Involvement of Conserved, Acidic Residues in the N-Terminal Domain of Troponin C in Calcium-Dependent Regulation†

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ABSTRACT: We have mutated eight conserved, charged amino acid residues in the N-terminal, regulatory domain of troponin C (TnC) so we could investigate their role in troponin-linked Ca^{2+} regulation of muscle contraction. These residues surround a hydrophobic pocket in the N-terminal domain of TnC which, when Ca^{2+} binds to regulatory sites in this domain, is exposed and interacts with the inhibitory region of troponin I (TnI). We constructed three double mutants (E53A/E54A, E60A/E61A, and E85A/D86A) and two single mutants (R44A and R81A) of rabbit fast skeletal muscle troponin C (TnC) in which the charged residues were replaced with neutral alanines. All five of these mutants retained TnC's ability to bind TnI in a Ca^{2+}-dependent manner, to neutralize TnI's inhibition of actomyosin S1 ATPase activity, and to form a ternary complex with TnI and troponin T (TnT). Ternary complexes formed with TnC(R44A) or TnC(R81A) regulated actomyosin S1 ATPase activity normally, with TnI-based inhibition in the absence of Ca^{2+} and TnT-based activation in the presence of Ca^{2+}. TnC(E53A/E54A) and TnC(E85A/D86A) interacted weakly with TnT, as judged by native gel electrophoresis. Ternary complexes formed with these mutants inhibited actomyosin S1 ATPase activity in both the presence and absence of Ca^{2+}, and did not undergo Ca^{2+}-dependent structural changes in TnI which can be detected by limited chymotryptic digestion. TnC(E60A/E61A) interacted normally with TnT. Its ternary complex showed Ca^{2+}-dependent structural changes in TnI, inhibited actomyosin S1 ATPase in the absence of Ca^{2+}, but did not activate ATPase in the presence of Ca^{2+}. This is the first demonstration that selective mutation of TnC can abolish the activating effect of troponin while its inhibitory function is retained. Our results suggest the existence of an elaborate network of protein−protein interactions formed by TnI, TnT, and the N-terminal domain of TnC, all of which are important in the Ca^{2+}-dependent regulation of muscle contraction.

The proteins troponin (Tn)[1] and tropomyosin (Tm) regulate contraction in vertebrate striated muscle by transmitting Ca^{2+} signals. Tn is composed of three subunits: the Ca^{2+}-binding subunit TnC, the inhibitory subunit TnI, and the Tm-binding subunit TnT (for reviews, see refs *1*−*3*). The crystal structure of vertebrate fast skeletal muscle TnC with two of its four Ca^{2+}-binding sites occupied was determined some time ago (*4*, *5*), and the structure of TnC with all four Ca^{2+}-binding sites occupied was recently determined by NMR (*6*) and X-ray crystallography (*7*). Although no high-resolution structure of the Tn complex is yet available, the TnC structures have provided a starting point for detailed examination of the molecular mechanism of troponin-linked regulation. Of particular interest are the events immediately after Ca^{2+} binding by the regulatory sites of TnC (*8*−*10*).

Protein structural comparisons have shown that amino acid residues which stabilize native protein conformations tend to be highly conserved (*11*−*13*). Amino acid sequence comparisons of families of related proteins have proven to be useful in predicting ligand interaction sites, which generally are located on the surfaces of the molecules (*14*, *15*). Sequence comparisons of the N-terminal, regulatory domains of TnCs from various species and muscle types reveal that the following charged amino acid residues are highly conserved (*16*, *17*) and therefore may be functionally important: Arg-44, Glu-53, Glu-54, Glu-60, Glu-61, Arg-81, Glu-85, and Asp-86. As shown in Figure 1, these conserved, charged residues are located on the protein surface and surround a hydrophobic pocket in the N-terminal, regulatory domain of TnC which, upon Ca^{2+} binding to the regulatory sites, is exposed and forms new interactions with TnI and/or TnT. These Ca^{2+}-dependent changes in Tn subunit interactions are communicated to the actin−myosin interface,

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[1] Abbreviations: Tn, troponin; Tm, tropomyosin; Tris, tris(hydroxymethyl)aminomethane; MOPS, 4-morpholinoethanesulfonic acid; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IAEDANS, *N*-(iodoacetyl)-*N*′-(1-sulfo-5-naphthyl)ethylenediamine; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); TLCK, N^{α}-tosyl-L-lysyl chloromethyl ketone; SDS, sodium dodecyl sulfate; S1, myosin subfragment-1.

