The Highly Conserved COOH Terminus of Troponin I Forms a Ca^{2+}-Modulated Allosteric Domain in the Troponin Complex†

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ABSTRACT: The primary structure of the COOH-terminal region of troponin I (TnI) is highly conserved among the cardiac, slow, and fast skeletal muscle TnI isoforms and across species. Although no binding site for the other thin filament proteins is found at the COOH terminus of TnI, truncations of the last 19−23 amino acid residues reduce the activity of TnI in the inhibition of actomyosin ATPase and result in cardiac muscle malfunction. We have developed a specific monoclonal antibody (mAb), TnI-1, against the conserved COOH terminus of TnI. Using this mAb, isolation of the troponin complex by immunoaffinity chromatography from muscle homogenate and immunofluorescence microscopic staining of myofibrils indicate that the COOH terminus of TnI forms an exposed structure in the muscle thin filament. Binding of this mAb to the COOH terminus of cardiac TnI induced extensive conformational changes in the protein, suggesting an allosteric role of this region in the functional integrity of troponin. In the absence of Ca^{2+}, the binding of troponin C and troponin T to TnI had very little effect on the conformation of the COOH terminus of TnI as indicated by the unaffected mAb affinity for the TnI-1 epitope. However, Ca^{2+} significantly increased the accessibility of the TnI-1 epitope on TnI in the presence of troponin C and troponin T. The results provide evidence that the COOH terminus is an essential structure in TnI and participates in the allosteric switch during Ca^{2+} activation of contraction.

Contraction of vertebrate striated muscle is regulated by intracellular Ca^{2+} via the thin filament-based troponin−tropomyosin system. The troponin complex contains three subunits: the Ca^{2+}-binding subunit troponin C (TnC),[1] the tropomyosin-binding subunit troponin T (TnT), and the inhibitory subunit troponin I (TnI) (see refs *1*−*3* for reviews). During muscle contraction, TnI interacts with TnC, TnT, tropomyosin, and actin by means of a series of allosteric conformational changes (*4*). These thin filament protein interactions translate the triggering signal (elevation of cytosolic [Ca^{2+}]) into a response of the contractile system (activation of actomyosin ATPase and development of force).

The structure−function relationship of TnI has been extensively studied. Three TnI genes (cardiac, fast skeletal muscle, and slow skeletal muscle) have evolved in vertebrates to encode the muscle type-specific TnI isoforms (*5*). The expression of TnI isoforms is regulated in different muscle types and during development (*6*, *7*). Primary structures of cardiac, fast, and slow skeletal muscle TnI isoforms have been determined in a number of species (*8*−*17*). The main structural difference between cardiac and skeletal muscle TnI is an NH_2-terminal extension unique to cardiac TnI (cTnI), containing phosphorylation sites for cAMP-dependent protein kinase (PKA) (*18*).

Several models have been proposed for the three-dimensional structure of full-length TnI and of TnI fragments complexed with TnC. Upon the basis of specific interactions with other thin filament regulatory proteins, structure−function mapping has identified subdomains of TnI. Summarized in a recent review (*4*), the TnC binding sites are located in the region of amino acids 83−134; the TnT binding site is in the region of amino acids 30−80; and amino acids 98−116 and amino acids 140−148 bind actin. A segment at amino acids 96−116 of fast skeletal muscle TnI (fsTnI) carries TnI's core function of inhibiting actomyosin ATPase. This inhibitory peptide forms a β-hairpin structure in the TnC−TnI complex (*19*) and is conserved in all TnI isoforms. The COOH terminus of TnI shows a strongly conserved primary structure among the three muscle type-specific TnI isoforms and across species. This conserved region is encoded by the exon 8 in the cTnI (*14*, *20*), fsTnI (*21*), and slow skeletal muscle TnI (ssTnI) (*22*) genes, corresponding to the COOH-terminal 27, 31, or 34 residues of human cTnI, fsTnI, and ssTnI, respectively. Although this

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[1] Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid); BSA, bovine serum albumin; buffer A, 0.1 M KCl, 3 mM $MgCl_2$, 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), pH 7.0; buffer T, buffer A containing 0.05% Tween 20; cTnI, cardiac TnI; ELISA, enzyme-linked immunosorbant assay; fsTnI, fast skeletal muscle TnI; HRP, horseradish peroxidase; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PKA, cAMP-dependent protein kinase; ssTnI, slow skeletal muscle TnI; TBS, Tris-buffered saline; TCEP, tris(carboxyethyl)phosphine; TnC, troponin C; TnI, troponin I; TnT, troponin T.

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region did not have a stable contact with TnC, TnT, and actin as shown by cross-linking experiments (*23*), deletion of the COOH-terminal 17−26 amino acids from fsTnI or 23 amino acids from cTnI weakens the inhibitory effect of TnI on actomyosin ATPase activity (*24, 25*).

Proteolytic removal of the COOH-terminal 19 amino acids from cTnI was found during myocardial ischemia and reperfusion injury (*26*). In transgenic mouse hearts, expression of cTnI lacking the COOH-terminal 19 amino acids produced myocardial stunning (*27*). A truncation of the COOH-terminal 8 amino acids due to a point mutation in human cTnI gene has been found in familial hypertrophic cardiomyopathy (*28*). Therefore, the function of the conserved COOH-terminal region of TnI needs to be further investigated in order to understand the structure−function relationship of TnI in the contraction of muscle and its significance in the pathogenesis of myocardial diseases.

A major limitation in the investigation of the structure−function relationship of the COOH-terminal domain of TnI is the lack of high-resolution three-dimensional structural information for full-length TnI. Since antibody−antigen recognition is based on the complementary fit in three-dimensional structures of protein (*29*), specific antibodies can provide useful tools to investigate the conformation of proteins (*30, 31*). This can be achieved by analysis of epitope affinity in order to monitor conformational changes during structural and functional modulation. Hybridoma monoclonal antibody (mAb) technology (*32*) has provided a powerful approach to generate homogeneous antibodies against specific epitopes.

In the present study, we have developed and characterized a specific anti-TnI mAb (named TnI-1) which recognizes an epitope in the highly conserved COOH-terminal domain of TnI. Using TnI-1, immunoaffinity isolation of the troponin complex and immunofluorescence staining of myofibrils indicate that the COOH-terminal region of TnI forms an exposed structure in troponin and in the muscle thin filament. Conformational analysis of epitopes shows that binding of TnI-1 mAb to the COOH terminus of cardiac TnI induces a secondary conformational change. This supports a critical role of this domain in the functional integrity of troponin. In the presence of TnC and TnT, Ca^{2+} significantly enhances the accessibility of the COOH terminus of TnI. The results provide evidence that the COOH-terminal domain of TnI is an essential structure in allosteric transitions during Ca^{2+} regulation of contraction.

