A Proteolytic NH$_2$-terminal Truncation of Cardiac Troponin I That Is Up-regulated in Simulated Microgravity

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In a tail suspension rat model, we investigated changes in myofilament protein during cardiac adaptation in simulated microgravity. Contractile force and velocity of cardiac muscle were decreased in the tail suspension rats as compared with the control. Ca$^{2+}$-dependent actomyosin ATPase activity was also decreased; however, sensitivity of cardiac muscle to Ca$^{2+}$ activation was unchanged. There was no change in expression of myosin heavy chain, tropomyosin, troponin T, or troponin I isoforms in hearts of tail suspension rats. A novel finding is a fragment of cardiac troponin I (cTnI) that had increased amounts in the heart of tail suspension rats. Binding of this cTnI fragment by a monoclonal antibody that specifically recognizes the COOH terminus indicates an intact COOH terminus. NH$_2$-terminal sequence analysis of the cTnI fragment revealed truncations primarily of amino acids 1–26 and 1–27 and smaller amounts of 1–30, including Ser$^{23}$ and Ser$^{24}$, which are substrates of protein kinase A phosphorylation. This cTnI fragment is present in normal cardiac muscle and incorporated into myofibrils, indicating a role in regulating contractility. This proteolytic modification of cTnI up-regulated during simulated microgravity suggests a potential role of the NH$_2$-terminal segment of cTnI in functional adaptations of cardiac muscle.

In microgravity, a significant stress on the cardiovascular system is the redistribution of body fluid toward the head due to the lack of hydrostatic pressure. Through neurohumoral regulations, this fluid redistribution induces reductions of blood volume and central venous pressure (1). Although decreased intrapleural pressure during space flight may assist filling of the heart (2), prolonged exposure to microgravity results in decreases in cardiac preload and function, evident by echocardiography of astronauts showing decreases in left ventricular end diastolic volume and ventricular stroke volume (3, 4). Rats flown in space for 14 days showed decreased average cross-sectional area of the myocytes in left ventricular muscle, indicating myocardial atrophy (5). These observations suggest that prolonged exposure to microgravity induces a decrease in cardiac function. However, the regulation of cardiac muscle contractility in microgravity is unclear. In addition to the health and safety of astronauts during and after long space flight, a thorough understanding of the adaptation of cardiac muscle in microgravity will also contribute to the prevention and treatment of myocardial dysfunction in chronic bedridden, paraplegic, and heart failure patients, since similar changes are seen in their hearts (6–8).

The adaptation of myocardial contractility in microgravity may involve structural and functional modifications of contractile proteins. The contraction of cardiac muscle is based on actin-myosin interactions regulated by intracellular Ca$^{2+}$ via the thin filament-based troponin-tropomyosin system (9). The regulation of thin filament proteins may play a role in the functional adaptation of cardiac muscle. The troponin complex contains three subunits: the Ca$^{2+}$-binding subunit troponin C (TnC),$^{1}$ the tropomyosin (Tm)-binding subunit troponin T (TnT), and the inhibitory subunit troponin I (TnI) (10, 11). During muscle contraction, Ca$^{2+}$-induced interactions between TnC and TnI, TnT, Tm, and actin result in a series of allosteric conformational changes in the thin filament, translating the signal into the activation of actomyosin ATPase and development of force (10). A key step in this signaling mechanism is the release of inhibition of TnI on actin-myosin interaction (12).

Three homologous TnI genes (cardiac, fast skeletal muscle, and slow skeletal muscle) have evolved in vertebrates to encode the muscle type-specific Tnl isoforms (13). Expression of TnI isoforms is regulated during development. The embryonic heart expresses exclusively slow skeletal muscle TnI. During perinatal heart development, the expression level of slow TnI decreases, and expression of cardiac TnI (cTnI) increases and becomes the only TnI isoform in the adult heart (14–16). Primary structures of cardiac, slow, and fast skeletal muscle TnI isoforms have been determined from cDNA and genomic cloning and sequencing (15, 17–23). Post-translational regulation of TnI structure and function has been found to involve both amino acid side chain modification and cleavage of the primary structure. A proteolytic truncation of 19 amino acids of the COOH terminus of cTnI has been found during myocardial ischemia and reperfusion injury (24). Expression of this cTnI fragment in transgenic mice produced myocardial stunning (25). A significant difference between cTnI and skeletal muscle TnI is an NH$_2$-terminal extension of 32 amino acids in cTnI (12). The $\beta$-adrenergic signaling pathway controls phosphoryl-