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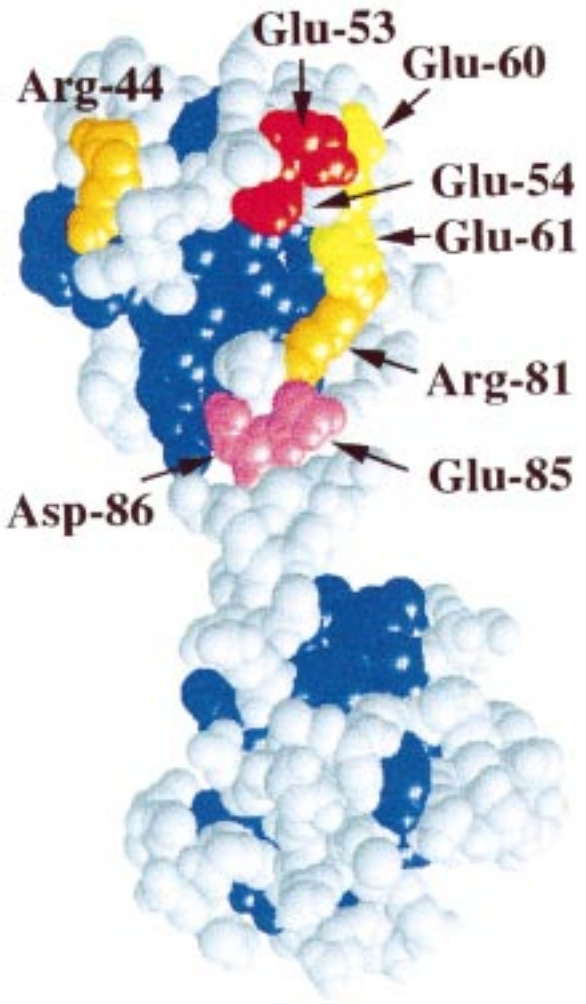

FIGURE 1: Molecular model of TnC. A space-filling model of the 4-Ca^{2+} form of rabbit skeletal muscle TnC (PDB file name 1TN4; *7*), generated using RasMol, is shown. Hydrophobic residues are blue. Arg-44 and Arg-81 are orange. Glu-53 and Glu-54 are red. Glu-60 and Glu-61 are yellow. Glu-85 and Asp-86 are pink.

resulting in the activation of actomyosin ATPase (*18*−*21*). It would not be surprising, therefore, to find that mutation of conserved, charged TnC residues adjacent to the hydrophobic pocket would alter interactions among the three Tn subunits and affect the ability of Tn to regulate actomyosin ATPase. To explore this possibility, we examined the functional properties of several mutant TnCs in which one or two of the eight charged residues identified above were replaced by neutral alanines. We found that mutation of either of the basic residues (Arg-44 and Arg-81) had no discernible effect on function, but mutation of pairs of adjacent acidic residues in the N-terminal domain of TnC affected the structural transitions and regulatory properties of troponin.

EXPERIMENTAL PROCEDURES

Proteins. All myofibrillar proteins, except TnC, used in this study were prepared from rabbit fast skeletal muscle. TnI and TnT were purified using a modification of the method of Ebashi et al. (*22*) with an additional, final purification step by reversed-phase HPLC with a Resource RP column (3 mL, Pharmacia). Tm, actin, and myosin subfragment-1 (S1) were prepared as described previously (*23*−*25*). The expression system for TnC used in this study was designed as follows. A *Bst*EII site was introduced into the *Hinc*II site of RF-M13mp19 which contains a rabbit skeletal muscle TnC cDNA sequence (26), using the *Bst*EII linker GCGGTCACCCG and CGGGTGACCGC. The region which encodes TnC was subcloned into the *Bst*EII−*Bam*HI site of a pTrc99c expression vector. Mutation reactions were carried out using the Sculptor *in vitro* Mutagenesis System (Amersham). The oligonucleotides used for mutagenesis were as follows (mutation codons are underlined):