EXPERIMENTAL PROCEDURES

Purification of Troponin Subunits. Chicken fsTnI and fsTnT were purified from adult breast muscle, as described previously (*33*). Briefly, following homogenization and extraction of fresh muscle with 50 mM KCl to remove soluble proteins, troponin was extracted by 0.75 M $LiCl_2$ at pH 4.5. The supernatant was adjusted to pH 7.5 and then lowered to pH 4.5 to precipitate tropomyosin. Troponin in the supernatant was then fractionated by $(NH_4)_2SO_4$ at 35−65% saturation and the precipitate was dialyzed against 0.01 N HCl until conductivity was less than 2 mS/cm. Following dialysis, the pH of the solution was raised to 4 and the supernatant clarified by centrifugation. Solid urea and sodium acetate were added to the supernatant to 6 and 0.01 M, respectively, and the TnI−TnT mixture was fractionated by CM52 cation-exchange chromatography at pH 4. The column was developed by a 0−500 mM NaCl gradient. The TnI and TnT peaks were identified by 180:1 14% SDS−polyacrylamide gel electrophoresis (PAGE). The peak fractions were pooled and dialyzed against 0.1% formic acid to remove urea and salt and lyophilized. To further purify TnI and TnT to homogeneity, the lyophilized powders were dissolved in 6 M urea, 20 mM Tris-HCl, pH 8, for fractionation by DE52 anion-exchange chromatography. Pure TnI and TnT that eluted from the columns were dialyzed against diluted formic acid, lyophilized, and stored at −20 °C until used. All steps were performed at 4 °C and included 0.1 mM EDTA and 0.5 mM phenylmethanesulfonyl fluoride to inhibit proteases and 5 mM β-mercaptoethanol as a reducing agent.

Chicken fsTnC was prepared by expressing the cloned cDNA in *E. coli* (*34*). Bacterial culture was transformed by the pET expression plasmid and inducted as described previously (*30*). The bacterial cells harvested were lysed in 6 M urea, 30 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$ by three passes through a French press. The lysate was clarified by centrifugation and loaded onto a DE52 ion-exchange column equilibrated in the same buffer and eluted by a 0−300 mM KCl gradient. The fractions containing the TnC peak were identified by SDS−PAGE, collected, and dialyzed against double distilled water. The sample was then concentrated by lyophilization for further fractionation on a gel filtration column (Sephadex G75, Pharmacia-Amersham) in 0.5 M KCl, 20 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$. The purified TnC was dialyzed and lyophilized as above.

Development of a Mouse Hybridoma Cell Line Producing Anti-TnI mAb. The purified chicken fsTnI was used to immunize 7-week-old female Balb/c mice by intraperitoneal injection of 100 μg of chicken fsTnI in 100 μL of 0.15 M NaCl mixed with an equal volume of Freund's complete adjuvant and boosted twice at 3-week intervals using the same dosage of immunogen with Freund's incomplete adjuvant. Four days before fusion, a mouse with a positive immune response, as determined by a strong titer of antibodies against chicken fsTnI in serum with enzyme-linked immunosorbant assay (ELISA), was further boosted by intravenous injection of 200 μg of fsTnI in sterilized 0.15 M NaCl. The cell fusion with SP2/0-Ag14 myeloma cell line (American Type Culture Collection) and hybridoma-selective culture were performed as described previously (*35*).

The hybridoma clones were screened for the production of anti-TnI mAb. ELISA was used to test the culture supernatant from wells containing visible hybridoma colonies as described previously (*35*). Briefly, the purified chicken TnI (2 μg/mL) was coated on microtiter plate (100 μL/well) in 50 mM sodium carbonate, pH 9.6, by incubation at 4 °C overnight. After washing and blocking the plate with phosphate-buffered saline containing 0.05% Tween 20, the hybridoma supernatant was added (100 μL/well) and incubated at room temperature for 2 h. The anti-TnI antibodies were then detected using horseradish peroxidase (HRP)-labeled goat anti-mouse immunoglobulin second antibody (Sigma) via H_2O_2−2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) substrate reaction.

The positive hybridomas were subcloned three times by the limiting dilution method (*35*), using young Balb/c mouse

spleen cells as the feeder. The stable hybridoma cell lines established were introduced into the peritoneal cavity of 2,6,-10,14-tetramethylpentadecane (pristane)-primed Balb/c mice to produce mAb-enriched ascites fluid.

To determine the mAb immunoglobulin subclass, the hybridoma culture supernatant was tested using a mouse immunoglobulin isoform typing kit (Pharmingen, San Diego, CA) according to the manufacturer's instructions.

Western Blot Determination of mAb Specificity to TnI Isoforms. Western blotting was performed as described previously (*30*) to examine the specificity of the mAb. Total protein was extracted from cardiac and skeletal muscles of fish, toad, turtle, chicken, mouse, rat, rabbit, cat, dog, bovine, monkey, and human by homogenization in SDS−gel sample buffer containing 1% SDS. The samples were resolved by 12% SDS−PAGE with acrylamide/bisacrylamide ratio of 29:1. The protein bands resolved by SDS−PAGE were electrophoretically transferred to nitrocellulose membrane (0.45-μm pore size) for Western blotting. The interaction of the anti-TnI mAb was detected using alkaline phosphatase labeled anti-mouse IgG second antibody (Sigma), as described previously (*30*).

Analysis of Peptide Fragment Specificity. The peptide binding specificity of the anti-TnI mAb was analyzed by Western blotting of several TnI fragments. Chemically synthesized or bacterially expressed chicken fsTnI fragments of amino acids 1−30, 1−40, 1−98, 96−116, 96−148, and 104−116 were generously provided by Dr. Larry Smillie (University of Alberta) and a human cTnI fragment of amino acids 1−192 was a gift from Dr. Jennifer van Eyk (Queen's University). Western blotting on the 1−192 fragment of cTnI was carried out as described above. A small-pore SDS−PAGE in Tris-Tricine buffer (*36*) was used in the Western blotting analysis of the smaller TnI fragments. The concentration of the resolving gel was 14% and the acrylamide:bisacrylamide ratio was 20:1. SDS was omitted from the resolving gel. The TnI fragments were transferred to nitrocellulose membrane with a pore size of 0.22 μm, and Western blotting was carried out as described above. Chicken breast muscle protein extract and intact human cTnI (Sigma) were blotted as controls. A rabbit anti-TnI polyclonal antibody (*7*) was used as a positive control in the mapping of anti-TnI mAb peptide specificity.

Phylogenetic Analysis. Amino acid sequences of human cTnI (*9*), rat cTnI (*11*), mouse cTnI (*18*), quail cTnI (*37*), frog cTnI (*38*), human ssTnI (*39*), rat ssTnI (*8*), human fsTnI (*40*), rat fsTnI (GenBank/EBI Data Bank accession number: M73701), chicken fsTnI (*41*), and salmon fsTnI (*16*) were analyzed using the MegAlign computer program from DNAStar by the Clustal Method for the sequence distances. In addition to the exon 8-encoded COOH-terminal segment, whole TnI and the adjacent exon 7-encoded segment (amino acids 125−183 in the human cTnI sequence) that contains most of the identified functional sites, including the inhibitory peptide (Figure 3), were analyzed as controls. In contrast to comparing sequences of a selected functional site, the analysis of intact TnI and its exon building blocks more closely reflects the molecular evolution and structure−function relationship of TnI.

