Identification of a Region of Troponin I Important in Signaling Cross-bridge-dependent Activation of Cardiac Myofilaments*

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Force generating strong cross-bridges are required to fully activate cardiac thin filaments, but the molecular signaling mechanism remains unclear. Evidence demonstrating differential extents of cross-bridge-dependent activation of force, especially at acidic pH, in myofilaments in which slow skeletal troponin I (ssTnI) replaced cardiac TnI (cTnI) indicates the significance of a His in ssTnI that is an homologous Ala in cTnI. We compared cross-bridge-dependent activation in myofilaments regulated by cTnI, ssTnI, cTnI(A66H), or ssTnI(H34A). A drop from pH 7.0 to 6.5 induced enhanced cross-bridge-dependent activation in cTnI myofilaments, but depressed activation in cTnI(A66H) myofilaments. This same drop in pH depressed cross-bridge-dependent activation in both ssTnI myofilaments and ssTnI(H34A) myofilaments. Compared with controls, cTnI(A66H) myofilaments were desensitized to Ca2+, whereas there was no difference in the Ca2+-force relationship between ssTnI and ssTnI(H34A) myofilaments. The mutations in cTnI and ssTnI did not affect Ca2+ dissociation rates from cTnC at pH 7.0 or 6.5. However, at pH 6.5, cTnI(A66H) had lower affinity for cTnT than cTnI. We also probed cross-bridge-dependent activation in myofilaments regulated by cTnI(Q56A). Myofilaments containing cTnI(Q56A) demonstrated cross-bridge-dependent activation that was similar to controls containing cTnI at pH 7.0 and an enhanced cross-bridge-dependent activation at pH 6.5. We conclude that a localized N-terminal region of TnI comprised of amino acids 33–80, which interacts with C-terminal regions of cTnC and cTnT, is of particular significance in transducing signaling of thin filament activation by strong cross-bridges.

In experiments described here we investigated the significance of a near N-terminal region of troponin I (TnI)4 in molecular signaling by which strongly bound cross-bridges activate cardiac thin filaments. Cooperative activation of the thin filament by strongly bound cross-bridges is generally accepted to play a prominent role in cardiac muscle contraction (1–4). The idea that bound cross-bridges might activate the thin filaments independently of Ca2+ came from seminal studies of Bremel and Weber (5), who reported that nucleotide-free (rigor) cross-bridges are able to activate actomyosin ATPase activity in reconstituted systems in the absence of Ca2+. Further biochemical evidence suggested that although Ca2+ triggers activation, Ca2+ alone cannot fully activate the thin filament, and that strong cross-bridge binding is required for full activation (6). Structural data generally support the biochemical data demonstrating that whereas Ca2+ is able to move Tm partially on the thin filament, complete movement of Tm away from myosin binding sites on actin requires strongly bound cross-bridges (7). The results of these studies indicate that binding of a cross-bridge to a structural unit (7 actins; 1 Tn; 1 Tm) can affect near neighbor structural units by cooperative spread of activation.

There is evidence that cardiac muscle may depend more on this mode of activation than skeletal muscle (8, 9). For example, strongly bound cross-bridges enhance the affinity of cTnC for Ca2+ (10), an effect unique to cardiac myofilaments (11). Yet, there is little information related to the molecular basis for these differences or the protein–protein interactions critical to the feedback effects of strong cross-bridges on activation. An important clue came from studies of Morimoto et al. (12), who compared the effects of pH on the ability of strongly bound rigor cross-bridges to activate tension at pCa 9.0 in skinned cardiac fiber bundles reconstituted with Tn complexes containing either cTn or ssTnI. The acidic pH induced reduction in cross-bridge-dependent activation of skinned fiber bundles was significantly blunted when cTnI was replaced with ssTnI. Moreover, skinned fiber bundles in which cTnC had been specifically extracted demonstrated the same cross-bridge-dependent activation at neutral and acidic pH. Inasmuch as cTnI-(33–80) forms a binding interface with the C-lobe of cTnC (13) with the tightest interaction between cTnI and cTnC being a peptide comprised of amino acids 39–58 (14), the

cTnC, cardiac troponin C; MOPS, 3-(N-morpholino)propanesulfonic acid; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; GST, glutathione S-transferase; NEM, N-ethylmaleimide; S1, myosin head; PIPES, 1,4-piperazinediethanesulfonic acid; HR, high relax buffer.
Cloning, Expression, and Purification of Cardiac Troponin and Slow Skeletal Troponin I and Mutant Proteins—Human cardiac TnC, adult mouse cardiac TnT, and wild-type mouse cardiac TnI were expressed and purified as previously described (17). Adult rat ssTnI cDNA (which differs from mouse ssTnI by only 1 residue found at the very C-terminal end) was previously cloned into a PET3a vector (generously provided by Dr. Anne F. Martin) and selectively mutated using the QuikChange site-directed mutagenesis kit (Stratagene). All plasmid constructs were verified by DNA sequencing. For protein expression, each GST-TnI plasmid was transformed into BL21(DE3) cells and grown in 100 ml of Luria broth containing 50 μg/ml carbenicillin at 37 °C, which was induced after 3 h with 0.1 mM isopropyl β-D-thiogalactopyranoside. The cell culture was centrifuged and the supernatant fraction discarded. The remaining pellet was resuspended in Buffer A containing 0.1 M KCl, 3 mM MgCl₂, 10 mM PIPES, pH 7.0, with 1 mM DTT and 0.1 mM benzamidine added. The cells were lysed by sonication on ice and the cell lysate was centrifuged. The supernatant fraction containing the protein was saved and stored at 4 °C up to 1 week for further use.