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E53A/E54A:  GAT GGC GTC CAG CGC CGC TTT GGT GGG TGT C
E60A/E61A:  CCA TCC TCA TCC ACC GCC GCG ATG ATA GCG TC
E85A/D86A:  CTT GCC CTT GGC GCC CCC TTT CAT CTG GCG
R44A:       GGG TGT CTG GCC CAG CAT GGC CAT GAC TGT GCC
R81A:       GTC CTC TTT CAT CTG GGC CAC CAT CAT GAC C
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Expression and purification of recombinant TnCs were carried out as described previously (*26*−*28*). Purification steps include Ca^{2+}-dependent hydrophobic interaction chromatography on phenyl-Sepharose columns (*27*); TnC binds to phenyl-Sepharose in the presence of Ca^{2+} and is released in the presence of EGTA or EDTA. Concentrations of purified Tn subunits and Tm were determined by the method of Gill and von Hippel (*29*).

Reconstitution of Binary and Ternary Complexes. Equimolar amounts of Tn subunits were combined in a solution containing 6 M urea, 1 M NaCl, 1 mM $CaCl_2$, 1 mM dithiothreitol, and 20 mM Tris-HCl (pH 8.0). The solution was then dialyzed successively against 0.6, 0.3, and 0.1 M NaCl solutions, each containing 1 mM $CaCl_2$, 1 mM dithiothreitol, and 20 mM Tris-HCl (pH 8.0). After dialysis, solutions were applied to a Mono-Q HR5/5 column (Pharmacia) equilibrated with 0.1 M NaCl, 1 mM $CaCl_2$, and 20 mM Tris-HCl (pH 8.0). The complex was eluted with a linear gradient of 0.1 to 0.5 M NaCl in the same solution. The identity of pure Tn subunits in the complex was confirmed by SDS gel electrophoresis. These methods have previously been described in detail (*30*).

Actomyosin S1 ATPase Activity Measurements. To measure release of inhibition by TnC, ATPase activity measurements were carried out in 50 mM KCl, 3.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM dithiothreitol, and 20 mM MOPS (pH 7.0) at 25 °C. The concentrations of myosin S1, actin, Tm, and TnI were 0.23, 6, 1, and 2 μM, respectively. For Ca^{2+}-dependent activity measurements for the ternary complexes, the assay conditions were 20 mM KCl, 3.5 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM MOPS, and either 0.1 mM $CaCl_2$ or 1 mM EGTA (pH 7.0). In both cases, the reactions were initiated by adding 2 mM ATP. ATPase activity was determined from the time course of P_i liberation for 5 min. Every 1 min, a 50 μL aliquot was removed and the reaction was terminated by the addition of 450 μL of 0.2 M perchloric acid. The amount of released phosphate was determined by the malachite green method (*31*).

Binding Experiments. The affinity of binding between TnI and wild-type or mutant TnCs was determined by measuring fluorescence changes of IAEDANS attached to TnC residue Cys-98 (*28*). The excitation and emission wavelengths were 340 and 490 nm, respectively. The signal was averaged for 20 s. Dissociation constants were obtained from a nonlinear least-squares fit to the following equation as a root of a

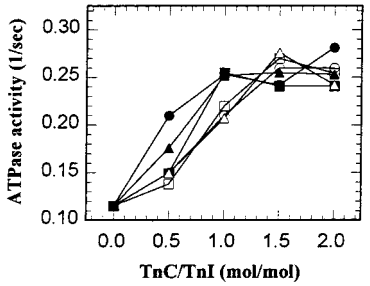

FIGURE 2: Neutralization of TnI's inhibition of actomyosin S1 ATPase activity by mutant TnCs. The conditions were as follows: 50 mM KCl, 3.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM dithiothreitol, 2 mM ATP, and 20 mM MOPS (pH 7.0) at 25 °C. The concentrations of myosin S1, actin, Tm, and TnI were 0.23, 6, 1, and 2 μM, respectively. The concentrations of TnC (○), TnC(R44A) (●), TnC(E53A/E54A) (□), TnC(E60A/E61A) (■), TnC(R81A) (△), and TnC(E85A/D86A) (▲) are varied from 0 to 4 μM. A representative data set is shown.

quadratic equation for binary complex formation:

$$\Delta Y_i = \Delta Y_{max}\{X_i + P + K - [(X_i + P + K)^2 - 4PX_i]^{1/2}\}/(2P) \quad (1)$$

where P is the fixed molar concentration of labeled TnCs (corrected for dilution), X_i is the variable molar concentration of TnI, ΔY_i is the fluorescence signal change after the ith addition of TnI, ΔY_{max} is the maximum fluorescence signal change, and K is the dissociation constant. The association constant was then obtained as the reciprocal of the dissociation constant. Fluorescence measurements were taken at 15 °C in 0.1 M NaCl, 10 mM PIPES (pH 7.0), and either 1 mM $CaCl_2$ or 5 mM $MgCl_2$ with 1.5 mM EGTA. The protein concentrations that were used ranged from 0.02 to 0.5 μM.