Immunofluorescence Microscopy. Chicken breast muscle myofibrils were prepared as described previously (*36*). Indirect immunofluorescence microscopy was carried out to investigate the localization of the TnI epitope recognized by the anti-TnI mAb. Briefly, the myofibrils were sedimented on glass slides and fixed in cold acetone for 10 min. After blocking in 1% bovine serum albumin (BSA), the slides were incubated with 1:200 dilutions of the anti-TnI mAb ascites fluid or normal mouse serum control at room temperature for 2 h. This was followed by washing and incubation with tetramethylrhodamine isothiocyanate-conjugated anti-mouse IgG second antibody (Sigma) at room temperature for 1 h. After three final washes, the slide was mounted with a cover slip and viewed under a Zeiss Axiovert 100H phase contrast-epifluorescence microscope. A Plan-Neo phase fluorescence 100× objective lens (oil, numerical aperture 1.30) was used for photography of both phase-contrast and fluorescence images.

Isolation of Troponin Complex by Immunoaffinity Column Chromatography. The TnI-1 mAb (mouse IgG1κ) was purified from the hybridoma ascites fluid using a Sepharose-Protein G (Pharmacia) column. The IgG peak that eluted from the Protein G column was identified by OD_{280nm}. The anti TnI-1 mAb was then coupled to CNBr-activated Sepharose 4B (Pharmacia), according to the manufacturer's protocol. The immunoaffinity isolation of troponin complex was carried out using a 0.25-mL Sepharose 4B-TnI-1 mAb minicolumn. Rat ventricular muscle was homogenized in 20 volumes (w/v) of 10 mM Tris-HCl, pH 8.0, containing 1 M KCl and 0.2 mM phenylmethanesulfonyl fluoride. After spinning at 12000*g* for 20 min, the supernatant was diluted with 4 volumes of Tris-buffered saline, pH 7.5 (TBS), and centrifuged as above to remove any precipitates. The supernatant equivalent to ~0.1 g of the ventricular muscle was loaded onto the TnI-1 mAb affinity column. After washing with TBS to remove nonspecific proteins, the bound TnI was eluted with 50 mM glycine, pH 2.7, and neutralized immediately with 1 M Tris-HCI, pH 8.0, containing 1 mM EDTA. SDS−PAGE and Western blot were used to examine the recovery of the troponin subunits.

ELISA Analysis of TnI Conformational Changes Induced by the Binding of TnI-1 mAb to the COOH Terminus. Using monoclonal and polyclonal anti-TnI antibodies raised in different species, we were able to detect changes in the conformation of TnI resulting from a structural reconfiguration of the COOH-terminal domain by the binding of mAb TnI-1. In a dual-antibody ELISA (*30*), purified chicken fsTnI or human cTnI (Sigma) was dissolved at 2 μg/mL in a buffer containing 0.1 M KCl, 3 mM $MgCl_2$, 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), pH 7.0 (buffer A), containing 0.1 mM tris(carboxyethyl)phosphine (TCEP; used as a reducing agent) for coating on microtiter plates at 100 μL/well. After incubation at 4 °C overnight, the TnI solution was removed and the wells blocked with buffer A containing 0.05% Tween 20 (buffer T). The coated TnI was first incubated for 5 min with serial dilutions of the mouse mAb TnI-1 in buffer T containing 0.1% BSA, followed by the addition of a rabbit polyclonal antiserum against TnI (RATnI; *7*) at a pretitrated dilution (1:4000, which gives a presaturation binding under the ELISA conditions). After a further incubation at room temperature (22 °C) for 1 h and washing with buffer T, two parallel sets of the assay were incubated with HRP-conjugated anti-rabbit or anti-mouse immunoglobulin second antibody (Sigma), respectively, at room temperature for 45 min to selectively detect the bound RATnI

or TnI-1 first antibodies. After final washes with buffer T, the plates were developed with H_2O_2−ABTS substrates and A_{405nm} was measured at a series of time points using a BioRad Benchmark automated microplate reader. The relationship between the binding of mAb TnI-1 to the COOH terminus and TnI reactivity to the RATnI polyclonal antibody was plotted using data from triplicate assay wells.

ELISA-Mediated Protein Binding and Epitope Affinity Analysis. ELISA-mediated protein binding and mAb affinity analyses were carried out to examine the relationship between the TnI-1 epitope and interaction of TnI with TnT and TnC under Ca^{2+} regulation. Microtiter plates were coated at 100 μL/well with purified chicken fsTnI (5 μg/mL) in buffer A containing 0.1 mM TCEP at 4 °C overnight. After three washes with buffer T as above, the plates were incubated at room temperature (22 °C) for 2 h with the buffer alone or chicken ffsTnC and/or fsTnT (2 μM each) in buffer T containing 0.1% BSA and 0.1 mM TCEP in the presence of 0.1 mM EGTA or 0.1 mM $CaCl_2$. The plates were then washed with buffer T three times and incubated with serial dilutions of anti-TnI mAb TnI-1 ascites fluid, 100 μL/well in buffer T plus 0.1% BSA, at room temperature for 1 h. After another three buffer T washes, HRP-conjugated goat anti-mouse immunoglobulin second antibody (Sigma) in buffer T containing 0.1% BSA was added to the plate (100 μL/well) and incubated at room temperature for 45 min. The plates were finally washed three times with buffer T, and H_2O_2−ABTS substrate was added (100 μL/well) for the color development at room temperature. The A_{405nm} of each assay well was recorded at a series of time points by the microplate reader, as above, and data from a time point within the linear range of the color development were chosen to plot the mAb TnI-1 affinity titration curves. The assays were done in triplicates. The mAb dilution required for 50% maximum binding and the maximum level of binding under the different experimental conditions were compared by Student *t*-test to determine differences in TnI-1 epitope affinity.

RESULTS

High Affinity mAb Against the Conserved COOH Terminus of cTnI, ssTnI, and fsTnI Across the Vertebrate Phyla. Using intact chicken fsTnI as the immunogen, we have established a mouse hybridoma cell line (6C7) secreting anti-TnI mAb (TnI-1). Immunoglobulin isotyping showed that the subclass of this mouse mAb is IgG1κ. The 6C7 hybridoma cells grew well in the peritoneal cavity of Balb/c mice and produced mAb-enriched ascites fluid with an ELISA titer higher than 10^{-6}. Western blots on total muscle protein extracts showed that this mAb is specific to TnI, with no cross-reaction to any other proteins in muscle. More importantly, this mAb recognizes fsTnI, ssTnI, and cTnI from all of the species tested across the vertebrate phyla with a similar strength (Figure 1A,B). On mouse muscle samples, results of Western blot analysis further show that the TnI-1 mAb reacts with the three TnI isoforms with equal strength (Figure 1C). These results indicate that the TnI-1 mAb binds an epitope formed by a conserved structure of TnI.