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$^{1}$ The abbreviations used are: TnC, troponin C; cTnI, cardiac troponin I; EDL, extensor digitorum longus; mAb, monoclonal antibody; MHC, myosin heavy chain; PAGE, polyacrylamide gel electrophoresis; pCa$_{50}$, pCa required for half-maximal activation; PKA, cAMP-dependent protein kinase; TBS, Tris-buffered saline; Tm, tropomyosin; TnI, troponin I; TnT, troponin T.
ation of two serine residues (Ser23 and Ser24) in the NH2-terminal region of cTnI by cAMP-dependent protein kinase (PKA) (26). This phosphorylation of cTnI decreases myofilament Ca2+ sensitivity by reducing the Ca2+-binding affinity of TnC (27). This mechanism plays an important role in the functional adaptation of cardiac muscle to physiological or pathological stress (28, 29).

The present study investigated the role of myofilament proteins in the adaptation of myocardial contractility in a rat tail suspension model of simulated microgravity (30). 4 weeks of tail suspension resulted in decreases of cardiac muscle contractility without change in the expression of contractile and regulatory protein isoforms. However, a novel finding is an NH2-terminal truncated cTnI fragment with increased amounts in the heart of tail suspension rats. This proteolytic NH2-terminal modification of cTnI removes the two serine residues that are PKA substrates. This post-translational regulation in simulated microgravity suggests a role of the NH2-terminal domain of cTnI in functional adaptations of cardiac muscle.

EXPERIMENTAL PROCEDURES

Animal Model—Male Harlan Sprague-Dawley rats weighing 180–210 g were randomly divided into control and tail suspension groups. The rats were housed in a 22 ± 2 °C environment, subjected to 12-h light/dark cycles, and fed water and Rat Chow ad libitum. Tail suspension was carried out by a modified Morey-Holton method (30) for 1, 2, 3, or 4 weeks. Care was taken to protect the tail tissue, and the movement of the rats was not restricted during the procedure.

Mechanical Recordings—Rats were anesthetized with ether. The heart was rapidly excised and rinsed in oxygenated Krebs-Henseleit solution (120 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 20 mM NaHCO3, 6.5 mM. The solution was maintained at 30 °C and oxygenated with 95% O2, 5% CO2. The nontendinous end of the papillary muscle was held by a spring clip. The tendinous end was tied to a stainless steel hook connected to an isometric force transducer (TB-651; Kohden, Japan).

The muscles were electrically stimulated by square wave pulses (10-ms duration) at 0.2 Hz. The length-tension relationship was recorded after equilibration at a resting tension of 1 g for 60–90 min. The length of the muscle was increased until a maximum developed force was obtained (Lmax). The length-tension relationship was then measured by reducing the muscle length at 2% intervals from the Lmax to 85% of Lmax while recording the resting and developed forces. Each of the steps change in length was in a reproducible sequence of 6–15 consecutive steps to minimize effects of hysteresis.

At the end of each experiment, the Lmax was measured again, and the muscle was then blotted dry and weighed. The cross-sectional area of the muscle was calculated assuming the geometry of a cylinder with a specific gravity of 1.0 (31). Tension was normalized by cross-sectional area. The unloaded maximum velocity of shortening (Vmax) was extrapolated from zero loading condition according to the Maxwell model of muscle (32).

Skinned Cardiac Muscle Preparations—Small cardiac muscle bundles (~0.4 mm in diameter and 2.5–2.8 mm in length) were dissected from the papillary muscle of left ventricle under a dissection microscope. The bundles were mounted as above for the measurement of unloaded maximum velocity of shortening at 4 °C for 20 min, the supernatant containing mainly myosin was removed. The pellet was extracted in 20 volumes (1 ml 5 M KCl, 10 mM Tris-HCl, pH 8.0, 0.1 mM phenylmethylsulfonyl fluoride) by stirring on ice for 30 min. After centrifugation as above, the extract was diluted 5-fold in TBS and loaded on the Tn1-1 mAb affinity column equilibrated in TBS. The column was washed with TBS, and the proteins bound to the Tn1-1 mAb affinity column were eluted with 50 mM glycine-HCl, pH 2.7. 0.5-ml fractions were collected into tubes containing 0.1 ml of neutralizing buffer containing 1 mM Tris-HCl, 0.5 M NaCl, 1 mM EDTA, pH 8.0. The fractions were analyzed by SDS-PAGE and Western blotting as described above to identify the cTnI peak.