Whole Troponin Complex Exchange into Detergent-skinned Myofilaments—Male CD-1 mice (age 3–4 months) were anesthetized with sodium pentobarbitol (5 mg/kg body weight) by intraperitoneal injection. Hearts were quickly excised and left ventricular papillary muscles were dissected into fiber bundles (4–5 mm long and 150–250 μm in diameter) in high relax buffer (HR) containing 20 mM MOPS, pH 7.0, 10 mM EGTA, 1 mM free Mg²⁺, 5 mM MgATP²⁻, 12 mM creatine phosphate, 10 IU/ml creatine phosphokinase (Sigma) with free Ca²⁺ concentration at 10⁻⁵ m and ionic strength adjusted to 150 mM with KCl with concentrations calculated by a computer program (20). To extract membranes, the skinned fiber bundles were placed for 4 h at 4 °C in HR with 1% Triton X-100 added and then transferred to exchange buffer containing 20 mM MOPS, pH 6.5, 200 mM KCl, 5 mM EGTA, 5 mM MgCl₂, 1 mM DTT, with −18 μM recombinant Tn complex and incubated overnight at 4 °C. All solutions contained a mixture of protease inhibitors as previously described (21).

Measurements of Cross-bridge and Ca²⁺-dependent Activation of Force at pH 7.0 and pH 6.5—The skinned fiber bundles were mounted between a micromanipulator and force transducer with cellulose-acetate glue. Resting sarcomere length was set at 2.0 μm as determined using laser diffraction patterns, cross-sectional area measurements were made, and isometric tension was recorded on a chart recorder (22). All experiments were carried out at 22 °C. The myofilaments were initially placed in HR and then subjected to sequential decreases in [MgATP] (10⁻³–10⁻⁸ M) or increasing Ca²⁺ concentration (10⁻⁸–10⁻⁴.5 M) and force was recorded. HR buffer (10⁻⁹ M Ca²⁺) contained 20 mM MOPS, pH 7.0 or 6.5, 10 mM EGTA, 1 mM free Mg²⁺, 5 mM MgATP²⁻, 12 mM creatine phosphate, 10 IU/ml creatine phosphokinase (Sigma), and ionic strength was adjusted to 150 mM with KCl. For force-Ca²⁺ measurements, Ca²⁺ was varied by mixing HR with HR to which CaCl₂ had been added to achieve 10⁻⁴.5 M free Ca²⁺. For MgATP-force
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measurements, Mg and ATP were varied in HR buffer to achieve a range of concentrations at ionic strength 0.15 M and 10^{-9} M Ca^{2+}. All solutions included a mixture of protease inhibitors: 1 μg/ml pepstatin, 5 μg/ml leupeptin, and 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride and brought to a final pH of 7.0 or 6.5 with KOH. The multiequilibria in the solutions used in these experiments were computed using binding constants given by Godt and Lindley (20) as previously described. Extra fiber bundles were removed from exchange buffer, rinsed in HR solution, then placed in 10 μl of 1% SDS and stored at −20 °C for later analysis by SDS-PAGE and Western blotting.

Ca^{2+} Activation with the Addition of NEM-S1—The effects of NEM-S1 on Ca^{2+}-activated force have been previously described by Swartz and Moss (23). We isolated myosin S1 from rabbit fast skeletal muscle and modified it with NEM as previously described (23). Control or whole Tn-exchanged fiber bundles were mounted between a force transducer and micro-manipulator as described above then initially bathed in HR, switched to a maximally activating Ca^{2+} solution (10^{-4.5} M), and then fully relaxed in HR. The first series of Ca^{2+} activations (10^{-8}–10^{-4.5} M) was done without NEM-S1; then the fiber bundles were immersed in a HR bath containing 6 μM NEM-S1 for 15 min. This incubation was followed with a second full series of Ca^{2+} activations.