Western blots of various TnI fragments further located this conserved TnI-1 epitope to the most COOH-terminal region of TnI. The results in Figure 2 demonstrate that TnI-1 does not recognize any TnI fragment lacking the COOH terminus.

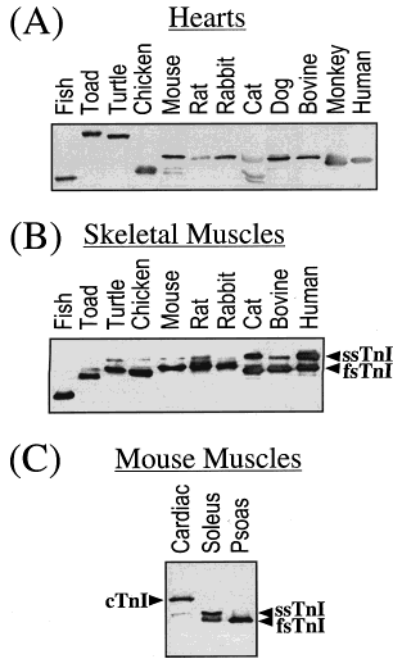

FIGURE 1: Western blots of mAb TnI-1 on total protein extracts of cardiac and skeletal muscle samples from various species. Total muscle homogenates of adult heart (A) and skeletal muscle (B) of fish, toad, turtle, chicken, mouse, rat, rabbit, cat, dog, bovine, monkey, and human were resolved by 12% SDS−PAGE with an acrylamide:bisacrylamide ratio of 29:1 and examined by Western blotting using the TnI-1 mAb. The results show that the TnI-1 mAb binds to all three TnI isoforms in all of the species examined. Some cTnI degradation products are also recognized by mAb TnI-1. Mouse heart, soleus, and psoas muscles were also examined (C) to identify the cTnI, ssTnI, and fsTnI isoforms. Note the molecular weight order (from high to low) of cTnI, ssTnI, and fsTnI isoforms, in which the cTnI-specific NH_2-terminal extension resulted in the significantly larger size.

Removal of the COOH-terminal 19 amino acids from human cTnI completely abolished TnI-1 recognition (Figure 2B). The binding of TnI-1 to some cTnI fragments (Figure 1A) indicates that the lack of binding of this mAb to human cTnI 1−192 fragment is not due to a loss of just any integrity of the TnI molecule. This 19-amino acid COOH-terminal segment in mammalian cTnI corresponds to the last 25 amino acids in avian and amphibian cTnIs, 26 amino acids in mammalian ssTnI, 22 amino acids in mammalian fsTnI, 23 amino acids in avian fsTnI, and 21 amino acids in fish fsTnI (Figure 3). Sequence alignment shows that despite the variation among the last few residues, this region of the TnI polypeptide chain corresponds to a segment highly conserved among the muscle type-specific TnI isoforms and during evolution (Figure 3). Therefore, the TnI-1 mAb epitope is based on this conserved primary structure in the COOH-terminal region of TnI. Genomic structure reveals that this conserved segment is encoded by a single exon (exon 8) in the TnI genes (*20*−*22*). This arrangement suggests that the COOH terminus of TnI may represent a modular structure and a functional unit. The sequence distances among TnI isoforms in Figure 4 demonstrate that the adjacent segment encoded by exon 7, which contains most of the identified functional sites including the inhibitory peptide (Figure. 3), has been conserved during evolution to an extent similar to that of the whole TnI protein. In contrast, the COOH-terminal segment encoded by exon 8 has been more conserved during

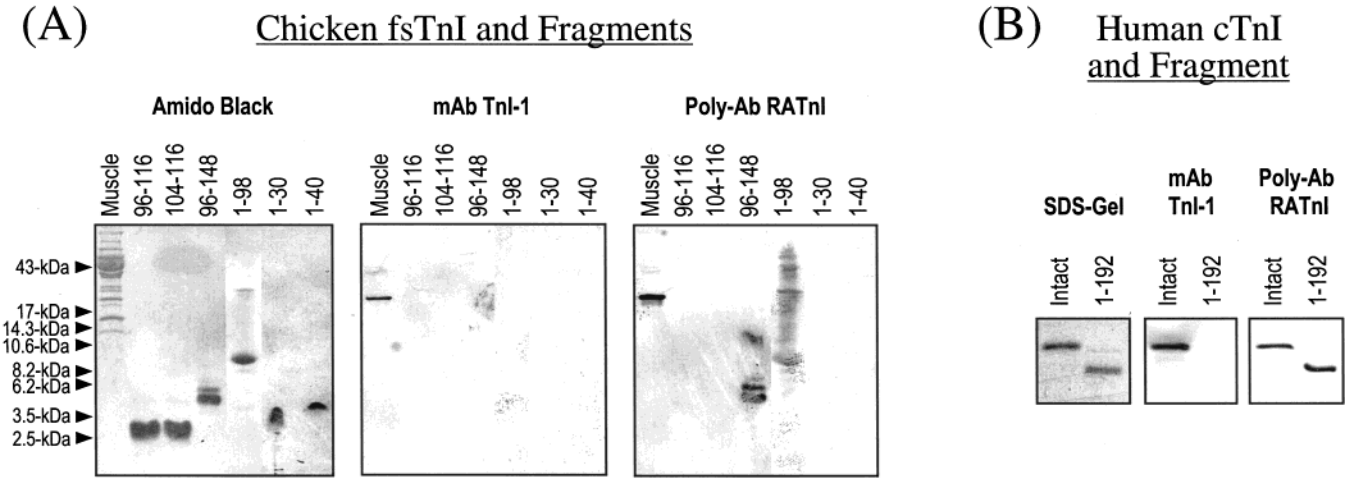

FIGURE 2: Peptide specificity of mAb TnI-1. Chicken fsTnI fragments containing amino acids 1−30, 1−40, 1−98, 96−116, 96−148, 104−116 (A) and human cTnI fragment 1−192 (B) were resolved by SDS−PAGE together with intact TnI controls. After transfer to nitrocellular membrane, Western blotting was carried out using the TnI-1 mAb or RATnI polyclonal antiserum. The results demonstrated that the COOH terminus is essential for the binding of mAb TnI-1. Note that not all of the TnI fragments were recognized by the anti-TnI polyclonal antibody, reflecting the distribution of immunological determinants.