NH2-terminal Sequencing of cTnI Fragment—The cTnI fragments isolated by immunofinity chromatography were pooled and dialyzed against 5 mM Tris-HCl, pH 7.5, and lyophilized. The concentrated protein was dissolved in a buffer containing 50 mM KCl, 2 mM MgCl2, 1 mM EGTA, 2 mM NaN3, 1 mM CaCl2, 20 mM Hepes, pH 7.0. The reaction was initiated by the addition of an equal volume of cold 10% (w/v) trichloroacetic acid. After removing the precipitate, inorganic phosphate (P) released was quantified as described previously (36).

Western Blot Analysis of Myosin Heavy Chain, Tm, TnT, and TnI Isoforms—As described previously (37), total protein was extracted from rat ventricular muscle by homogenization in SDS-PAGE SDS-PAGE sample buffer containing 1% SDS. The myocardial protein extracts were resolved by SDS-PAGE using Laemmli gels. 6% gel with an acrylamide/bisacrylamide ratio of 180:1 was used for the analysis of myosin heavy chain (MHC); 14% gel with an acrylamide/bisacrylamide ratio of 29:1 was used for the examination of TnT and TnI, and 12% gel with an acrylamide/bisacrylamide ratio of 29:1 was used for the examination of TnI. The protein bands resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose membrane (0.45-μm pore size) at 5 mA/cm2 for 25 min. The blotted nitrocellulose membrane was blocked in Tris-buffered saline (TBS; 137 mM NaCl, 5 mM KCl, 25 mM Tris-HCl, pH 7.4) containing 1% bovine serum albumin at room temperature for 1 h. The blocked membrane was incubated with anti-MHC monoclonal antibodies (mAbs) (41), an anti-actin mAb 1D4 (42), an anti-α-tubulin (43) (Sigma), an anti-cardiac TnT mAb CT7 (39), an anti-TnI mAb TnI-1 (40), a rabbit anti-TnI polyclonal antiserum (16), an anti-TnI-1 mAb CH1 (a gift from Dr. Jim Lin, University of Iowa (41)) in TBS containing 0.1% bovine serum albumin at 4 °C overnight. After washes with TBS plus detergents (0.5% Triton X-100 and 0.05% SDS) and TBS rinses, the membrane was incubated with alkaline phosphatase-conjugated anti-mouse or anti-rabbit IgG second antibody (from Sigma) in TBS containing 0.1% bovine serum albumin at room temperature for 1.5 h. After washes as above, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrozolium substrate reaction was carried out as described previously (40) to reveal the expression of MHC, TnT, TnI, and TnI isoforms in the cardiac muscle.

Immunofinity Chromatographic Isolation of cTnI—Rat cTnI was isolated by immunofinity chromatography using the Tn1-1 mAb against an epitope at the COOH terminus of TnI (40). The Tn1-1 mAb (IgG1) was purified from hybridomas ascites fluid using a Protein G column (Amersham Pharmacia Biotech) and coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer’s protocol. The immunofinity isolation of cTnI was then carried out with 1 ml 5 M KCl, 10 mM Tris-HCl, pH 8.0, 0.1 mM phenylmethylsulfonyl fluoride. The left ventricular muscle was minced into 1–2-mm3 pieces and extracted by 20 volumes (w/v) of Guba-Staub solution containing 300 mM KCl, 100 mM K-HPO4, 50 mM KH2PO4, 2.5 mM MgCl2, 1 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride, pH 6.5, on ice for 15 min. After centrifugation at 16,000 × g at 4 °C for 20 min, the supernatant containing mainly myosin was removed. The pellet was extracted in 20 volumes (1 ml 5 M KCl, 10 mM Tris-HCl, pH 8.0, 0.1 mM phenylmethylsulfonyl fluoride) by stirring on ice for 30 min. After centrifugation as above, the extract was diluted 5-fold in TBS and loaded on the Tn1-1 mAb affinity column equilibrated in TBS. The column was washed with TBS, and the proteins bound to the Tn1-1 mAb affinity column were eluted with 50 mM glycine-HCl, pH 2.7. 0.5-ml fractions were collected into tubes containing 0.1 ml of neutralizing buffer containing 1 mM Tris-HCl, 1.5 mM NaCl, 1 mM EDTA, pH 8.0. The fractions were analyzed by SDS-PAGE and Western blotting as described above to identify the cTnI peak.