SDS-PAGE and Western Blot Analysis—Fiber bundles were prepared for SDS-PAGE and Western blot analysis to determine the extent of exchange of endogenous for recombinant Tn complex as previously described (24). Exchange of Tn was determined by the presence of recombinant cTnT, which contained a 9-amino acid c-Myc tag at the N terminus of the recombinant cTnT that retarded its mobility in 15% SDS-PAGE (acylamide: bisacrylamide ratio 200:1) and separated the exogenous cTnT from the endogenous cTnT. Proteins were transferred to a nitrocellulose membrane (0.45 μm) and probed with a mouse monoclonal primary antibody (Sigma CT-3) against cTnT followed by a secondary goat anti-mouse antibody (Sigma, A2304). The signal was detected using an ECL-plus Western blotting system (Amersham Biosciences). Fig. 1 shows examples of the exchange of cTnI with exogenous Tn complexes. Standards were a recombinant Tn complex containing c-Myc-tagged cTnT (run in lane 1) and a mouse heart ventricular homogenate (run in lane 2). Lanes 3–6 contained skinned fiber bundles in which the native Tn complex was exchanged with Tn reconstituted with c-Myc-tagged cTnT and with cTnI, cTnI(A66H), ssTnI, or ssTnI(H34A). Exchange of the native complexes containing cTnI(A66H), ssTnI, or ssTnI(H34A) demonstrated an 80–90% replacement. On the other hand, exchange of native cTnI with exogenous Tn containing cTnI indicated about a 50% replacement. We have done control experiments demonstrating no difference in the Ca^{2+}-force relationships of fiber bundles treated with exchange buffer alone or with exchange buffer containing cTnI-cTnTmyc-cTnC. As previously reported (17), we found a similar difference in the ability of complexes containing native cTnI to replace endogenous Tn complexes, but no difference in the Ca^{2+}-tension relationship, as we would expect in view of the replacement of native complexes with endogenous complexes.

ELISA and Binding Assay—To determine the affinity of cardiac TnT for the various TnIs, the ELISA-based solid-phase protein binding assay was carried out similar to that previously described (25, 26). The concentration of Escherichia coli cell lysates containing GST-tagged fusion proteins of cardiac, slow skeletal, or mutant TnI required to completely saturate the well of Reacti-bind glutathione-coated plates (Pierce) was first determined by standard ELISA (27) carried out with the anti-Tn specific monoclonal antibody C5 (Research Diagnostics Inc.). The affinity of TnT for TnI was then determined by incubating 100 μl of GST-TnI E. coli lysate at an excess concentration to ensure complete saturation of glutathione on buffer A (0.1 M KCl, 3 mM MgCl_2, 10 mM Pipes, pH 7.0) washed Reacti-bind glutathione-coated plates (Pierce) at room temperature for 2 h. Following a wash with buffer A to remove unbound Tn and contaminants, the unoccupied plastic surface was blocked by incubation with 150 μl of 1% bovine serum albumin in buffer A for 1 h at room temperature. The plate was then washed three times in buffer A and TnT binding to the immobilized GST-TnI was carried out by incubation with serial dilutions of cTnI in buffer A from 13.5 nM. Following TnT incubation for 2 h at room temperature, the plate was washed three times with buffer A and the TnT that remained bound to TnI quantified by the anti-TnT monoclonal antibody CT-3 (Developmental Studies Hybridoma Bank, Iowa City, IA) as previously described (24, 25). All experiments were carried out in triplicate and the EC_{50} for cTnI binding to TnI determined from titration curves relating [cTnI] to percent bound cTnT.

Determination of Ca^{2+} Dissociation Rates—Ca^{2+} dissociation (k_{off}) rates from recombinant Tn complexes containing cTnI, ssTnI, cTnI(A66H), or ssTnI(H34A) at either pH 7.0 or 6.5 were measured in a stopped-flow apparatus using the fluorescent Ca^{2+} chelator Quin-2 as previously described (28). Each recombinant complex (~6 μM) was prepared in the following buffer: 10 mM MOPS, pH 7.0 or 6.5, 150 mM KCl, 1 mM DTT, 3 mM Mg^{2+}. This solution was rapidly mixed with an equal volume of Quin-2 (150 μM) in the same buffer at 15 °C. As Ca^{2+} dissociated from cTnI, it was chelated by Quin-2 resulting in an increased fluorescent signal. The fluorescence was monitored through a 510-nm broad band-pass interference filter with excitation at 330 nm. Each trace represents the average of five individual shots (which was repeated multiple times) and fit with a single exponential from which the k_{off} for Ca^{2+} exchange with cTnI was calculated.

