function. A consensus change found in the previous studies due to the presence of the NH_2-terminal truncated TnT is the decrease in the maximum Ca^{2+}-activated actomyosin ATPase and myofibril force (*14*−*16*). Such a decrease of Ca^{2+} activation by deleting the NH_2-terminal domain of cTnT during myocardial ischemia reperfusion could contribute to the depressed function after ischemia but may also provide a mechanism against Ca^{2+} overload-induced contractures (*54*). The hypothesis that the NH_2-terminal truncation of cTnT is not simply destructive is supported by our observation that transgenic mice overexpressing high levels of $cTnT\text{-}ND_{72-291}$ in the cardiac muscle do not show apparent cardiac dysfunc-

tion (*37*). Using the transgenic mouse hearts overexpressing the NH_2-terminal truncated cTnT, detailed functional characterization is currently underway.

Myofibril-Associated Calpain Activity as a Rapid Regulation of Myocardial Function. Triton X-100 extraction of cardiac muscle fibers induces the production of cTnT-ND, indicating a myofibril-associated proteolytic activity. Exogenous calpain inhibitors suppressed the production of cTnT-ND in Triton-extracted myofibrils. This endogenous calpain activity is independent of the presence or absence of Ca^{2+} in the incubation buffers (data not shown). Ca^{2+} concentration in living cardiac muscle cells rises periodically during the systole of each cardiac cycle to reach a level sufficient for μ-calpain activation. Therefore, the myofilament-associated μ-calpain may have been Ca^{2+}-primed in the myocytes before skinning. Triton extraction may have removed an endogenous calpain inhibitor or altered the myofilament conformation to activate the specific cleavage of cTnT. Consistent with a regulation by the substrate conformation, μ-calpain treatment of cardiac myofibrils reproduced the cTnT-ND_{72-291} fragment, in contrast to the treatment of purified cTnT that resulted in nonspecific degradation. Although the specific production of cTnT-ND is seen in the μ-calpain treatment of the isolated cardiac troponin complex, the protection of the TnT core structure was much less effective than that in the intact myofibril (Figures 8 and 10). Therefore, the specific modification of TnT by μ-calpain cleavage is based on the physiological structure of the myofilament. Despite sequence differences, similar NH_2-terminal truncation of chicken fast skeletal muscle TnT is produced by endogenous calpain proteolysis in transgenic mouse cardiac muscle (Figure 11), further supporting the myofilament structure-based specific calpain cleavage of the TnT NH_2-terminal variable region.

With the functional effects of removing the NH_2-terminal variable region of TnT, the μ-calpain-mediated proteolytic modification of TnT presents a rapid short-term mechanism to adjust muscle function under stress conditions. The cleavage occurs within minutes after myocardial ischemia reperfusion, which is apparently much faster than regulations by altering TnT isoform gene expression and/or alternative RNA splicing as that seen in the adaptation of skeletal muscle to unloading (*24*). Since myofilamental TnT only has a half-life of 4−5 days (*55*), the functional effect would be transient, suitable for an acute response to the usually short-lived ischemia-reperfusion stress conditions.

Proteolytic modifications of cTnT and cTnI have been shown with pathological effects on myocardial contractility (*28*, *56*). A caspase-catalyzed fragmentation of cardiac TnT has been found to reduce force production (*29*). Ca^{2+} overload in cardiomyocytes caused by ischemia reperfusion has been proposed to activate proteolytic cleavage of cTnI at amino acid 192 to remove the COOH terminus (*57*). The $cTnI_{1-192}$ fragment reduces the maximal isometric tension of the myocardium and causes a stunning phenotype in the hearts of transgenic mice (*28*). However, the production of cTnT-ND_{72-291} by μ-calpain modification is most likely a functional regulation rather than a detrimental destruction. The presence of low amounts of cTnT-ND_{72-291} in normal cardiac muscle also supports the hypothesis that the myofibril-associated μ-calpain activity functions in the physiological regulation of contractility.