NH2-terminal Sequencing of cTnI Fragment—The cTnI fractions isolated by immunofinity chromatography were pooled and dialyzed against 5 mM Tris-HCl, pH 7.5, and lyophilized. The concentrated protein was dissolved in SDS-PAGE sample buffer, resolved on 14% SDS-PAGE, and then electrophoretically transferred onto a polyvinylidene difluoride membrane as described above. The membrane was stained with 0.5% Amido Black, and a stained protein band representing the cTnI fragment was identified by Edman degradation using an automated amino acid sequencer (44).
muscle (42, 43). The calculated tail suspension rats, similar to that seen in the failing cardiac tension relationship was preserved in the cardiac muscle of tail suspension rats, similar to that seen in the failing cardiac tension relationship was preserved in the cardiac muscle of tail suspension rats. However, the length-pCa curves of skinned cardiac muscle preparations showed no difference between the pCa50 values obtained from the 4-week tail suspension and control rats (Fig. 2A). The Hill coefficient n obtained from the pCa50 values was also not changed. However, consistent with the results from intact cardiac muscle, the maximal isometric force (Fmax) of the skinned cardiac muscle was decreased in the tail suspension rats (p < 0.05) (Fig. 2B). Correspondingly, the Ca2+-activated ATPase activity of the cardiac myofibrils of 4-week tail suspension rats was significantly lower than that of the control rats (Fig. 2C, p < 0.05).

No Change in the Expression of Cardiac MHC, Tm, and TnT Isoforms in the Heart of Tail Suspension Rats—Expression of isoforms of the contractile and regulatory proteins was examined to determine whether any isoform switching could contribute to the decrease in the cardiac muscle contractility in the tail suspended rats. Using mAbs FA1 (specific to α-MHC) and FA2 (recognizing both α- and β-MHC) (38), the Western blots in Fig. 3 show no decrease in the normally predominant α-MHC or detectable expression of β-MHC in the ventricular muscle of 4-week tail suspension rats.

The results in Fig. 6 show that the Western blots using the anti-Tm mAb CH1 that recognizes both α- and β-Tm detected only the normally occurring α-Tm in the heart of 3- and 4-week tail suspension rats with no difference from the controls. The Western blots using the anti-cardiac and slow skeletal muscle TnT mAb CT3 showed only adult cardiac TnT, indicating no change in the expression of alternative RNA splicing-generated cardiac TnT isoforms (45) in the heart of 3- and 4-week tail suspension rats. Western blots using the RATnT polyclonal antibody against cardiac and skeletal muscle TnTs showed no expression of fast or slow skeletal muscle TnT isoforms in the heart of tail suspension rats. No proteolytic fragment of Tm and TnT was detected in the tail suspension rat hearts by Western blot analysis.

An NH2-terminal Truncated cTnI Fragment Up-regulated in the Heart of Tail Suspension Rats—Western blots using mAb TnI-1 recognizing all three TnI isoforms (40) did not detect fast and slow skeletal muscle TnI isoforms in the heart of tail suspension rats (Fig. 4). A novel finding was a 22-kDa TnI fragment with increased amounts in the heart of tail-suspended rats (Fig. 4). In addition to the mAb TnI-1 from mouse ascites fluid, supernatant from TnI-1 hybridoma cell culture recognizes this band on Western blot, excluding nonspecific reaction from other immunoglobulins in the mouse ascites fluid. This band is also recognized by a rabbit polyclonal anti-TnI antibody, RATnI (16), confirming that it is indeed a TnI fragment. The Western blots of hearts of tail-suspended and control rats plus rat extensor digitorum longus (EDL; a fast skeletal muscle), soleus (a slow skeletal muscle), and neonatal cardiac muscle controls showed that the low M1 cTnI band migrated faster than the slow skeletal muscle TnI but more slowly than the fast skeletal muscle TnI (Fig. 4). Although slow skeletal muscle TnI is expressed in embryonic and postnatal cardiac muscles (14–16), the gel mobility indicates that this low M1 TnI is not a reexpression of slow skeletal muscle TnI in the adult heart. Although phosphorylation may also slightly

![Figure 1](http://www.jbc.org/)

**Fig. 1.** Tension-length relationship and velocity of shortening in intact papillary muscle of control and tail suspension rats. Lmax was determined as the muscle length with a resting tension of 1 g/mm². n = 10 for each group. A, the results showed decreased developed tension (DT) for the 4-week tail suspension rat (SUS) cardiac muscle, while resting tension (RT) was not changed. The tension-length relationship was preserved in the cardiac muscle of tail suspension rats. B, a lowered Vmax was seen in the cardiac muscle of tail suspension rats. Values are means ± S.E. *p < 0.05; **p < 0.01 compared with the control (CON).

Data Analysis—Values are presented as means ± S.E. The statistical significance of differences between the mean values was analyzed by Student’s t test. The theoretical molecular weight and isoelectric point of rat cTnI and fragments were calculated from amino acid sequences using the DNASTar computer program.