Statistical Analysis—The relationship between [MgATP] or [Ca^{2+}] and tension were fit to the Hill equation using nonlinear least squares regression to obtain [MgATP]_{50} or [Ca^{2+}]_{50} and
**RESULTS**

**Rigor Cross-bridge-dependent Activation**—Our aim was to determine whether signals generated by strongly bound cross-bridges that affect cooperative activation of the thin filament are modified by the N-terminal region of cTnl. One approach to this aim involved activating skinned cardiac fiber bundles with rigor cross-bridges in the absence of Ca\(^{2+}\) (pCa 9.0) by varying the concentration of MgATP. As shown in Fig. 2, the relationship between [MgATP] and tension was not significantly different between myofilaments containing cTnl ([MgATP]\(_{50}\) = 16.54 ± 0.78 \(\mu\)M) and myofilaments containing cTnl(A66H) ([MgATP]\(_{50}\) = 16.10 ± 1.59 \(\mu\)M) at pH 7.0. A decrease from pH 7.0 to 6.5 induced an inhibition of cross-bridge-dependent activation in both preparations as indicated by the leftward shift of the tension-[MgATP] relationship. However, inhibition of cross-bridge-dependent activation at pH 6.5 was significantly greater in myofilaments containing cTnl(A66H) ([MgATP]\(_{50}\) = 6.30 ± 0.09 \(\mu\)M) than in myofilaments containing cTnl ([MgATP]\(_{50}\) = 10.27 ± 0.36 \(\mu\)M).

Data in Fig. 3 compare results at pH 7.0 and 6.5 in myofilaments containing either ssTnl or ssTnl(H34A) in place of cTnl. In this series of experiments, activation of tension by rigor cross-bridges in myofilaments containing ssTnl at pH 7.0 ([MgATP]\(_{50}\) = 10.6 ± 0.74 \(\mu\)M) was diminished at pH 6.5 ([MgATP]\(_{50}\) = 6.42 ± 0.26 \(\mu\)M). However, tension developed by myofilaments containing ssTnl(H34A) were less sensitive to rigor cross-bridge activation than those containing ssTnl at both pH 7.0 ([MgATP]\(_{50}\) = 8.78 ± 0.47 \(\mu\)M) and 6.5 ([MgATP]\(_{50}\) = 4.13 ± 0.13 \(\mu\)M). The Hill coefficient was significantly increased for all the myofilament preparations at pH 6.5 compared with pH 7.0.

**Ca\(^{2+}\)-dependent Activation**—We also compared the tension-[Ca\(^{2+}\)] relationship at pH 7.0 and 6.5 in myofilaments containing cTnl, ssTnl, mutant cTnl(A66H), or mutant cTnl(H34A). Data in Fig. 4 show that at both pH 7.0 and 6.5, myofilaments containing cTnl(A66H) were desensitized to Ca\(^{2+}\) (pH 7.0 [Ca\(^{2+}\)]\(_{50}\) = 2.60 ± 0.15 \(\mu\)M; pH 6.5 [Ca\(^{2+}\)]\(_{50}\) = 10.53 ± 1.28 \(\mu\)M) compared with cTnl (pH 7.0 [Ca\(^{2+}\)]\(_{50}\) = 1.85 ± 0.03 \(\mu\)M; pH 6.5 [Ca\(^{2+}\)]\(_{50}\) = 6.86 ± 0.15 \(\mu\)M). In a separate series of experiments shown in Fig. 5, ssTnl myofilaments ([Ca\(^{2+}\)]\(_{50}\) = 2.37 ± 0.10 \(\mu\)M) and ssTnl(H34A) myofilaments ([Ca\(^{2+}\)]\(_{50}\) = 2.57 ± 0.14 \(\mu\)M) were more sensitive to Ca\(^{2+}\) acti-
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![Diagram](image)

**FIGURE 5.** Force-Ca\(^{2+}\) relationship in skinned myofilaments containing cTnI, ssTnI or ssTnI(H34A). A comparison of cTnI myofilaments at pH 7.0 ([Ca\(^{2+}\)])\(_{50}\) = 4.12 ± 0.23 μM) showed that myofilaments containing either ssTnI or ssTnI(H34A) were more sensitive to Ca\(^{2+}\) to the same extent (ssTnI [Ca\(^{2+}\)])\(_{50}\) = 2.37 ± 0.10 μM; ssTnI(H34A) [Ca\(^{2+}\)])\(_{50}\) = 2.57 ± 0.14 μM). At pH 6.5 both ssTnI and ssTnI(H34A) demonstrated the same degree of resistance to acidic deactivation (ssTnI, [Ca\(^{2+}\)])\(_{50}\) = 7.78 ± 0.30 μM; ssTnI(H34A), [Ca\(^{2+}\)])\(_{50}\) = 7.62 ± 0.84 μM) compared with cTnI myofilament ([Ca\(^{2+}\)])\(_{50}\) = 10.68 ± 0.25 μM). Data are presented as mean ± S.E., n = 5–8 fiber bundles from 3 different hearts.

As a value in the inset of each graph to demonstrate the relative ability of NEM-S-1 to activate tension in myofilaments regulated by different TnI preparations. Thus, the -fold increase for cTnI at pH 7.0 was 4.7, but increased significantly under acidic pH to 15.6. For cTnI(A66H) at pH 7.0, the -fold increase was 7.4, but at pH 6.5 the -fold increase declined to 5.7. The ability of NEM-S1 to recruit cross-bridges over a range of submaximal Ca\(^{2+}\) concentrations is shown in Fig. 9. Fig. 9A demonstrates that at pH 7.0 there was no significant difference in the ability of NEM-S1 to recruit additional cross-bridges in cTnI(A66H) myofilaments compared with cTnI myofilaments. However, at pH 6.5 (Fig. 9B), the -fold increase in tension developed after NEM-S1 treatment was significantly diminished in cTnI(A66H) myofilaments, which indicates that the addition of protonated His affected the ability of these myofilaments to recruit cross-bridges under these conditions.