The binding of wild-type and mutant TnCs to TnT was examined by polyacrylamide gel electrophoresis in the absence of SDS. Solutions containing 5 μM wild-type or mutant TnC and 5 μM TnT in 0.1 M NaCl, 5 mM $MgCl_2$, 20 mM MOPS (pH 7.0), and either 1 mM $CaCl_2$ or 1 mM EGTA were prepared and analyzed on 12% polyacrylamide gel electrophoresis in the absence of SDS.

Limited Proteolysis of Ternary Tn Complexes. Ternary Tn complexes (3 μM) in 0.1 M NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM Tris-HCl, and either 1 mM $CaCl_2$ or 1 mM EGTA (pH 8.0) were digested with a 1:2000 weight ratio of TLCK-treated α-chymotrypsin at 37 °C for 40 min. The reaction was terminated by adding phenylmethanesulfonyl fluoride to a final concentration of 1 mM.

RESULTS

Interaction between TnC and TnI. We first examined the ability of our mutant TnCs to neutralize TnI's inhibition of actomyosin S1 ATPase activity in the absence of TnT. Figure 2 shows the relationship between the concentration of TnC and the neutralization of this inhibition. ATPase activities were measured in the presence of Ca^{2+} and 2 μM TnI. The maximal effect was obtained when TnC was added in a 1:1 molar ratio to TnI, reflecting the association of TnC and TnI in a tight binary complex. Since the binding constant for binding of wild-type TnC to TnI is estimated to be approximately 10^9 M^{-1} in the presence of Ca^{2+}, it is possible that some TnC mutants may have a reduced affinity for TnI that we could not detect under the conditions used for the ATPase activity measurements. To examine this possibility, we estimated the TnI binding affinities of TnC(E53A/E54A), TnC(E60A/E61A), and TnC(E85A/D86A) by measuring fluorescence intensity changes of IAEDANS attached at Cys-98 of TnC. As summarized in Table 1, these three mutants were, within the accuracy limitations of the binding measurements, equivalent to wild-type TnC in their affinity for TnI. We were not able to detect any differences among the TnC mutants in their affinity for TnI by native gel electrophoresis in the presence and absence of Ca^{2+}.

Table 1: Binding of TnCs to TnI[a]

TnC type	condition	K_a (M^{-1})[b]
wild-type	calcium	$(7.7 \pm 1.3) \times 10^9$
	magnesium	$(4.0 \pm 0.4) \times 10^6$
TnC(E53A/E54A)	calcium	$(5.3 \pm 1.7) \times 10^9$
	magnesium	$(3.5 \pm 0.6) \times 10^6$
TnC(E60A/E61A)	calcium	$(7.0 \pm 0.3) \times 10^9$
	magnesium	$(5.5 \pm 0.9) \times 10^6$
TnC(E85A/D86A)	calcium	$(4.8 \pm 1.3) \times 10^9$
	magnesium	$(2.2 \pm 0.1) \times 10^6$

[a] Estimated from fluorescence intensity changes of IAEDANS attached at Cys-98 of TnC. The protein concentrations that were used ranged from 0.02 to 0.5 μM. [b] K_a (association constant) values represent means ± the standard deviation from four to eight data sets.