RESULTS

Decreased Myocardial Function in the Tail Suspension Rats—The body weights of the tail-suspended rats were similar to age-matched controls. The heart rate, mean arterial pressure, and maximal left ventricular pressure of the 4-week tail suspension rats were not significantly different from the controls. The heart weight/body weight ratio and the size of papillary muscle of the tail suspension rats were also not significantly different from those of the control rats (data not shown).

Isometric force measurements on the papillary muscles of tail-suspended and control rats showed no difference in resting tension (RT) (Fig. 1A). In contrast, developed tension (DT) was decreased in the cardiac muscle of the tail suspension rats (Fig. 1A, p < 0.05–0.01). This decrease began at 2 weeks of tail suspension (data not shown) and became significant at 4 weeks of tail suspension. However, the length-tension relationship was preserved in the cardiac muscle of tail suspension rats, similar to that seen in the failing cardiac muscle (42, 43). The calculated Vmax of the papillary muscle was also decreased after 4 weeks of tail suspension (p < 0.05) (Fig. 1B) together with a prolonged time to peak tension development (data not shown).

Despite the decreased force and velocity, the response curve and EC50 of intact papillary muscle to the extracellular Ca2+ concentration were not different from controls (data not shown), indicating that the Ca2+ handling of cardiomyocyte membranes was not significantly affected in the tail suspension rats (44). The force-pCa curves of skinned cardiac muscle preparations showed no difference between the pCa50 values obtained from the 4-week tail suspension and control rats (Fig. 2A). The Hill coefficient n obtained from the pCa50 values was also not changed. However, consistent with the results from intact cardiac muscle, the maximal isometric force (Fmax) of the skinned cardiac muscle was decreased in the tail suspension rats (p < 0.05) (Fig. 2B). Correspondingly, the Ca2+-activated ATPase activity of the cardiac myofibrils of 4-week tail suspension rats was significantly lower than that of the control rats (Fig. 2C, p < 0.05).

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alter the mobility of TnI in SDS gel, we have confirmed by amino acid sequencing that this low Mr TnI is a cTnI fragment. While a slight increase was detected in the heart of 3-week tail suspension rats as compared with controls, amounts of this cTnI fragment significantly increased in the heart of 4-week tail suspension rats. The relative levels of the low Mr cTnI band in hearts of 4-week tail suspension rats was quantified by two-dimensional densitometry of the TnI-1 mAb Western blots. The results demonstrate an increase of the cTnI fragment from 13.1 to 16.3% of the total cTnI (**p < 0.05), reflecting an increase of its ratio to intact cTnI from 1:6.61 to 1:5.15. Although the cTnI fragment increased 24.4%, this portion only represents 3.2% of the total cTnI. Therefore, no significant change in the amounts of intact cTnI was detected by densitometric analysis of the Western blots (Fig. 5).

Protein degradation during the post-mortem handling of the sample could contribute to the different amounts of the cTnI fragment. To exclude this possibility, Western blot analysis of mouse heart samples taken at a series of post-mortem time points showed no difference in the levels of the cTnI fragment during a period of up to 8-h storage at room temperature (Fig. 6). We further showed that 1-h perfusion of rat heart in vitro as described previously (46) did not change the

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**Fig. 2.** Ca\(^{2+}\) activation of skinned cardiac muscle preparations. A, the force-pCa curves show no difference in pCa\(_{50}\) and Hill coefficient (n) between the two groups. F\(_{max}\) (B) and Ca\(^{2+}\)-activated MgATPase activity (C) of the left ventricular myofibrils were decreased in the tail suspension rats (SUS) as compared with the control (CON). n = 6 rats for each group. Values are means ± S.E. **p < 0.05.

**Fig. 3.** Expression of cardiac myosin heavy chain in the heart of control and tail suspension rats. SDS-PAGE (top panel) and Western blots using FA1 (against α-MHC) and FA2 (against both α- and β-MHC) mAbs (middle and lower panels) detected no difference between the expression of cardiac MHC isoforms in the left ventricle of 4-week tail suspension and control rats (two samples each).