Fig. 7 shows data for experiments in which we measured Ca\(^{2+}\)-activated tension before and after NEM-S1 treatment in myofilaments regulated by ssTnI (Fig. 7, A and B) and ssTnI(H34A) (Fig. 7, C and D). For both ssTnI and ssTnI(H34A) myofilaments, there was significant dependence on cross-bridge activation at pH 7.0 as shown by the -fold increase values of 11.4 and 8.5, respectively (Fig. 7, A and C). Under acidic conditions, the -fold increase was significantly diminished in both ssTnI and ssTnI(H34A) myofilaments with values of 6.2 and 3.4, respectively (Fig. 7, B and D).

To further identify the N-terminal region of cTnI as significant in cross-bridge-dependent activation and to further define a special role of Ala-66, we tested the influence on cross-bridge activation of a second mutant, cTnI(Q56A). Our previous studies (13) on the solution structure of the C-terminal domain of cTnC free and bound to cTn-I(33–80) identified the binding interface as predominantly hydrophobic. Moreover, predictions based on the crystal structure of the core domain of cardiac Tn indicated that cTnI residues 43–65 form an amphiphilic region of binding to the C-lobe of cTnC (31). Comparison of the primary structures of cTnI and ssTnI shows a substitution in cTnI Gln-56, a hydrophilic residue, with a hydrophobic Ala in the homologous residue of ssTnI. Fig. 8 shows data from a second series of experiments in which we compared tension of myofilaments containing either cTnI or cTnI(Q56A) before and after treatment with NEM-S1 over a range of submaximal Ca\(^{2+}\) concentrations. As was the case in the control data reported in Fig. 6, A and B, a drop in pH from 7.0 (Fig. 8A) to 6.5 (Fig. 8B) was associated with enhanced ability of NEM-S1 to recruit force-generating cross-bridges in myofilaments regulated by cTnI. At pH 7.0, replacement of cTnI with cTnI(Q56A) also was associated with a significant enhancement of NEM-S1-induced recruitment of force-generating cross-bridges similar to that of the controls (Fig. 8C). However, in contrast to the case with myofilaments containing cTnI(A66H), in which there was a suppression of S-1 NEM-induced activation of submaximal tension at pH 6.5 compared with controls, the presence of cTnI(Q56A) in the myofilaments enhanced cross-bridge-dependent activation at pH 6.5 (Fig. 8D). Data illustrated in Fig. 9 emphasize this difference in effect of cTnI(A66H) and cTnI(Q56A) on cross-bridge-dependent activation. In Fig. 9, we summarized the data on -fold activation of force induced by...
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FIGURE 6. Tension developed at submaximal Ca\(^{2+}\) concentrations in myofilaments containing cTnI or cTnI(A66H) activated with Ca\(^{2+}\) before and after NEM-S1. In A and B, cTnI myofilaments before NEM-S1 treatment (white bars) showed decreased tension development at pH 6.5. After NEM-S1 treatment (black bars), there was increased Ca\(^{2+}\) sensitivity due to enhanced cross-bridge activation. In C and D, replacement of cTnI with cTnI(A66H) showed deactivation with acidic pH before NEM-S1 (white bars). After NEM-S1 treatment (black bars), there was increased Ca\(^{2+}\) sensitivity due to enhanced cross-bridge activation, however, the effect was less than that seen with cTnI myofilaments (cf. B and D). The tension developed before NEM-S1 treatment at a given [Ca\(^{2+}\)] (0.32 μM used throughout for comparison) compared with the -fold increase in that tension after NEM-S1 is given as a value in the inset of each graph. Asterisks denote significant difference (p < 0.05) in tension development after NEM-S1 treatment. Data are presented mean ± S.E., n = 5–6 fiber bundles from 3 different hearts, with a separate set of fiber bundles used for each experiment at pH 7.0 or 6.5.