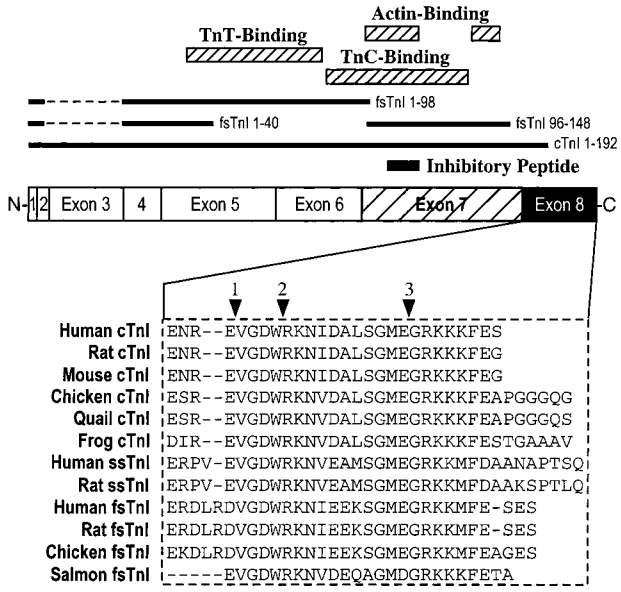

FIGURE 3: Exon organization, structure−function relationship, and conserved sequence in the COOH-terminal region of TnI. A primary structure map and exon organization of human cTnI is shown together with the TnI fragments tested for the epitope specificity of mAb TnI-1 and the binding sites for TnC, TnT, actin and the inhibitory peptide. The cTnI-specific NH_2-terminal segment is encoded by exon 3 of the cTnI gene. Regions encoded by exons 7 and 8 in the COOH-terminal domain are indicated by filled boxes. Amino acid sequences of the exon 8-encoded COOH terminus of TnI isoforms from a wide range of vertebrate species are aligned. The data demonstrate a highly conserved primary structure of the COOH terminus of TnI. The arrowheads 1, 2, and 3 indicate the end of the cardiac TnI fragment 1−188 (*25*), the proteolytic cleavage site for the generation of human cTnI fragment 1−192 in the stunning heart (*27*), and the deletion site found in human familial hypertrophic cardiomyopathy (*28*), respectively.

evolution to a greater extent and shows a much lower divergence and higher sequence similarity among the TnIs from a wide range of vertebrates (Figure 4). For example, human cTnI (HC), salmon fsTnI (SF), and their exon 7-encoded segments have both diverged 75.2% with sequence similarities of 46.5% and 45.8%, respectively. However, their exon 8-encoded segments show only 25.7% divergence with 72.0% sequence similarity. The evolutionary conservation data suggest that the COOH terminus of TnI is an essential structure in the thin filament regulatory system of striated muscle.

COOH Terminus of TnI Forms an Epitope Structure Exposed in the Troponin Complex and Myofibrils. In a physiological buffer, immunoaffinity chromatography using the TnI-1 mAb effectively recovered the whole troponin complex from the total protein extract of rat cardiac muscle (Figure 5). The three subunits of troponin recovered using the TnI-1 mAb immunoaffinity column remained near 1:1:1 stoichiometry as determined by densitometry of the Coomassie Brilliant Blue R250-stained gel. The fact that the TnC band is lighter may be because TnC does not stain as well as TnI and TnT by the dye and its smaller size may cause greater loss from the fixed gel. Effective affinity isolation of TnI by the TnI-1 epitope indicates that the COOH terminus of TnI is exposed in the troponin complex. At a relatively high ionic strength (>0.2 M) which is not optimal for the integrity of the thin filament assembly, the presence of some tropomyosin, actin, and myosin in the troponin fraction isolated by the affinity column suggests that anchoring of troponin on the thin filament was not significantly affected by the binding of a mAb to the COOH terminus of TnI. Immunofluorescence microscopy on intact chicken breast muscle myofibrils showed that the TnI-1 mAb clearly stained the I-band of the sarcomere (Figure 6). This result indicates that the epitope formed by the COOH-terminal region of TnI is exposed in the myofilament assembly. Altogether, the results demonstrate that the integration of TnI into the myofibril structure does not mask the COOH terminus of TnI and the binding of TnI-1 mAb to the COOH terminus of TnI does not abolish the structural and functional integrity of the troponin complex. Therefore, we can conclude that the COOH-terminal region of TnI forms an exposed structure in the troponin complex and in the muscle thin filament.

Effect of the Binding of Anti-COOH Terminus mAb TnI-1 on the Conformation of TnI. Epitope analysis by dual-antibody ELISA was carried out to examine the conformational changes in TnI induced by the binding of TnI-1 mAb

Whole TnI

Percent Similarity

Percent Divergence

	HC	RC	MC	QC	FC	HS	RS	HF	RF	CF	SF	
HC		91.4	91.4	60.1	67.6	58.8	59.9	52.2	53.3	53.6	46.5	HC
RC	7.5		99.1	60.6	69.7	58.3	59.4	50.5	51.6	53.0	47.1	RC
MC	7.5	1.0		61.1	69.7	58.3	58.8	50.5	51.1	53.0	46.5	MC
QC	45.4	46.9	46.0		60.1	50.8	50.8	45.1	44.5	44.8	41.3	QC
FC	34.5	33.5	33.5	47.1		62.0	60.4	56.0	56.0	55.7	45.3	FC
HS	49.3	50.4	50.4	65.7	46.2		96.3	54.9	54.4	57.4	45.9	HS
RS	47.3	48.3	49.3	65.7	49.2	3.8		54.4	54.9	57.4	45.3	RS
HF	62.3	64.7	64.7	78.4	53.2	57.5	57.5		97.8	81.3	46.5	HF
RF	59.8	62.3	63.5	79.9	53.2	58.7	56.3	2.2		80.8	45.9	RF
CF	58.7	58.7	58.7	77.6	52.8	52.6	52.6	20.1	20.8		50.6	CF
SF	75.2	73.7	75.2	83.9	75.3	74.4	75.9	78.3	79.9	67.4		SF
	HC	RC	MC	QC	FC	HS	RS	HF	RF	CF	SF	

Exon 7

Percent Similarity

Percent Divergence

	HC	RC	MC	QC	FC	HS	RS	HF	RF	CF	SF	
HC		94.9	94.9	50.9	67.8	72.9	72.9	72.9	72.9	74.6	45.8	HC
RC	5.3		100.0	50.9	67.8	71.2	71.2	69.5	69.5	71.2	45.8	RC
MC	5.3	0.0		50.9	67.8	71.2	71.2	69.5	69.5	71.2	45.8	MC
QC	73.1	73.1	73.1		52.6	56.1	56.1	57.9	57.9	57.9	38.6	QC
FC	33.7	33.7	33.7	53.7		67.8	67.8	74.6	74.6	74.6	45.8	FC
HS	33.7	36.3	36.3	57.3	33.7		98.3	81.4	81.4	84.7	52.5	HS
RS	33.7	36.3	36.3	57.3	33.7	1.7		81.4	81.4	84.7	52.5	RS
HF	33.7	39.1	39.1	61.0	23.8	21.5	21.5		100.0	93.2	47.5	HF
RF	33.7	39.1	39.1	61.0	23.8	21.5	21.5	0.0		93.2	47.5	RF
CF	31.1	36.3	36.3	57.3	23.8	17.1	17.1	7.1	7.1		49.2	CF
SF	75.2	75.2	75.2	96.0	77.6	58.8	58.8	70.8	70.8	66.6		SF
	HC	RC	MC	QC	FC	HS	RS	HF	RF	CF	SF	