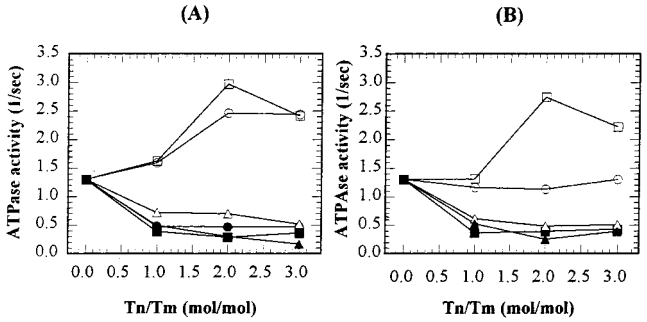

FIGURE 3: Effects of the ternary Tn complexes on actomyosin S1 ATPase activity. The conditions were as follows: 20 mM KCl, 3.5 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM ATP, and 20 mM MOPS (pH 7.0) at 25 °C. The white and black symbols indicate the presence of 0.1 mM $CaCl_2$ and 1 mM EGTA, respectively. The concentrations of myosin S1, actin, and Tm were 0.23, 6, and 1 μM, respectively: (A) TnT−TnI−TnC (circles), TnT−TnI−TnC(R44A) (squares), and TnT−TnI−TnC(E53A/E54A) (triangles) and (B) TnT−TnI−TnC(E60A/E61A) (circles), TnT−TnI−TnC(R81A) (squares), and TnT−TnI−TnC(E85A/D86A) (triangles).

Interaction between TnC and TnT. We used native gel electrophoresis to examine the Ca^{2+}-dependent binding of our TnC mutants to TnT because previous fluorescence titration studies by others (*32*, *33*) failed to detect a Ca^{2+} dependency for interactions between TnT and fluorescently labeled TnC. As shown in Figure 4, the wild-type TnC and all mutant TnCs form binary complexes in the presence of Ca^{2+}. The bands for the complexes which contained TnC(E53A/E54A) and TnC(E85A/D86A), however, were relatively faint, indicating that these two mutant TnCs have a lower affinity for TnT.

Effects of Ternary Tn Complexes on Actomyosin S1 ATPase Activity. All five of the mutant TnCs used in this study formed ternary complexes with TnI and TnT. The reconstituted complexes were purified by anion-exchange chromatography, and their subunit composition was confirmed by SDS gel electrophoresis as described previously (*30*). The Ca^{2+}-dependent regulatory functions of the ternary

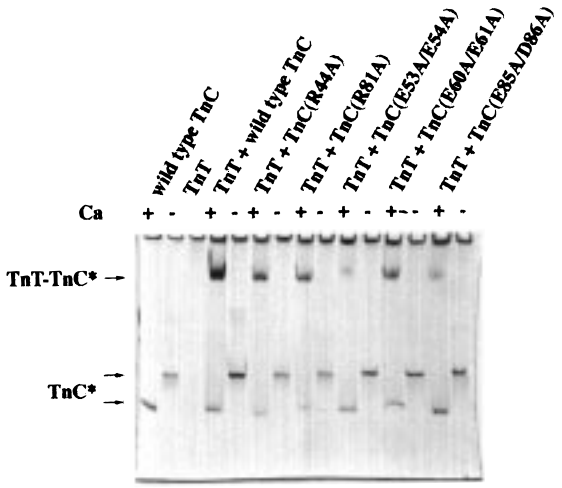

FIGURE 4: TnC−TnT complex formation investigated by native gel electrophoresis. Each TnC was combined with TnT (to a concentration of 5 μM for both proteins) in 0.1 M NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM Tris-HCl (pH 8.0), and either 1 mM $CaCl_2$ (+) or 1 mM EGTA (−). The binding of TnC to TnT was analyzed with 12% polyacrylamide gel electrophoresis. Note that the bands correspond to TnT−TnC(E53A/E54A) and TnT−TnC(E85A/D86A) are faint compared to those of other TnT−TnC complexes in the presence of Ca^{2+}. The mobility of TnC is Ca^{2+}-dependent; the double arrows for TnC denote its location in the presence or absence of Ca^{2+}.

