Mutations of Hydrophobic Residues in the N-terminal Domain of Troponin C Affect Calcium Binding and Exchange with the Troponin C-Troponin I\textsubscript{96–148} Complex and Muscle Force Production*

Received for publication, December 23, 2003, and in revised form, February 4, 2004
Published, JBC Papers in Press, February 16, 2004, DOI 10.1074/jbc.M314095200

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Interactions between troponin C and troponin I play a critical role in the regulation of skeletal muscle contraction and relaxation. We individually substituted 27 hydrophobic Phe, Ile, Leu, Val, and Met residues in the regulatory domain of the fluorescent troponin C\textsuperscript{F29W} with polar Gln to examine the effects of these mutations on: (a) the calcium binding and dynamics of troponin C\textsuperscript{F29W} complexed with the regulatory fragment of troponin I (troponin I\textsubscript{96–148}) and (b) the calcium sensitivity of force production. Troponin I\textsubscript{96–148} was an accurate mimic of intact troponin I for measuring the calcium dynamics of the troponin C\textsuperscript{F29W}-troponin I\textsubscript{complexes.}

The calcium affinities of the troponin C\textsuperscript{F29W}-troponin I\textsubscript{96–148} complexes varied 243-fold, whereas the calcium association and dissociation rates varied 38- and 33-fold, respectively. Interestingly, the effect of the mutations on the calcium sensitivity of force development could be better predicted from the calcium affinities of the troponin C\textsuperscript{F29W}-troponin I\textsubscript{96–148} complexes than from that of the isolated troponin C\textsuperscript{F29W} mutants. Most of the mutations did not dramatically affect the affinity of calcium-saturated troponin C\textsuperscript{F29W} for troponin I\textsubscript{96–148}. However, the Phe\textsubscript{26} to Gln and Ile\textsubscript{62} to Gln mutations led to 10-fold lower affinity of calcium-saturated troponin C\textsuperscript{F29W} for troponin I\textsubscript{96–148} causing a drastic reduction in force recovery, even though these troponin C\textsuperscript{F29W} mutants still bound to the thin filaments. In conclusion, elucidating the determinants of calcium binding and exchange with troponin C in the presence of troponin I provides a deeper understanding of how troponin C controls signal transduction.

Troponin C (TnC)\textsuperscript{3} regulates striated muscle contraction and relaxation through the binding and release of Ca\textsuperscript{2+} (for review see Refs. 1–3). Skeletal muscle TnC (18 kDa) consists of globular N- and C-terminal domains connected by a 31-residue \alpha-helix (for review see Refs. 4 and 5). Both domains bind two Ca\textsuperscript{2+} ions through a pair of EF hand Ca\textsuperscript{2+}-binding motifs. Each pair of EF hands interacts with one another through a short antiparallel \beta-sheet connecting the two Ca\textsuperscript{2+}-binding loops (Ref. 6 and references within). The EF hands are numbered I–IV, and the helices flanking the loops are designated A–H, with an additional N-terminal 14-residue \alpha-helix (Fig. 1, N-helix), which is absent in the closely related EF hand Ca\textsuperscript{2+}-binding protein calmodulin.

Much is known about the cation binding properties of TnC in solution. Each EF hand system binds Ca\textsuperscript{2+} and Mg\textsuperscript{2+} competitively, with the two C-terminal EF hands possessing higher Ca\textsuperscript{2+} and Mg\textsuperscript{2+} affinities (6–8). In fact, the Ca\textsuperscript{2+}-binding sites of the C-domain of TnC possess 10-fold higher Ca\textsuperscript{2+} affinity with a greater than 100-fold slower Ca\textsuperscript{2+} dissociation rate compared with those in the N-domain (6, 9). In part because of its high Ca\textsuperscript{2+} and Mg\textsuperscript{2+} affinities and slow Ca\textsuperscript{2+} exchange rates (as compared with the kinetics of muscle contraction and relaxation), the C-domain is thought to play a structural role in muscle function by anchoring TnC into the Tn complex. In contrast, the Ca\textsuperscript{2+} exchange rates of the N-domain of TnC are rapid enough to be involved in the dynamic Ca\textsuperscript{2+}-dependent regulation of muscle mechanics (for review see Refs. 3 and 10).