Exon 8

Percent Similarity

Percent Divergence

	HC	RC	MC	QC	FC	HS	RS	HF	RF	CF	SF	
HC		96.3	96.3	88.9	88.9	66.7	66.7	74.1	74.1	70.4	72.0	HC
RC	3.8		100.0	88.9	85.2	66.7	66.7	70.4	70.4	70.4	72.0	RC
MC	3.8	0.0		88.9	85.2	66.7	66.7	70.4	70.4	70.4	72.0	MC
QC	7.8	12.1	12.1		75.8	63.6	60.6	58.1	58.1	59.4	76.0	QC
FC	16.6	16.6	16.6	29.3		60.6	57.6	64.5	64.5	59.4	76.0	FC
HS	27.6	33.4	33.4	40.4	51.6		91.4	58.1	58.1	59.4	68.0	HS
RS	33.4	33.4	33.4	45.8	57.8	9.1		58.1	58.1	62.5	68.0	RS
HF	26.4	31.8	31.8	40.0	46.0	38.3	43.9		100.0	87.1	64.0	HF
RF	26.4	31.8	31.8	40.0	46.0	38.3	43.9	0.0		87.1	64.0	RF
CF	26.4	31.8	31.8	33.0	56.6	42.1	47.8	6.8	6.8		64.0	CF
SF	25.7	25.7	25.7	29.0	35.1	41.6	41.6	36.9	36.9	36.9		SF
	HC	RC	MC	QC	FC	HS	RS	HF	RF	CF	SF	

FIGURE 4: Sequence conservation of TnI. Amino acid sequences of human cTnI (HC), rat cTnI (RC), mouse cTnI (MC), quail cTnI (QC), frog cTnI (FC), human ssTnI (HS), rat ssTnI (RS), human fsTnI (HF), rat fsTnI (RF), chicken fsTnI (CF), and salmon fsTnI (SF) were analyzed using the DNAStar computer program. The sequences of whole TnI were analyzed together with the segments encoded by exons 7 and 8. Although the fsTnI and ssTnI genes lack a counterpart to the cTnI gene exon 3 (Figure 3), they both contain an additional 5′-noncoding exon 1 (*21*, *22*). Therefore, the three TnI genes all contain 8 exons, and their exons 7 and 8 encode homologous segments. The results demonstrate that the exon 7-encoded segment containing most of the identified functional sites (Figure 3) is conserved with an extent similar to that of whole TnI. In contrast, the exon 8-encoded COOH-terminal segment is more conserved with a much lower divergence and higher sequence similarity.

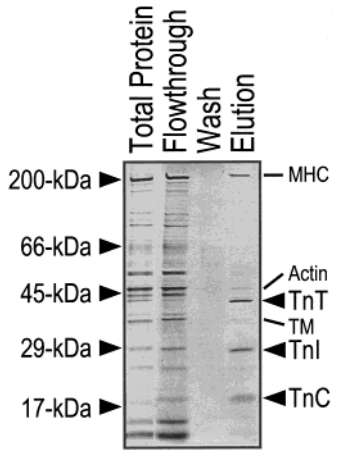

FIGURE 5: Immunoaffinity isolation of troponin complex from rat cardiac muscle. Total protein extracted from rat ventricular muscle was loaded on an anti-TnI mAb affinity column. SDS−PAGE showed that after wash, TnI plus TnC, TnT, and trace amounts of other myofibril proteins bound to the column in the physiological buffer and eluted together by acidic pH. MHC, myosin heavy chain; TM, tropomyosin.

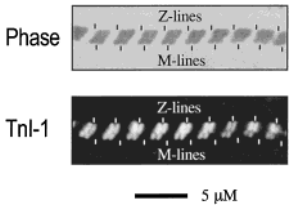

FIGURE 6: Immunofluorescence localization of the TnI-1 epitope in the sarcomere. Chicken breast muscle myofibrils were examined by immunofluorescence microscopy for the reactivity and location of the anti-TnI mAb TnI-1 via rhodamine-conjugated anti-mouse IgG second antibody. The phase contrast (upper panel) and fluorescence (lower panel) micrographs of a myofibril are aligned with the Z-lines and M-lines of the sarcomeres indicated by the vertical lines. The results show that mAb TnI-1 had a clear I-band staining in the sarcomere.

to the COOH terminus. The ELISA binding curves demonstrate that as the binding of TnI-1 to the COOH terminus of TnI increased, the level of RATnI binding (to multiple epitopes on the TnI molecule) became weaker. The results show that saturated binding of TnI-1 mAb to chicken fsTnI resulted in a moderate decrease (15% of the maximum) in the binding of RATnI polyclonal antibody (Figure 7A). In parallel experiments, saturated binding of the anti-TnI COOH terminus mAb to human cTnI resulted in a very significant decrease (97% of the maximum) in the binding of RATnI polyclonal antibody (Figure 7B). In contrast to the strong response of cTnI, the similar and slightly stronger binding of TnI-1 mAb to chicken fsTnI induced only a moderate change in the binding of RATnI under identical ELISA conditions. This result serves as a control to exclude any significant contribution to the cTnI results by a competition for the COOH-terminal epitope between TnI-1 and RATnI or by steric blocking of other epitopes due to the binding of TnI-1 mAb. Therefore, the changes in the binding of RATnI reflect the decrease in the affinity of this polyclonal antibody to multiple epitopes on TnI molecule. The results indicate an extensive secondary conformational change in TnI induced by the binding of TnI-1 mAb to the COOH terminus. The conformational changes induced by the binding of a protein (IgG) to the COOH terminus of TnI, which is more readily detectable in cTnI (Figure 7B), suggest that structural modifications in the COOH-terminal domain, such as the truncations by proteolysis or genetic mutation resulting in pathological changes (*27*, *28*), may affect the molecular conformation and function of TnI.

Ca^{2+}-Regulated Modulation of the COOH-Terminal Conformation of TnI in the Presence of TnC and TnT. By means of changes in the affinity for TnI-1 mAb, the ELISA protein binding and the conformation analyses can be used to evaluate the conformational changes in the TnI COOH terminus induced by the interactions with TnT, TnC, and Ca^{2+}. The results show that the binding of TnT to TnI does not affect the affinity of mAb TnI-1 (Figure 8A). The binding of TnC to TnI results in a slight decrease in the affinity of