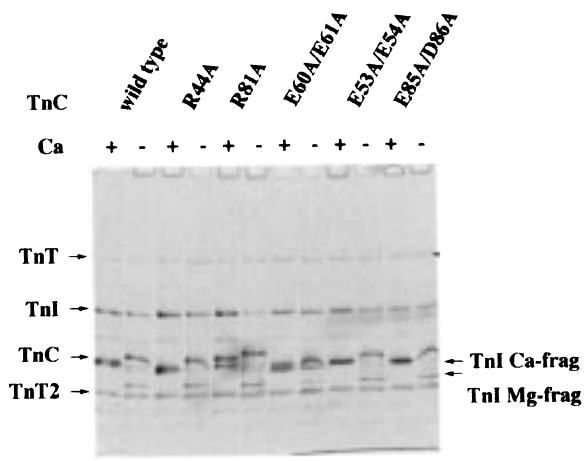

FIGURE 5: Chymotryptic digestion of the ternary Tn complexes. Tn complexes (3 μM) in 0.1 M NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM Tris-HCl (pH 8.0), and either 1 mM $CaCl_2$ (+) or 1 mM EGTA (−) were digested with a 1:2000 weight ratio of α-chymotrypsin at 37 °C for 40 min. The digests were analyzed with 15% polyacrylamide gel electrophoresis in the presence of SDS. Bands correspond to TnT, TnT2 (residues 159−259), TnI, $TnI_{Ca-frag}$ (residues 1−134 and/or 1−140), $TnI_{Mg-frag}$ (residues 1−116), and TnC are denoted by arrows. $TnI_{Ca-frag}$, which migrates just ahead of TnC, is missing in the digests of Tn complexes that contain TnC(E53A/E54A) or TnC(E85A/D86A). Note that the bands for TnC and $TnI_{Ca-frag}$ migrate close together and appear as a doublet on the gel. Bands migrate a little more slowly in the TnC(R81A) sample, but both the TnC and $TnI_{Ca-frag}$ bands are clearly present.

complexes were then examined. Figure 3 shows the relationship between the concentration of the Tn complex and actomyosin S1 ATPase activities. Three different outcomes were observed. Complexes formed with wild-type TnC, TnC(R44A), or TnC(R81A) inhibited actomyosin S1 ATPase activity in the absence of Ca^{2+} and activated the enzyme in the presence of Ca^{2+}. The complex formed with TnC(E60A/E61A) inhibited actomyosin S1 ATPase activity in the absence of Ca^{2+}, but failed to activate the enzyme in the presence of Ca^{2+}. The complexes formed with TnC(E53A/E54A) or TnC(E85A/D86A) inhibited ATPase activity in both the presence and absence of Ca^{2+}.

Ca^{2+}-Dependent Structural Transitions of the Tn Complexes. We prepared native gels (not shown) of ternary Tn complexes containing wild-type TnC, TnC(E53A/E54A), TnC(E60A/E61A), or TnC(E85A/D86A), each prepared in the presence or absence of Ca^{2+}. As we expected, all four samples formed complexes in both the presence and absence of Ca^{2+}. The only observable difference in the behavior of the various samples was that the ternary C−I−T complex containing TnC(E60A/E61A) showed a slightly larger amount of dissociation into C−I or C−T binary complexes in the presence of Ca^{2+}. Recently, we showed that limited chymotryptic digestion of the ternary Tn complex can be used to detect Ca^{2+}-dependent structural changes in TnI (*30*). Both TnT and TnI, but not TnC, were cleaved, and the location of the cleavage sites in TnI was Ca^{2+}-dependent. In the presence of Ca^{2+} and Mg^{2+}, chymotrypsin produced two N-terminal fragments collectively designated $TnI_{Ca-frag}$ (residues 1−134 and 1−140). In the presence of EGTA and Mg^{2+}, chymotrypsin produced the smaller N-terminal fragment $TnI_{Mg-frag}$ (residues 1−116). TnC remained intact in both cases, while TnT was cleaved to produce the C-terminal fragment TnT2 (residues 159−259). $TnI_{Ca-frag}$, $TnI_{Mg-frag}$, and TnT2 can be identified on 15% polyacrylamide gels after electrophoresis of degraded complexes in the presence of SDS. $TnI_{Ca-frag}$ migrates slightly faster than TnC, while $TnI_{Mg-frag}$ migrates between $TnI_{Ca-frag}$ and TnT2. Electrophoretic patterns obtained in this study after limited chymotryptic digestion of ternary Tn complexes are shown in Figure 5. The patterns obtained from Tn complexes containing TnC(E53A/E54A) and TnC(E85A/D86A) differed from those of other complexes in that they did not show any $TnI_{Ca-frag}$ after digestion in the presence of Ca^{2+} and Mg^{2+}.