DISCUSSION
Although our prediction that the presence of a His at position 34 in ssTnI may be important in Ca\(^{2+}\) and pH-dependent activation did not bear out, our data are the first to specifically identify the significance of an N-terminal region of cTnI in the process by which cross-bridges signal the activation of cardiac thin filaments. Our approach involved two modes of activation of the thin filament by strong cross-bridges. In the first approach, we activated the thin filament with rigor cross-bridges at Ca\(^{2+}\) concentrations below the threshold for binding to the cTnC regulatory site. Under conditions of replacing cTnI with ssTnI, cTnI myofilaments were more sensitive to rigor cross-bridge-dependent activation at pH 7.0 than ssTnI myofilaments. Moreover, increasing hydrogen ion concentration from pH 7.0 to 6.5 demonstrated that the ability of strong rigor cross-bridges to activate the thin filament at pCa 9.0 was depressed to a greater extent in myofilaments regulated by cTnI.
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![Graphs showing tension development at submaximal Ca²⁺ concentrations](Image)

**FIGURE 7.** Tension developed at submaximal Ca²⁺ concentrations in myofilaments containing either ssTnI or ssTnI(H34A) activated with Ca²⁺ before and after NEM-S1. In A and B, ssTnI myofilaments before NEM-S1 treatment (white bars) showed resistance to deactivation at pH 6.5. After NEM-S1 treatment (black bars), there was a significant decrease in Ca²⁺ sensitivity due to a diminished ability of NEM-S1 to induce cross-bridge activation. In C and D, myofilaments containing ssTnI(H34A) demonstrated a sensitization to Ca²⁺ at pH 7.0 before NEM-S1 (white bars) and resistance to acidic deactivation at pH 6.5 similar to that seen in ssTnI. After NEM-S1 treatment (black bars) at pH 7.0, there was an increased ability of ssTnI to induce cross-bridge activation in both ssTnI and ssTnI(H34A) myofilaments (cf. A and C). However, at pH 6.5, the relative reliance on cross-bridge activation was significantly diminished for both ssTnI and ssTnI(H34A) myofilaments (cf. B and D). The tension developed before NEM-S1 treatment at a given [Ca²⁺] (0.32 μM used throughout for comparison) compared with the fold increase in that tension after NEM-S1 is given as a value in the inset of each graph. Asterisks denote significant difference (p < 0.05) in tension development after NEM-S1. Data are presented as mean ± S.E., n = 5–6 fiber bundles from 3 different hearts, with a separate set of fiber bundles used for each experiment at pH 7.0 or 6.5.

(Δ[MgATP]₅₀ = 6.27 μM) than myofilaments regulated by ssTnI (Δ[MgATP]₅₀ = 4.18 μM). This blunting of the effect of acidic pH on cross-bridge-dependent activation may be, in part, responsible for the relative lack of effect of acidic pH induced by hypercapnia (32, 33) and ischemia/reperfusion injury (34) on function in hearts of transgenic mice expressing ssTnI in place of cTnI (35). Our data are also relevant to the generation of ischemic contracture, which is associated with rigor cross-bridge connections to the thin filament (36, 37). As pH is lowered, there is a decrease in ATP production with concomitant increases in ADP and Pᵢ. This shifts the equilibrium of cross-bridges initially to the strongly bound MgADP state but, as the ability of the cell to generate sufficient ATP is diminished and ATPase activity increases, eventually rigor contraction occurs. Our data indicate that the extent of ischemic contracture may be influenced by the state of cTnI. Data in support of this idea were reported by MacGowan et al. (38), who demonstrated that the onset of ischemic contracture was altered in hearts in which cTnI was partially replaced with cTnI(S43A,S45A).

These differences between ssTnI and cTnI with regard to cross-bridge-dependent activation appear to be due to part in differences in N-terminal amino acids especially the existence of an Ala at position 66 in cTnI, which is replaced with a His at the homologous position 34 in ssTnI. Compared with myofilaments regulated by cTnI, cTnI(A66H) myofilaments demonstrated a significantly decreased sensitivity to rigor cross-bridge-dependent activation at pH 6.5 although there was no significant effect of this mutation on rigor-dependence thin filament activation at pH 7.0 (Fig. 2). Replacement of His with Ala in ssTnI(H34A) myofilaments also resulted in decreased sensitivity to rigor activation at both pH 7.0 and 6.5 (Fig. 3).

Our second approach involved using NEM-S1, a strongly binding myosin analogue, to promote cooperative cross-bridge binding in myofilaments activated over a range of submaximal Ca²⁺ concentrations where cross-bridge activation is most prevalent. The relative insensitivity of myofilaments regulated by ssTnI compared with those regulated by cTnI to activation by NEM-S1 was evident especially at pH 6.5. As illustrated in Fig. 6B, NEM-S1 induced a significantly greater increase in tension over a range of low Ca²⁺ ion concentrations at pH 6.5 in cTnI-regulated myofilaments than in ssTnI myofilaments (Fig. 7B). More importantly, the substitution of Ala with
An important question in our understanding of muscle regulation is: how do force generating cross-bridges activate the thin filament? A corollary to the first question that arises from our data is: how do modifications in TnI alter this mechanism? An appealing theory for the mechanism of strong cross-bridge-dependent activation of the thin filament is that force-generating myosin heads induce a movement of a highly flexible Tm that promotes the activation of longitudinal and possibly radial near neighbor units in the thin filament (3, 42, 43). Although movements of Tm are initiated by Ca\(^{2+}\) binding to cTnC, full activation requires the isomerization of weakly bound cross-bridges to a strongly bound state that moves Tm further into the actin helical groove. The strong head to tail interaction between contiguous Tm molecules extends activation along the thin filament. There is also evidence that strongly bound cross-bridges are able to increase the affinity of cTnC for Ca\(^{2+}\), an effect that is predicted on the basis of detailed balance (46). In view of our finding of an effect of TnI modification on cross-bridge-dependent activation in the absence of Ca\(^{2+}\) and our finding that the Ala/His substitution in cTnI had no effect on Ca\(^{2+}\) off-rate, we favor the hypothesis that modification of the N-terminal region of cTnI alters cross-bridge-dependent activation. A relationship between cTnI interactions with thin filament actin and the position of Tm on the thin filament is well accepted as a key mechanism in striated muscle activation. Moreover, direct TnI-Tm interactions have also been proposed (47). The question here is: how does substitution of a His for Ala at amino acid 66 affect the process?