**Fig. 4.** Expression of thin filament regulatory proteins in the heart of tail suspension rats and a cTnI fragment. Total protein extracts from the control and tail suspension (3 and 4 weeks) rat hearts and rat neonatal heart, adult soleus, and adult EDL controls were resolved by SDS-PAGE and analyzed by Western blotting for the expression of TnT, Tm, and TnI. While no difference in the expression of TnT and Tm isoforms was found between the hearts of tail suspension and control rats, a low Mr TnI band was detected in the rat hearts with increased amounts in the 4-week tail suspension rats. The gel mobility of the low Mr cTnI differs from that of fast (fsTnI) and slow (ssTnI) skeletal muscle TnIs expressed in the control muscle samples. ssTnT and fsTnT, slow and fast skeletal muscle TnT, respectively; cTnTe and cTnTa, embryonic and adult cardiac TnT, respectively.
amount of the cTnI fragment (Fig. 6). These results indicate that the cTnI fragment detected with an increased level in the heart of 4-week tail suspension rats is not a product of nonspecific protein degradation. In contrast, it may reflect a physiologically regulated proteolysis of cTnI in the cardiac muscle and may play a role during the functional adaptation in simulated microgravity.

The isolation of the cTnI fragment by the anti-COOH terminus mAb was as effective as that for the intact cTnI (Fig. 7). Since the TnI-1 mAb does not recognize the cTnI-(1–192) fragment (40), this result indicates that COOH terminus in the cTnI fragment. NH2-terminal sequence analysis showed that there were three main species of cTnI fragments generated by truncations at the COOH-end of amino acids Asn26, Tyr27, or Tyr30 (Fig. 8). The main products were the deletions of amino acids 1–26 and 1–27 (48 and 36%, respectively). These truncation sites do not correspond to any exon boundaries of the cTnI gene (Fig. 8). Therefore, the cTnI fragment is not generated by alternative RNA splicing but, instead, by proteolytic cleavage. This cleavage of cTnI polypeptide chain removes the exon 1 and 2-encoded short segments at the very NH2 terminus and a large portion of the exon 3-encoded cTnI-specific NH2-terminal extension. The calculated Mr of the three NH2-terminal truncated cTnI fragments (Table I) is in agreement with the size range shown in the SDS-PAGE (Figs. 4, 6, and 7) and further supports the preservation of an intact COOH terminus.

Preserved Core Structure and Myofibril Incorporation of the NH2-Terminal Truncated cTnI—The primary structural alignment of cardiac, slow, and fast skeletal muscle TnIs (Fig. 8) shows that the NH2-terminal truncated cTnI preserves the regions homologous to the skeletal muscle TnIs containing all of the identified binding sites for other thin filament proteins. It has been shown that deletion of the NH2-terminal 32 amino acid did not produce a significant change in the inhibitory function of cTnI in vitro (47). Therefore, it may be assumed that the cTnI fragment truncated by the NH2-terminal 26–30 amino acids would preserve its core function in the thin filament regulatory system. Consistent with this hypothesis, Western blot analysis of extensively washed rat cardiac myofibrils showed that the incorporation of the NH2-terminal truncated cTnI fragment into the myofilaments is comparable with that of intact cTnI, proportional to their total levels detected in the cardiac muscle cells (Fig. 9A). The truncated cTnIs have very similar molecular weights and isoelectric points (Table I), implying similarity in their three-dimensional structural and functional features.

The Western blot in Figs. 6 and 8 demonstrate that normal rat and mouse cardiac muscles contain significant amounts of the NH2-terminal truncated cTnI. Using the anti-TnI COOH terminus mAb TnI-1, Western blot analysis of cardiac muscle samples from a wide spectrum of vertebrate species revealed similar cTnI fragments in all of the hearts examined (Fig. 9B). These data suggest that the proteolytic NH2-terminal truncation of cTnI occurs under physiological conditions, implying a post-translational regulation of myocardial contraction. Together with the removal of the NH2-terminal extension, an important structural feature of the truncated cTnI is the loss of Ser23 and Ser24 (Fig. 8), two PKA phosphorylation sites in C-terminal, implying similarity in their three-dimensional structural and functional features.

**DISCUSSION**

The Tail Suspension Rat Model of Simulated Microgravity Produces Changes in Myocardial Function—The present study investigated the adaptation of cardiac muscle in simulated microgravity using the tail suspension rat model. The 30° head down tilt of the tail-suspended rats produces a redistribution of body fluid toward the head. The hemodynamic changes in the tail suspension rats are similar to those found in astronauts during space flight (1). As a convenient model of simulated weightlessness on the ground, tail-suspended rats have been...
FIG. 7. Immunoaffinity chromatographic isolation of rat cTnI and cTnI fragment. A, thin filament proteins extracted from rat ventricular muscle were fractionated on an anti-TnT mAb affinity column. The proteins bound to the column were eluted with low pH and analyzed by SDS-PAGE and Western blotting using anti-TnI (TnI-1), TnT (CT3), and TnC (CH1) mAbs. The results show that both intact cTnI and the cTnI fragment were recovered by the anti-TnI COOH terminus mAb affinity column together with TnT, TnC, and trace amounts of TnC and other myofilament proteins. B, the cTnI fractions eluted from the affinity column were concentrated, resolved by SDS-PAGE, and transferred to polyvinylidene difluoride membrane. Amido Black staining revealed a good yield of the cTnI fragment, and the band was sliced out for NH2-terminal amino acid sequencing.