DISCUSSION

The amino acids mutated in this study are all located in the N-terminal domain of TnC and should not destroy TnC's ability to form a binary complex with TnI or a ternary complex with TnI and TnT. Although the N-terminal, regulatory domain of TnC binds to the inhibitory and C-terminal regions of TnI in a Ca^{2+}-dependent manner, the C-terminal domain of TnC remains tightly attached to the N-terminal region of TnI regardless of whether Ca^{2+} is absent or present (*34*−*36*). The N-terminal domain of TnC also takes part in Ca^{2+}-dependent interactions with the C-terminal region of TnT, although these are weaker than its interactions with TnI (*37*−*39*). Much more critical to the structural integrity of the ternary complex are stronger, Ca^{2+}-independent interactions between the C-terminal region of TnT and the N-terminal region of TnI (*36*, *40*, *41*). The N-terminal region of TnT can activate actomyosin ATPase activity in the presence of Tm, but the globular region of the Tn complex that contains TnC, TnI, and the C-terminal region of TnT blocks this activation in the absence of Ca^{2+} (*36*).

All of our mutations involve conserved, charged amino acids which surround a hydrophobic pocket in the N-terminal, regulatory domain of TnC. This pocket becomes exposed

upon Ca^{2+} binding and interacts with TnI, strengthening TnC−TnI binding and reversing TnI's inhibition of actomyosin ATPase activity. Although our mutations might be expected to alter the exposure of this pocket, we were not able to detect any resulting differences in the behavior of the mutants. The wild-type TnC and all five mutant TnCs could be purified by Ca^{2+}-dependent hydrophobic interaction chromatography on phenyl-Sepharose columns. In this procedure, exposure of the hydrophobic pocket in the presence of Ca^{2+} enables TnC to bind to phenyl-Sepharose, while other proteins are not retained. The phenyl-Sepharose column is then washed with an EGTA- or EDTA-containing solution, Ca^{2+} released from TnC, the hydrophobic pocket of TnC buried, and the TnC released from the column (*27*). The ability of our mutants to interact with phenyl-Sepharose in a Ca^{2+}-dependent manner shows that, while mutation may have altered some details of the Ca^{2+}-dependent conformational alteration which exposes the hydrophobic pocket, a change does take place. Further evidence for the retention of Ca^{2+}-dependent exposure of the N-terminal hydrophobic pocket is that our mutants exhibit a characteristic increase in affinity for TnI when Ca^{2+} binds to the N-terminal domain of TnC. The binding constants of our wild-type TnC and mutant TnCs for TnI is approximately 10^6 M^{-1} in the absence of Ca^{2+} and 10^9 M^{-1} in the presence of Ca^{2+}. Some of the TnC mutants have a moderately reduced (up to 45%) affinity for TnI (Table 1), but they still bind very tightly and are able to neutralize TnI's inhibition of actomyosin S1 ATPase activity in the absence of TnT (Figure 2), so the differences are not functionally significant.

Mutation of basic residues did not result in any measurable change in the functional properties of TnC. Most notably, ternary complexes containing wild-type TnC, TnC(R44A), or TnC(R81A) inhibited actomyosin S1 ATPase activity in the absence of Ca^{2+} and activated it in the presence of Ca^{2+}. On the other hand, mutation of acidic residues had a significant effect on Ca^{2+}-dependent regulation. Ternary Tn complexes containing TnC(E53A/E54A) or TnC(E85A/D86A) inhibited actomyosin S1 ATPase activity in both the absence and presence of Ca^{2+}. This loss of regulatory function appears to be due to weakened binding between TnC and TnT (Figure 4), since the high affinity of TnC for TnI was retained in these two mutants. These complexes also did not undergo the Ca^{2+}-dependent structural transitions that we normally observe after limited chymotryptic digestion (*30*). Digestion patterns obtained from Tn complexes containing TnC(E53A/E54A) and TnC(E85A/D86A), unlike those of complexes containing wild-type TnC or TnC(E60A/E61A), did not show any degradation of TnI after digestion of the complex in the presence of Ca^{2+} and Mg^{2+} (Figure 5). We interpret these findings to mean that, in ternary complexes containing TnC(E53A/E54A) or TnC(E85A/D86A), the fragment of residues 117−140 of TnI is no longer able to undergo Ca^{2+}-dependent structural changes which are important in regulation (*30*). Our results indicate that these structural changes in TnI occur only when native TnC−TnT interactions are preserved. The TnI segment of residues 117−140 is on the C-terminal side of the inhibitory region and is known to bind to the N-terminal hydrophobic pocket of TnC (*21*). The segment also includes Cys-133, an amino acid residue in TnI which moves away from actin, and toward TnC, when Ca^{2+} is bound to the regulatory sites on TnC (*42*, *43*). Our chemical cross-linking studies, carried out in the presence of Ca^{2+}, showed proximity of TnI Cys-133 to Cys-12 in a mutant TnC (*44*), and this was recently confirmed by fluorescence resonance energy transfer (*45*).