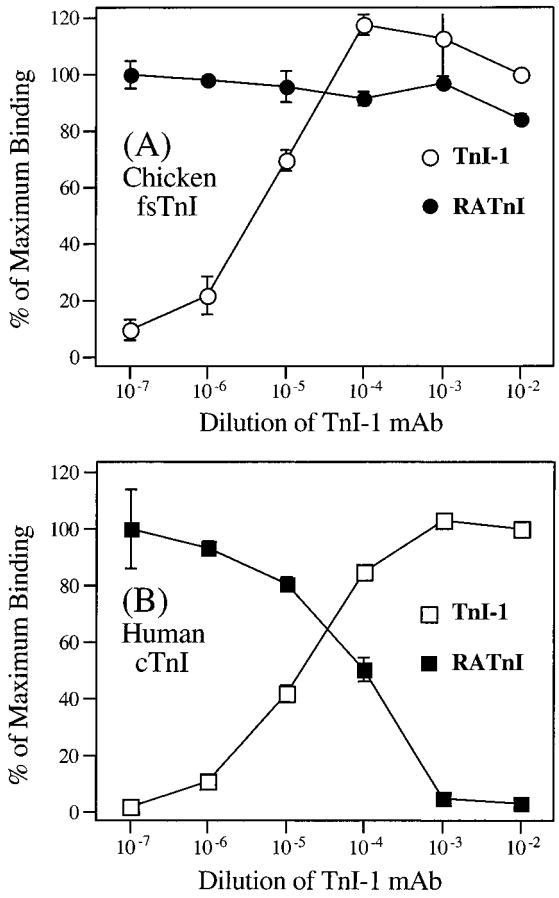

FIGURE 7: Effect of binding of mAb TnI-1 to the COOH terminus on the conformation of TnI. Dual-antibody ELISA epitope analyses were carried out to investigate the effect of mAb TnI-1 binding to the COOH terminus on the conformation of TnI. Parallel ELISA via HRP−anti-mouse and HRP−anti-rabbit immunoglobulin second antibodies showed a counter-relationship between the bindings of the mouse anti-TnI COOH terminus mAb TnI-1 and the rabbit polyclonal antiserum RATnI against multiple epitopes to the immobilized chicken fsTnI (A) and human cTnI (B). The results indicate that this protein interaction-induced reconfiguration of COOH-terminal structure resulted in extensive secondary conformational changes in TnI, more readily detectable in the cTnI.

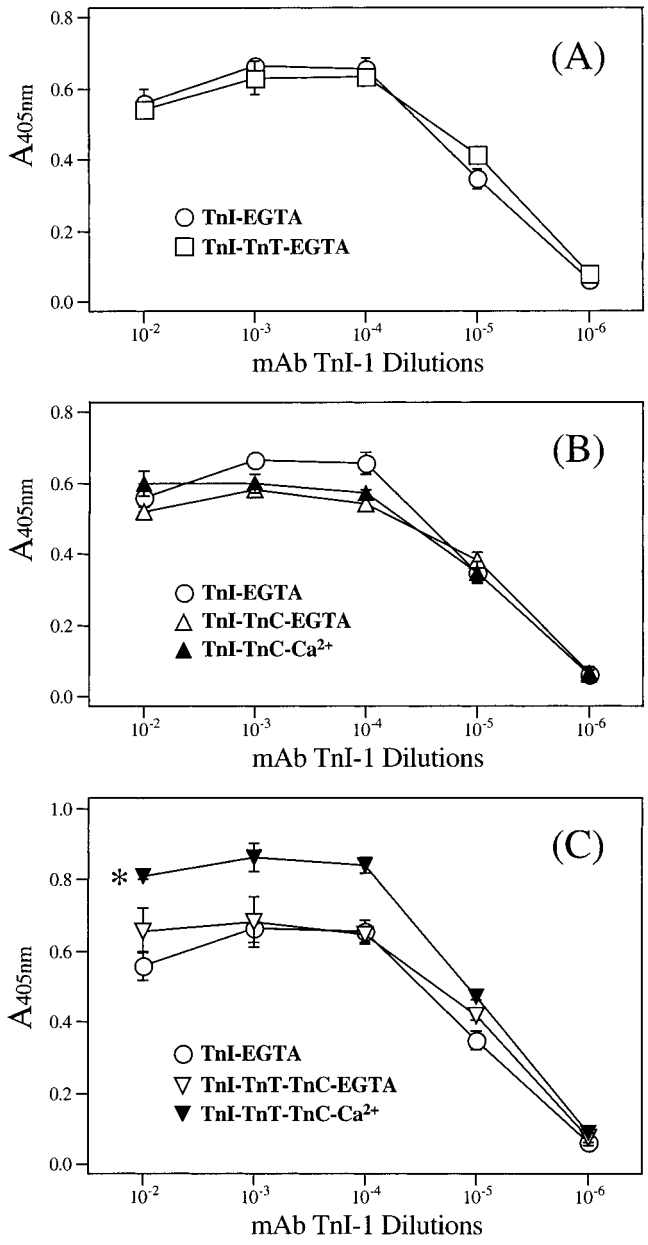

FIGURE 8: Effect of TnT binding, TnC binding, and Ca^{2+} on the accessibility of mAb TnI-1 epitope. In the ELISA protein binding conformation analyses, chicken fsTnI was coated on microtiter plates. After washing and blocking, the plates were incubated with chicken fsTnT and/or chicken fsTnC in the presence of 0.1 mM EGTA or $CaCl_2$. After washing away the unbound TnT and/or TnC, the plates were further incubated with serial dilutions of mAb TnI-1 followed by HRP-labeled second antibody reaction. After final washes, ABTS−H_2O_2 substrate reaction was carried out and A_{405nm} was recorded. The binding affinity of TnI-1 mAb to the COOH terminus of TnI under the different conditions is shown by the titration curves. The results demonstrate: (A) the binding of TnT did not affect the affinity of mAb TnI-1; (B) the binding of TnC to TnI resulted in a slight decrease in the affinity of mAb TnI-1 and Ca^{2+} had no significant effect; and (C) the addition of TnT minimized the effect of TnC and Ca^{2+} induced a significant increase in the accessibility of the TnI-1 mAb epitope in the presence of both TnC and TnT (*$P < 0.05$). The data suggest that the COOH terminus of TnI undergoes allosteric changes during the Ca^{2+} activation of contraction.

TnI-1 mAb, and Ca^{2+} has no significant effect (Figure 8B). The distinct effects of TnT and TnC binding on the TnI COOH-terminal epitope may reflect the relative distance of the TnT and TnC binding sites to the COOH terminus in the TnI polypeptide chain (Figure 3). When TnC and TnT are both present, the effect of TnC binding on TnI-1 affinity was minimized in the absence of Ca^{2+} (Figure 8C). Interestingly, Ca^{2+} induces a significant increase in the binding affinity of TnI-1 mAb to the COOH terminus of TnI when TnC and TnT are present (Figure 8C). This conformational response indicates a Ca^{2+}-induced allosteric change in the COOH-terminal domain of TnI in the troponin ternary complex.

These conformational changes indicate that the COOH terminus of TnI forms a defined tertiary structure in the troponin complex. The requirement for both TnC and TnT for the Ca^{2+}-induced allosteric change in the COOH terminus of TnI reflects the fact that the three subunits of troponin work as an interrelated complex in the regulation of muscle contraction. The Ca^{2+}-induced increase in accessibility of the TnI-1 epitope in the presence of TnC and TnT further suggests that the COOH terminus of TnI is more exposed in the troponin complex and/or its structure is more compliant when Ca^{2+} is bound to TnC. The evidence that the exposed COOH terminus of TnI undergoes an allosteric change during

the Ca^{2+}-dependent contractile cycle suggests that it is not only an essential structure but also an active domain in the thin filament-based Ca^{2+} regulatory system.