Tuning Thin Filament Function in Myocardial Response to Stress Conditions. The upregulation of the NH_2-terminal-truncated cTnT in ischemia reperfusion indicates that modification of the thin filament function may play a role in the adaptation of cardiac muscle in stress conditions. By decreasing maximum contractile activation, the deletion of the NH_2-terminal domain of cTnT may reduce the work of the cardiac muscle during ischemia reperfusion to prevent permanent damage. This observation suggests that reducing the thin filament Ca^{2+} activation may be a potential target for the prevention or reduction of myocardial infarction following ischemia reperfusion. Further studies are needed to investigate this hypothesis.

Posttranslational modification is a rapid mechanism to confer transient functional changes in a protein. Posttranslational regulation of the cTnT NH_2-terminal structure represents an effective immediate response for myocardial adaptation to functional demands and pathological conditions. To date, this level of myocardial regulation has been mainly studied on phosphorylation modifications (*25*). Study on restricted proteolytic modification of cTnT represents a new area of research and will provide valuable information to further understand the role of posttranslational regulation in cardiac muscle function and diseases. These studies may also contribute to the development of new preventative and therapeutic strategies for the management of acute coronary inefficiency. Beyond the widely recognized protein destruction in myocardial ischemia reperfusion, the present study laid a foundation for further investigations into the significance of cTnT modification in ischemic heart disease.

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REFERENCES

1. Leavis, P. C., and Gergly, J. (1984) Thin filament proteins and thin filament-linked regulation of vertebrate muscle contraction, *CRC Crit. Rev. Biochem. 16*, 235−305.
2. Zot, A. S., and Potter, J. D. (1987) Structural aspects of troponin-tropomyosin regulation of skeletal muscle contraction, *Annu. Rev. Biophys. Biophys. Chem. 16*, 535−559.
3. Tobacman, L. S. (1996) Thin filament-mediated regulation of cardiac contraction, *Annu. Rev. Physiol. 58*, 447−481.
4. Perry, S. V. (1998) Troponin T: genetics, properties and function, *J. Muscle Res. Cell Motil. 19*, 575−602.
5. Breitbart, R. E., and Nadal-Ginard, B. (1986) Complete nucleotide sequence of the fast skeletal troponin T gene. Alternatively spliced exons exhibit unusual interspecies divergence, *J. Mol. Biol. 188*, 313−324.
6. Jin, J.-P., Huang, Q.-Q., Yeh, H.-I., and Lin, J. J.-C. (1992) Complete nucleotide sequence and structural organization of rat cardiac troponin T gene. A single gene generates embryonic and adult isoforms via developmentally regulated alternative splicing, *J. Mol. Biol. 227*, 1269−1276.
7. Huang, Q.-Q., Chen, A., and Jin, J.-P. (1999) Genomic sequence and structural organization of mouse slow skeletal muscle troponin T gene, *Gene 229*, 1−10.
8. Jin, J.-P., and Lin, J. J.-C. (1988) Rapid purification of mammalian cardiac troponin T and its isoform switching in rat hearts during development, *J. Biol. Chem. 263*, 7309−7315.
9. Wang, J., and Jin, J.-P. (1997) Primary structure and developmental acidic to basic transition of 13 alternatively spliced mouse fast skeletal muscle troponin T isoforms, *Gene 193*, 105−114.