It is interesting to compare the effects of mutation of Glu-53 and Glu-54 with those obtained for Glu-60 and Glu-61. All of these residues are located on the same face at either end of the "C-helix" (residues 52−62) of TnC, which is known to interact with TnI in a Ca^{2+}-dependent manner (*1*, *21*, *27*, *37*, *46*, *47*). As noted above, TnC(E53A/E54A) interacts weakly with TnT, and its Tn complex inhibits actomyosin ATPase activity in both the presence and absence of Ca^{2+}. On the other hand, TnC(E60A/E61A) appeared to interact normally with TnT, but its Tn complex was not fully functional. Previous studies (*48*−*50*) showed that Ca^{2+} binding to the ternary Tn complex not only neutralizes inhibition by TnI, but also allows TnT to activate actomyosin ATPase activity. Our TnC(E60A/E61A)-containing Tn complex inhibited actomyosin ATPase activity in the absence but not in the presence of Ca^{2+}, but could not activate in the presence of Ca^{2+}. Since TnT1, the N-terminal segment (residues 1−156) of TnT, is responsible for the activation of actomyosin ATPase activity in the presence of Ca^{2+} (*36*), Glu-60 and/or Glu-61 of TnC may play an important role in transmitting the activation signal to TnT1. Our results show for the first time that the inhibition and activation functions of the troponin regulatory process can be separated by selective mutation of conserved acidic residues in the regulatory domain of TnC.

As noted above, our TnC(E85A/D86A)-containing ternary Tn complex has a diminished interaction with TnT and inhibits actomyosin S1 ATPase activity in both the presence and absence of Ca^{2+}. Glu-85 and Asp-86 of TnC are located in the linker region between the two domains of TnC, which in the crystal structure forms a long, central helix (*4*, *5*, *7*). In the NMR-derived solution structure of TnC, the linker region is flexible and the conformation of the segment which contains Glu-85 and Asp-86 cannot be determined (*6*). Both Glu-85 and Asp-86 are important for TnC function. Fujimori et al. (*51*) reported that a TnC mutant in which Glu-85 had been replaced by Lys was able to restore only half as much tension as wild-type TnC in TnC-depleted muscle fibers. Ramakrishnan and Hitchcock-DeGregori (*52*) reported that a TnC mutant in which Asp-86 had been replaced by Ala is defective in its ability to activate actomyosin ATPase activity in reconstituted thin filaments. This is consistent with our present finding that the Tn complex formed with TnC(E85A/D86A) inhibits actomyosin S1 ATPase activity in both the presence and absence of Ca^{2+}. Interestingly, mutant TnC(D86A) binds to TnT as strongly as wild-type TnC (*52*), whereas our TnC(E85A/D86A) does not, suggesting that Glu-85 of TnC may be particularly important for binding to TnT.

In summary, we have shown that our mutations of conserved, acidic residues in the N-terminal domain of TnC alter the subunit interactions, structural transitions, and regulatory properties of troponin. Our results indicate that two mutants, TnC(E53A/E54A) and TnC(E85A/D86A), when incorporated into ternary Tn complexes, inhibit actomyosin ATPase in both the presence and absence of Ca^{2+}. These TnC mutants exhibit a weakened Ca^{2+}-dependent binary interaction with TnT and a defective linkage in the

ternary complex to Ca^{2+}-dependent conformational changes in TnI. On the other hand, TnC(E60A/E61A) behaves normally in its Ca^{2+}-dependent binary interaction with TnT, and TnI exhibits normal Ca^{2+}-dependent conformational changes in the TnC(E60A/E61A)-containing ternary complex. Nevertheless, the TnC(E60A/E61A)-containing ternary complex, while able to relieve TnI-based inhibition in the presence of Ca^{2+}, cannot activate actomyosin S1 ATPase activity through TnT interaction. Further work is needed to comprehend the elaborate network of protein−protein interactions formed by TnI, TnT, and the N-terminal domain of TnC, all of which are important in the Ca^{2+}-dependent regulation of muscle contraction.

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