DISCUSSION

A Useful mAb Recognizing All Three TnI Isoforms Across the Vertebrate Phyla. The specific mAb TnI-1 developed and characterized in this study provides a very useful tool for the specific detection of TnI in all vertebrate muscles (Figure 1). Together with the SDS−PAGE system which clearly distinguishes the cTnI, ssTnI, and fsTnI by their size differences, Western blotting using mAb TnI-1 can readily identify the three TnI isoforms expressed in different muscle fiber types and at different developmental stages. The affinity of the TnI-1 mAb is sufficiently high to function very well in Western blotting, immunofluorescence assay, and ELISA experiments (Figures 1, 4, and 6, respectively). With a uniform affinity, it produced a sharp peak of TnI in immunoaffinity chromatography, providing an effective approach for rapid one-step isolation of TnI and troponin complex from total muscle homogenates (Figure 5).

A previous study on a large number of mAbs generated against human cTnI showed that most of them were against the cTnI-specific NH_2-terminal region. Very few of the mAbs recognized the COOH-terminal region of free cTnI, and none was reactive againt cTnI in the troponin complex (*42*). This was in agreement with the weak immunogenecity of the COOH-terminal region of TnI, a feature shared by many evolutionarily conserved epitopes. Therefore, short peptides are commonly used as immunogens to generate antibodies against a specific domain of a protein (*29, 43*). However, because immunological determinants are isolated from their original environment in the folded protein, the anti-peptide antibodies generated often have low affinity and inadequate specificity against the native protein. In the present study, the use of intact TnI as the immunogen for the generation of TnI-1 mAb may have been a key factor for the development of an antibody with high specificity and affinity for an epitope in intact native TnI (Figures 1, 5, and 6).

Because of both its homogeneity as a mAb and its unlimited supply, TnI-1 provides a standard reagent in the detection and identification of TnI isoforms. With a known antigenic epitope localized at the very COOH terminus of TnI (within the last 30 amino acids), this mAb also provides a tool to monitor the molecular conformation of TnI. As shown in Figure 8 and our previous studies on TnT (*30, 31*) and calponin (*44*), an analysis of changes in the binding affinity of a mAb to a specific epitope structure is a convenient way to quantitatively detect conformational modulations in a protein. We have demonstrated that the protein conformational changes detected by antibody epitope analysis correspond with the changes in molecular conformation and flexibility detected by fluorescence spectral analysis (*31*). This application is particularly useful in the studies of structure−function relationship of muscle thin filament regulatory proteins, since they naturally undergo allosteric transitions during the contraction and relaxation cycles. The conformational information obtained by the mAb ELISA epitope analysis will then help the determination of high-resolution structure using crystallography and NMR spectrometry.

COOH Terminus Is Critical to the Molecular Conformation of TnI. Effective TnI-1 mAb immunoaffinity isolation of whole troponin (Figure 5) indicates that the TnI-1 epitope is readily accessible in the troponin complex under the buffer conditions (~0.2 M salt at pH 7.5 in the absence of Ca^{2+}). Together with the strong immunofluorescence staining of TnI-1 in the I-band of the sarcomere (Figure 6), the data suggest that the COOH terminus of TnI is exposed in the troponin complex and in the myofibril assembly. This exposed position is in agreement with the previous findings that the COOH-terminal ~30 residues of TnI do not directly come into contact with other thin filament regulatory proteins (reviewed in ref *4*). On the other hand, sequence distance among cTnI, ssTnI, and fsTnI from fish, amphibians, birds, and mammals reveals that the sequence of the TnI COOH-terminal segment encoded by exon 8 is more conserved than the sequences of whole TnI and of the functionally important segment encoded by the 5′ adjacent exon 7 (Figure 4). The highly conserved structure indicates a high selection pressure during evolution and thus a low tolerance to structural variation of the COOH-terminal region of TnI. Indeed, when the binding of anti-TnI COOH terminus mAb TnI-1 was used to introduce a structural reconfiguration of the COOH terminus of TnI, the overall conformation of TnI was significantly altered (Figure 7). The results support the hypothesis that although it is an accessible epitopic structure in the thin filament regulatory system, the integrity of the COOH terminus of TnI is critical to the molecular conformation and function of TnI. Figure 7 shows that the COOH terminus-induced conformational effect is much more readily detected in cTnI than in fsTnI. One possible reason is that the lower affinity of the RATnI antiserum for cTnI than for its immunogen, chicken fsTnI, may have made its binding to cTnI more sensitive to conformational changes under the stringent ELISA conditions. However, it may also indicate that cTnI has a more allosteric structure than fsTnI and more susceptible to structural alterations. Therefore, a damage of the COOH-terminal integrity of cTnI has significant physiological and pathological effects on the function of cardiac muscle (*25*), such as that observed during myocardial stunning (*27*) and familial hypertrophic cardiomyopathy (*28*).

Participation of the COOH Terminus of TnI in the Thin Filament-Based Ca^{2+} Regulation of Contraction. The evolutionary conservation of the COOH-terminal structure of TnI indicates a stringent selection pressure on its physiological function. Deletion of as many as 60 of the COOH-terminal amino acids from cTnI did not affect binding to TnC, but removal of the COOH-terminal 23 amino acids weakened the inhibitory effect of TnI on actomyosin ATPase activity (*25*). Truncation of the COOH-terminal 19 amino acids from cTnI produces myocardial stunning in transgenic mice (*27*). A genetic mutation-caused deletion of as few as 8 of the COOH-terminal amino acids of cTnI is found in human familial hypertrophic cardiomyopathy (*26*). All these data support the functional importance of the COOH terminus of TnI. Our results show that when TnI is complexed with TnT and TnC, the binding affinity of TnI-1 mAb for TnI significantly increases in the presence of Ca^{2+} (Fgiure 8C). The increased accessibility of the TnI-1 epitope may reflect a further exposed position and/or an increased structural compliance/flexibility of the TnI COOH terminus. This result suggests that the COOH terminus of TnI undergoes a Ca^{2+}-

regulated allosteric transition during the activation of muscle contraction. A Ca^{2+}-induced movement of the TnI COOH terminus may contribute to the removal of TnI's inhibitory effect during muscle activation. This hypothesis is consistent with a previous observation that the COOH-terminal region of TnI switches positions in the thin filament under Ca^{2+} regulation (*45*). On the other hand, an increased flexibility of the TnI COOH-terminal structure in the presence of Ca^{2+} may facilitate the functional switch of the thin filament between the blocked and closed/open states (*46*). The conformational characterization of the COOH terminus of intact TnI using the TnI-1 mAb provides evidence for its role in the Ca^{2+} regulation of muscle contraction, contributing to our understanding of the structure−function relationship of TnI under physiological and pathological conditions.

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