10. Jin, J.-P., Chen, A., and Huang, Q.-Q. (1998) Three alternatively spliced mouse slow skeletal muscle troponin T isoforms: conserved primary structure and regulated expression during postnatal development, *Gene 214*, 121−129.
11. Pearlstone, J. R., and Smillie, L. B. (1982) Binding of troponin-T fragments to several types of tropomyosin. Sensitivity to Ca^{2+} in the presence of troponin-C, *J. Biol. Chem. 257*, 10587−10592.
12. Pearlstone, J. R., and Smillie, L. B. (1985) The interaction of rabbit skeletal muscle troponin-T fragments with troponin-I, *Can. J. Biochem. 63*, 212−218.
13. Hinkle, A., Goranson, A., Butters, C. A., and Tobacman, L. S. (1999) Roles for the troponin tail domain in thin filament assembly and regulation. A deletional study of cardiac troponin T, *J. Biol. Chem. 274*, 7157−7164.
14. Pan, B.-S., Gordon, A. M., and Potter, J. D. (1991) Deletion of the first 45 NH_2-terminal residues of rabbit skeletal troponin T strengthens binding of troponin to immobilized tropomyosin, *J. Biol. Chem. 266*, 12432−12438.
15. Fujita, S., Maéda, K., and Maéda, Y. (1992) Expression in Escherichia coli and a functional study of a beta-troponin T 25 kDa fragment of rabbit skeletal muscle, *J. Biochem. 112*, 306−308.
16. Chandra, M., Montgomery, D. E., Kim, J. J., and Solaro, R. J. (1999) The N-terminal region of troponin T is essential for the maximal activation of rat cardiac myofilaments, *J. Mol. Cell. Cardiol. 31*, 867−880.
17. Gomes, A. V., Guzman, G., Zhao, J., and Potter, J. D. (2002) Cardiac troponin T isoforms affect the Ca^{2+} sensitivity and inhibition of force development. Insights into the role of troponin T isoforms in the heart, *J. Biol. Chem. 277*, 35341−35349.
18. Anderson, P. A., Greig, A., Mark, T. A., Malouf, N. N., Oakeley, A. E., Ungerleider, R. M., Allen, P. D., and Kay, B. K. (1995) Molecular basis of human cardiac troponin T isoforms expressed in the developing, adult, and failing heart, *Circ. Res. 76*, 681−686.
19. Biesiadecki, B. J., and Jin, J.-P. (2002) Exon skipping in cardiac troponin T of turkeys with inherited dilated cardiomyopathy, *J. Biol. Chem. 277*, 18459−18468.
20. Biesiadecki, B. J., Elder, B. D., Yu, Z. B., and Jin, J.-P. (2002) Cardiac troponin T variants produced by aberrant splicing of multiple exons in animals with high instances of dilated cardiomyopathy, *J. Biol. Chem. 277*, 50275−50285.
21. Wang, J., and Jin, J.-P. (1998) Conformational modulation of troponin T by configuration of the NH_2-terminal variable region and functional effects, *Biochemistry 37*, 14519−14528.
22. Jin, J.-P., and Root, D. D. (2000) Modulation of troponin T molecular conformation and flexibility by metal ion binding to the NH_2-terminal variable region, *Biochemistry 39*, 11702−11713.
23. Jin, J.-P., Chen, A., Ogut, O., and Huang, Q.-Q. (2000) Conformational modulation of slow skeletal muscle troponin T by an NH(2)-terminal metal-binding extension, *Am. J. Physiol. Cell Physiol. 279*, C1067−C1077.
24. Yu, Z.-B., Gao, F., Feng, H., and Jin. J.-P. (2002) Role of myofilament protein isofrm regulation in the decreases in contractile force and tolerance to fatigue of slow skeletal muscles after unloading, *Biophys. J.* 82, 394a.
25. Sumandea, M. P., Burkart, E. M., Kobayashi, T., De Tombe, P. P., and Solaro, R. J. (2004) Molecular and integrated biology of thin filament protein phosphorylation in heart muscle, *Ann. N.Y. Acad. Sci. 1015*, 39−52.
26. Hirano, K., Hirano, M., and Kanaide, H. (2004) Regulation of myosin phosphorylation and myofilament Ca^{2+} sensitivity in vascular smooth muscle, *J. Smooth Muscle Res. 40*, 219−236.
27. Layland, J., Solaro, R. J., and Shah, A. M. (2005) Regulation of cardiac contractile function by troponin I phosphorylation, *Cardiovasc. Res. 66*, 12−21.
28. Murphy, A. M., Kogler, H., Georgakopoulos, D., McDonough, J. L., Kass, D. A., Van Eyk, J. E., and Marban, E. (2000) Transgenic mouse model of stunned myocardium, *Science 287*, 488−491.
29. Communal, C., Sumandea, M., de Tombe, P., Narula, J., Solaro, R. J., Hajjar, R. J. (2002) Functional consequences of caspase activation in cardiac myocytes, *Proc. Natl. Acad. Sci. U.S.A. 99*, 6252−6256.
30. Yu, Z.-B., Zhang, L. F., and Jin, J.-P. (2001) A proteolytic NH_2-terminal truncation of cardiac troponin I that is up-regulated in simulated microgravity, *J. Biol. Chem. 276*, 15753−15760.