![Diagram](image1)

FIG. 8. NH2-terminal amino acid sequence of the cTnI fragments. Structural maps of rat fast skeletal muscle TnI, slow skeletal muscle TnI, and cTnI are aligned with the regions for the binding of TnC, TnT, and actin as well as the inhibitory peptide indicated. The segments encoded by different exons of the three TnI genes are outlined by the boxes. The cTnI-specific exon 3 is shown by a filled box. The three NH2-terminal sequences determined from the purified rat cTnI fragment are shown and aligned with the sequence encoded by exons 1–3 of the rat cTnI gene (60). The arrowheads indicate the three clustered cleavage sites. The two PKA substrate serine residues (Ser23 and Ser24) are highlighted.

![Diagram](image2)

FIG. 9. Similar cTnI fragment in other vertebrate hearts and incorporation into myofibrils. A, TnI-1 mAb Western blots on protein extracts from intact cardiac muscle and extensively washed myofibrils from rat heart showed that the NH2-terminal truncated cTnI fragment was able to integrate into the myofibril. B, total muscle homogenates of adult heart of fish, turtles, mice, rats, rabbits, cats, dogs, and humans were resolved by SDS-PAGE and examined by Western blotting using the TnI-1 mAb against the COOH terminus of TnI. The results show low Mr cTnI bands in all of the hearts examined.

![Diagram](image3)
decreased myocardial contractility and volume preload may both contribute to the reduced cardiac function seen in long term exposure to microgravity.

No Change in Heart-specific and Developmental Expression of Cardiac Myofibril Protein Isoforms during the Adaptation to Simulated Microgravity—Although the Ca\(^{2+}\)-activated ATPase activity was reduced in the cardiac muscle of the tail suspension rats (Fig. 2C), there was no change in the expression of MHC isoforms (Fig. 3). Therefore, the decrease in contractility cannot be attributed to changes in myosin isoenzymes in the heart. In addition, no change in the expression of Tm, TnT, and TnI isoforms was found in the heart of tail-suspended rats (Fig. 4). It has been shown by numerous studies that these contractile and regulatory protein isoforms are sensitive markers for the change of muscle fiber types or developmental states (51). In contrast to the reexpression of fetal cardiac genes in the unloaded adult ventricular muscle in heterotopic transplanted heart (52), the lack of isoform switch of MHC, Tm, TnT, and TnI in the hearts of tail suspension rats indicates no change in cardiac muscle differentiation or developmental state during the functional adaptation in simulated microgravity.

A Proteolytic Truncation of the cTnI-specific NH\(_2\)-terminal Segment Regulated during Functional Adaptation of Cardiac Muscle—A novel finding of the present study is the NH\(_2\)-terminal truncated cTnI fragment in both normal and tail-suspended rat hearts (Figs. 4 and 6). Proteolytic modification of cTnI has been shown with pathological effects on myocardial contractility. For example, Ca\(^{2+}\) overload in cardiomyocytes caused by ischemia-reperfusion may activate proteolytic cleavage of cTnI at amino acid 192 to remove the COOH terminus (24). The cTnI(1–192) fragment reduces the maximal isometric tension of the myocardium and causes a stunning phenotype in the hearts of transgenic mice (25). The low molecular weight cTnI band identified in our study has an intact COOH terminus as shown by its reactivity to the anti-COOH terminus mAb Tnl-1 (Fig. 4) that does not recognize cTnI(1–192) (40). Together with its presence in normal cardiac muscle, the preserved core structure (Fig. 8) and apparently normal integration into the myofilament (Fig. 9A) of this NH\(_2\)-terminal modified form of cTnI suggest a physiological significance. This hypothesis is supported by the fact that a similar cTnI fragment is also found in other vertebrate hearts (Fig. 9B). Therefore, the increased amounts of the NH\(_2\)-terminal truncated cTnI in the heart of tail-suspended rats may represent a functional adaptation of cardiac muscle in simulated microgravity and a proteolytic regulation of cardiac myofibrillar proteins. The regulatory mechanism of the proteolytic NH\(_2\)-terminal truncation of cTnI requires further investigation. Calpain-catalyzed cTnI degradation that is regulated by PKA and protein kinase C phosphorylation of cTnI (53) may play an important role.