The recently solved core structure of the cardiac troponin complex provides insight into a possible mechanism of the influence of Ala-66 of cTnI on cross-bridge activation (31). The crystal structure shows N-terminal cTnI residues 43–65 interacting with the C-lobe of cTnC and residues cTnI residues 66–79 interacting with cTnT. However, close examination of the N-terminal region shows Ala-66 is at a position that does not make direct contact with either cTnC or cTnT. Our data indicate a possible mechanism for an effect of Ala-66 on cross-bridge-dependent activation involving a long range influence on a coiled coil region of cTnT and a resultant alteration in Tm. We show in Fig. 10A that replacement of Ala-66 with His depresses cTnI binding to cTnT at pH 6.5. Protonation of His at

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**FIGURE 8.** Tension developed at submaximal Ca\(^{2+}\) concentrations in myofilaments containing either cTnI or cTnI(Q56A) activated with Ca\(^{2+}\) before and after NEM-S1. In A and B, cTnI myofilaments before NEM-S1 treatment (white bars) showed decreased tension development at pH 6.5 ([Ca\(^{2+}\)\] 0 = 1.32 ± 0.05 μM for pH 7.0; [Ca\(^{2+}\)\] 0 = 6.42 ± 0.42 μM for pH 6.5). After NEM-S1 treatment (black bars), there was an increased Ca\(^{2+}\) sensitivity due to enhanced cross-bridge activation. In C and D, cTnI(Q56A) myofilaments demonstrated a significant decrease in tension development that was further inhibited under acidic conditions compared with cTnI myofilaments ([Ca\(^{2+}\)\] 0 = 2.08 ± 0.04 μM at pH 7.0; [Ca\(^{2+}\)\] 0 = 11.01 ± 0.83 μM at pH 6.5). After NEM-S1 treatment (black bars), there was a significant increase in NEM-S1-induced activation of tension in cTnI(Q56A) myofilaments in comparison to that seen with cTnI myofilaments (C), and this ability was significantly enhanced at pH 6.5 (D). The tension developed before NEM-S1 treatment at a given [Ca\(^{2+}\)] 0 (0.32 μM used throughout for comparison) compared with the fold increase in that tension after NEM-S1 is given as a value in the inset of each graph. Asterisks denote significant difference (p < 0.05) in tension development after NEM-S1 treatment. Data are presented as mean ± S.E., n = 5–6 fiber bundles from 3 different hearts, with a separate set of fiber bundles used for each experiment at pH 7.0 or pH 6.5.
pH 6.5 is likely to repel this cTnI region from positively charged amino acids (i.e., Arg-229) found in the cTnT heptad repeat. This cTnT region, which is highly conserved and important in activation, dimerizes with the heptad repeat region of cTnI to form the IT arm (31, 48). This altered interaction between cTnI and cTnT could be responsible in part for the effect of the A66H mutation to induce a relative inability of cross-bridges to move Tm under acidic conditions. There is also evidence that a 14-amino acid region (residues 165–178) of fsTnT N-terminal to the IT arm of fsTnT acts to stabilize this region through ionic interactions with the coiled coil of fsTnI (49).

Our data also add a new dimension to existing knowledge regarding the importance of modifications of cTnI in the physiology, pathology, and pharmacology of heart muscle. Covalent modifications of cTnI in the form of protein phosphorylation are of significance with regard to regulation of maximum tension, Ca$^{2+}$ sensitivity, and cross-bridge kinetics. In addition hyper-phosphorylation (50, 51) or dephosphorylation (50, 52, 53) may occur in maladaptive responses to cardiac stresses, which lead to heart failure (50). It is significant that hearts of mice expressing ssTnI in place of cTnI demonstrate remarkable resistance to acidic pH (33) and sepsis (54). These findings are important with regard to efforts directed at targeting myofilament proteins for inotropic agents (44, 45). This approach has met with some success with regard to the inotropic agent, levosimendan, which has fared well in clinical trials evaluating its use in acute heart failure (15). Levosimendan binds to TnC in a region that interacts with cTnI (39). Our finding of a region of cTnI, which when modified depresses Ca$^{2+}$ sensitivity with no apparent effect on Ca$^{2+}$ binding may be of significance with regard to therapeutic approaches in treatment of familial hypertrophic cardiomyopathies. Myofilaments in these hearts are sensitized to Ca$^{2+}$ and there is evidence that desensitization may be of benefit in management of these patients. Our data indicate an important target for induction of desensitization of the myofilaments to Ca$^{2+}$.