31. Barbato, J. C., Huang, Q.-Q., Hossain, M. M., Bond, M., and Jin, J.-P. (2005) Proteolytic N-terminal truncation of cardiac troponin I enhances ventricular diastolic function, *J. Biol. Chem. 280*, 6602−6609.
32. Ohtsuki, I., Shiraishi, F., Suenaga, N., Miyata, T., and Tanokura, M. (1984) A 26K fragment of troponin T from rabbit skeletal muscle, *J. Biochem. 95*, 1337−1342.
33. Kitamura, S., Muroya, S., Tanabe, S., Okumura, T., Chikuni, K., and Nishimura, T. (2005) Mechanism of production of troponin T fragments during postmortem aging of porcine muscle, *J. Agric. Food Chem. 53*, 4178−4181.
34. Goll, D. E., Thompson, V. F., Li, H., Wei, W., and Cong, J. (2003) The calpain system, *Physiol. Rev. 83*, 731−801.
35. Cong, J. Y., Goll, D. E., Peterson, A. M., and Kapprell, H. P. (1989) The role of autolysis in activity of the Ca^{2+}-dependent proteinases (mu-calpain and m-calpain), *J. Biol. Chem. 264*, 10096−10103.
36. Di Lisa, F., De Tullio, R., Salamino, F., Barbato, R., Melloni, E., Siliprandi, N., Schiaffino, S., and Pontremoli, S. (1995) Specific degradation of troponin T and I by mu-calpain and its modulation by substrate phosphorylation, *Biochem. J. 308*, 57−61.
37. Biesiadecki, B. J., and Jin, J.-P. (2005) N-terminal truncation of cardiac troponin T by restricted proteolysis in myocardial ischemia-reperfusion, *Biophys. J.* 88, 360a.
38. Huang, Q.-Q., Brozovich, F. V., and Jin, J.-P. (1999) Fast skeletal muscle troponin T increases the cooperativity of transgenic mouse cardiac muscle contraction, *J. Physiol. (London) 520*, 231−242.
39. Jin, J.-P., Brotto, M. A., Hossain, M. M., Huang, Q.-Q., Brotto, L. S., Nosek, T. M., Morton, D. H., and Crawford, T. O. (2003) Truncation by Glu180 nonsense mutation results in complete loss of slow skeletal muscle troponin T in a lethal nemaline myopathy, *J. Biol. Chem. 278*, 26159−26165.
40. Jin, J.-P., Wang, J., and Zhang, J. (1996) Expression of cDNAs encoding mouse cardiac troponin T isoforms: characterization of a large sample of independent clones, *Gene 168*, 217−221.
41. Jin, J.-P. (1995) Cloned rat cardiac titin class I and class II motifs. Expression, purification, characterization, and interaction with F-actin, *J. Biol. Chem. 270*, 6908−6916.
42. Guttmann, R. P., Elce, J. S., Bell, P. D., Isbell, J. C., and Johnson, G. V. W. (1997) Oxidation inhibits substrate proteolysis by calpain I but not autolysis, *J. Biol. Chem. 272*, 2005−2012.
43. Wang, K. K., Nath, R., Posner, A., Raser, K. J., Buroker-Kilgore, M., Hajimohammadreza, I., Probert, A. W., Jr., Marcoux, F. W., Ye, Q., Takano, E., Hatanaka, M., Maki, M., Caner, H., Collins, J. L., Fergus, A., Lee, K. S., Lunney, E. A., Hays, S. J., and Yuen, P. (1996) An alpha-mercaptoacrylic acid derivative is a selective nonpeptide cell-permeable calpain inhibitor and is neuroprotective, *Proc. Natl. Acad. Sci. U.S.A. 93*, 6687−6692.
44. Smillie, L. B. (1982) Preparation and identification of alpha- and beta-tropomyosins, *Methods Enzymol. 85*, 234−241.
45. Solaro, R. J., Pang, D. C., and Briggs, F. N. (1971) The purification of cardiac myofibrils with Triton X-100, *Biochim. Biophys. Acta 245*, 259−262.
46. Jin, J.-P., Yang, F.-W., Yu, Z.-B., Ruse, C., Bond, M., and Chen, A. (2001) The highly conserved COOH-terminus of troponin I forms a Ca^{2+}-modulated allosteric domain in the troponin complex, *Biochemistry 40*, 2623−2631.
47. Lin, J. J.-C., Chou, C.-S., and Lin, J. L.-C. (1985) Monoclonal antibodies against chicken tropomyosin isoforms: production, characterization, and application, *Hybridoma 4*, 223−242.
48. Wang, Q., Reiter, R. S., Huang, Q.-Q., Jin, J.-P., and Lin, J. J.-C. (2001) Comparative studies on the expression patterns of three troponin T genes during mouse development, *Anat. Rec. 263*, 72−84.
49. Murphy, A. M. (2006) Heart failure, myocardial stunning, and troponin: a key regulator of the cardiac myofilament, *Congestive Heart Failure 12*, 32−38 (quiz 39−40).
50. Ogut, O., and Jin, J.-P. (1998) Developmentally regulated, alternative RNA splicing-generated pectoral muscle-specific troponin T isoforms and role of the NH_2-terminal hypervariable region in the tolerance to acidosis, *J. Biol. Chem. 273*, 27858−27866.
51. Takeda, S., Yamashita, A., Maeda, K., and Maeda, Y. (2003) Structure of the core domain of human cardiac troponin in the Ca(2+)-saturated form, *Nature 424*, 35−41.
52. Vinogradova, M. V., Stone, D. B., Malanina, G. G., Karatzaferi, C., Cooke, R., Mendelson, R. A., and Fletterick, R. J. (2005)