Structure-Function Significance of the NH\(_2\)-terminal Truncated cTnI—The truncated cTnI has lost almost all of the cTnI-specific NH\(_2\)-terminal extension (Fig. 8). Accordingly, the functional effects of the NH\(_2\)-terminal truncated cTnI may be 2-fold: to mimic the property of skeletal muscle TnI as well as to remove the PKA phosphorylation-mediated regulation. Expression of slow skeletal muscle TnI in the heart of transgenic mice resulted in an increased sensitivity to Ca\(^{2+}\) activation, which was not reduced by in the \(\beta\)-adrenergic stimulated PKA treatment (54). Such an effect may increase the Ca\(^{2+}\) sensitivity of the cardiac muscle in the tail-suspended rats. However, the effects of the NH\(_2\)-terminal truncated cTnI on cardiac muscle contraction would not simply mimic that of slow skeletal muscle TnI, because over 50% sequence diversity is present between the homologous portion of cTnI and slow TnI (40), which may also result in differences in function. This is supported by the observation that a replacement of the NH\(_2\)-terminal half of the cTnI polypeptide chain with the NH\(_2\)-terminal domain of slow skeletal muscle TnI resulted in contractility features differing from those of either cTnI or slow TnI (55). Therefore, the NH\(_2\)-terminal truncated cTnI may confer a unique functional change in the cardiac thin filament. Nevertheless, the NH\(_2\)-terminal segment of cTnI contains the two serine residues, Ser\(^23\) and Ser\(^24\) (Fig. 8), which are substrates for PKA-catalyzed phosphorylation (26, 27). Phosphorylation of Ser\(^24\) in cTnI may occur constitutively, whereas phosphorylation of Ser\(^23\) produces a decrease in the sensitivity of the myofilaments to Ca\(^{2+}\) via a modulation of TnC-Ca\(^{2+}\) affinity (48). The presence of a dynamic pool of the NH\(_2\)-terminal truncated cTnI in the cardiac muscle may provide a basal level of function that is not regulated by the PKA signaling pathway. Therefore, the up-regulation of the NH\(_2\)-terminal truncated cTnI in the heart of tail suspension rats may modulate cardiac muscle contractility by counteracting the effect of PKA phosphorylation of Ser\(^23\) and Ser\(^24\) on the sensitivity of thin filament to Ca\(^{2+}\). While apparently complex neurohumoral regulation during cardiac deconditioning reduces the contractility of the cardiac muscle, this counteracting mechanism may have contributed to the preserved Ca\(^{2+}\) sensitivity in the cardiac muscle of 4-week tail suspension rats when the contractile force and velocity were decreased (Figs. 1 and 2).

Role of Thin Filament Regulation in the Functional Adaptation of Cardiac Muscle—The up-regulation of the NH\(_2\)-terminal truncated cTnI in the heart of tail suspension rats indicates that the function of thin filament may play a role in the adaptation of cardiac muscle in microgravity. For example, the proteolytic modification of cTnI may alter the responsiveness of the cardiac muscle to \(\beta\)-adrenergic regulation that plays an important role in myocardial adaptations to physiological and pathological stresses (56–58). It is worth noting that the arterial blood pressure and maximum left ventricular pressure are not changed in the tail suspension rats, and the afterload may stimulate a positive regulation at the myofilamental levels, while the systemic neurohumoral regulation in the tail-suspended rat would reduce cardiac function in simulated microgravity. The deletion of the NH\(_2\)-terminal PKA phosphorylation sites from cTnI may produce a \(\beta\)-adrenergic-independent domain of the cardiac muscle thin filament, which may be important in preserving cardiac function during prolonged exposure to microgravity. Therefore, the NH\(_2\)-terminal truncation of cTnI represents a novel proteolytic regulation of cardiac muscle contractility. This finding suggests that a selective inhibition of the NH\(_2\)-terminal phosphorylation of cTnI may be a potential target for the prevention of cardiovascular dysfunction of astronauts during and after long space flights. By increasing the sensitivity of cardiac myofilament to Ca\(^{2+}\) activation under elevated \(\beta\)-adrenergic stimulation, the proteolytic modification of cTnI may also play a role in myocardial adaptation under other physiological and pathological conditions, such as in chronic bedridden, paraplegic, and heart failure patients (7, 28, 29, 59).

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A Proteolytic NH₂-terminal Truncation of Cardiac Troponin I That Is Up-regulated in Simulated Microgravity

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