Our data also extend understanding of the diversity of regulatory mechanisms affected by a single protein operating in a

**Figure 9.** Relationship between tension developed at submaximal Ca$^{2+}$ concentrations in skinned fiber bundles before treatment with NEM-S1 and -fold increase in tension following treatment with NEM-S1. Top, at pH 7.0, the ability of NEM-S1 to induce cross-bridge recruitment is similar between cTnI, cTnI(A66H), and cTnI(Q56A) myofilaments. Bottom, at pH 6.5, the -fold increase in tension after NEM-S1 treatment is significantly lowered in cTnI(A66H) myofilaments compared with that seen in cTnI myofilaments. However, in cTnI(Q56A) myofilaments, tension development was enhanced compared with that seen in cTnI myofilaments.

**Figure 10.** ELISA solid-phase protein binding affinity assay between cTnT and GST-cTnI and GST-cTnI(A66H). GST fusion proteins (N terminus) of cTnI and cTnI(A66H) were engineered and coated onto glutathione microtiter plates. Serial dilutions of cTnT were bound to the proteins followed by binding of monoclonal antibody CT-3 against TnT and affinity determined by A$_{405}$ readings. A, showed that at pH 7.0 there was no difference in affinity of cTnT for either GST-cTnI (EC$_{50}$ = 1.39 ± 0.01 nM) or GST-cTnI(A66H) (EC$_{50}$ = 1.37 ± 0.06 nM). However at pH 6.5, affinity of cTnT for cTnI(A66H) (EC$_{50}$ = 2.08 ± 0.06 nM) was significantly diminished compared with GST-cTnI (EC$_{50}$ = 1.67 ± 0.01 nM). In B, the affinity of cTnT to either GST-ssTnI (EC$_{50}$ = 1.48 ± 0.11 nM at pH 7.0; EC$_{50}$ = 1.37 ± 0.06 nM at pH 6.5) or GST-ssTnI(H34A) (EC$_{50}$ = 1.30 ± 0.03 nM at pH 7.0; EC$_{50}$ = 1.48 ± 0.05 nM at pH 6.5) was unaltered by the switch in amino acid or the effect of acidic pH. Asterisk denotes significant difference ($p < 0.05$).
N-terminal Cardiac Troponin I Affects Cooperative Activation

FIGURE 11. Rate of Ca\(^{2+}\) dissociation from the regulatory site of cTnC in cTn complexes reconstituted with isomform-specific and mutant Tnls. The time course of increase in Quin-2 fluorescence is shown as Ca\(^{2+}\) was removed by Quin-2 from the regulatory site of cTnC in the various cTn complexes. Each cTn complex (~6 \mu M) in 10 mM MOPS, 150 mM KCl, 1 mM DTT, 3 mM MgCl\(_2\), at pH 7.0 or 6.5 was rapidly mixed with an equal volume of 150 \mu M Quin-2 in the same buffer at 15 °C. Quin-2 fluorescence was monitored through a 510-nm broad band-pass interference filter with excitation at 330 nm. All traces have been staggered and normalized for clarity. Each trace is an average of at least 5 traces fit with a single exponential equation (variance < 1.2 x 10\(^{-4}\) ).

hetero-oligomeric complex. In the case of Tnl, the regulatory mechanism includes a prominent role in switching on the thin filament and a prominent role in modulating the “on” state. The role of Tnl phosphorylation in modifying the on state is well accepted and an important element in homeostatic and pathological processes. Our data stress and illuminate a novel role of a region of cTnI in switching on the thin filament. Whereas the role of the actin binding sites of cTnl and the switch peptide in the regulatory head of Tnl are known with regard to switching on the thin filament by Ca\(^{2+}\), regions of cTnl important in switching on the thin filaments by cross-bridges have not been appreciated. This mode of activation is of particular significance in heart muscle in which cross-bridge-dependent activation is important in the Frank-Starling mechanism (1, 51). Cross-bridge-dependent activation also offers the advantage of promoting contraction of the heart while sparing the amounts of Ca\(^{2+}\) required to be released to the myofilament space. An ability to activate the thin filament with efficient use of Ca\(^{2+}\) is energy saving in that ATP is expended in returning Ca\(^{2+}\) to the sarcoplasmic reticulum. Moreover, elevations in cytoplasmic Ca\(^{2+}\) in cardiac myocytes lead to a threat of arrhythmias. Thus, our data have revealed that a near N-terminal region of cTnl appears specialized in the heart for ensuring a robust ability of cross-bridges to activate the thin filament.

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