Ca(2+)-regulated structural changes in troponin, *Proc. Natl. Acad. Sci. U.S.A. 102*, 5038−5043.
53. Jin, J.-P., and Samanez, R. A. (2001) Evolution of a metal-binding cluster in the NH(2)-terminal variable region of avian fast skeletal muscle troponin T: functional divergence on the basis of tolerance to structural drifting, *J. Mol. Evol. 52*, 103−116.
54. Piper, H. M., Meuter, K., and Schafer, C. (2003) Cellular mechanisms of ischemia-reperfusion injury, *Ann. Thorac. Surg. 75*, S644−S648.
55. Martin, A. F. (1981) Turnover of cardiac troponin subunits. Kinetic evidence for a precursor pool of troponin-I, *J. Biol. Chem. 256*, 964−968.
56. Colantonio, D. A., Van Eyk, J. E., and Przyklenk, K. (2004) Stunned peri-infarct canine myocardium is characterized by degradation of troponin T, not troponin I, *Cardiovasc. Res. 63*, 217−225.
57. Foster, D. B., Noguchi, T., VanBuren, P., Murphy, A. M., and Van Eyk, J. E. (2003) C-terminal truncation of cardiac troponin I causes divergent effects on ATPase and force: implications for the pathophysiology of myocardial stunning, *Circ. Res. 93*, 917−924.

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