Skeletal muscle contraction begins when cytoplasmic [Ca\textsuperscript{2+}]\textsuperscript{a} rises and binds to the N-terminal EF hands of TnC. The entire N-domain of TnC subsequently undergoes a large tertiary conformational change, in which helices B and C move away as a unit from helices N, A, and D, exposing a buried hydrophobic pocket to the solvent (Ref. 5 and references within). The newly formed hydrophobic pocket is thought to allow the N-domain of TnC to interact with the C-terminal of TnI transferring the inhibitory domain of TnI away from actin (11). Concurrently or subsequently, tropomyosin changes its position on the actin filament and myosin then binds cyclically to actin causing muscle contraction (for review see Refs. 1–3). As cytoplasmic [Ca\textsuperscript{2+}]\textsuperscript{a} lowers, the sequence of events above reverses (not necessarily in the same order), and the muscle relaxes. One of the steps that may influence the rate of muscle relaxation is Ca\textsuperscript{2+} dissociation from the N-domain of TnC.

The influence that TnC has on the kinetics of muscle relaxation is controversial and incompletely understood (Ref. 12 and references within). The actual rate that Ca\textsuperscript{2+} dissociates from TnC in muscle fibers has not been measured and thus must be inferred. Ca\textsuperscript{2+} dissociates from the regulatory domain of isolated TnC 20–30 times faster than skeletal muscle relaxation and thus has been speculated to be too rapid to influence the muscle relaxation process.
Ca$^{2+}$ Binding and Exchange with TnC in the Presence of TnI

rate of relaxation (for review see Ref. 3). However, the antiparallel binding of TnI to TnC increases the Ca$^{2+}$ sensitivity of the N-domain of TnC to 10-fold and slows the Ca$^{2+}$ dissociation rate to 30-fold, with little additional change upon the formation of the whole Tn complex (Refs. 8 and 13–16; for review see Ref. 10). Thus, the rate of Ca$^{2+}$ dissociation from the Tn complex and not TnC alone may be the more meaningful rate when considering factors that control muscle relaxation kinetics. Consistent with this idea, exchanging a TnC mutant with an -2-fold slower N-terminal Ca$^{2+}$ dissociation rate into skeletal muscle fibers slowed the rate at which the fibers relaxed -2-fold (12). However, in the same study exchange of an -1.5-fold faster TnC mutant into muscle did not statistically increase the rate of relaxation. Clearly, a broader range of Ca$^{2+}$ dissociation rates from TnC mutants is required to further probe the role of TnC in tuning the rate of striated muscle relaxation.

To better understand the regulation of muscle mechanics, it is important to elucidate the Ca$^{2+}$-dependent interactions of TnC with TnI because TnC regulates muscle contraction as a part of the Tn complex and not in isolation. TnI residues 96–116 (TnI$_{96-116}$) bind actin and are primarily responsible for the ability of TnI to inhibit the ATPase activity of actomyosin, which can be reversed upon TnC-Ca$^{2+}$ binding to TnI$_{96-116}$ (17–19). In conjunction with residues 96–116, residues 117–148 of TnI are required for the complete inhibitory activity and regulatory interactions with actin and TnC (20–23). Furthermore, the complete enhancement of the Ca$^{2+}$ sensitivity and the slowing of the Ca$^{2+}$ dissociation rate from the regulatory domain of TnC in the presence of intact TnI were mimicked by a peptide of TnI corresponding to residues 96–148 (TnI$_{96-148}$) (8). Thus, the Ca$^{2+}$-dependent binding of the regulatory domain of TnC to TnI$_{96-148}$ may be a good model system to study the Ca$^{2+}$-dependent interactions between TnI and TnC that regulate muscle mechanics.

Recently, we investigated the effect of hydrophobic residue substitutions on the Ca$^{2+}$ binding properties of the regulatory domain of TnC with the Phetrp mutation (TnC$_{Phetrp}$). The global N-terminal Ca$^{2+}$ affinities of the TnC$_{Phetrp}$ mutants varied 2340-fold, whereas the Ca$^{2+}$ association and dissociation rates varied less than 70-fold and more than 45-fold, respectively (6). In the present study we have therefore determined how these mutations affect the Ca$^{2+}$ binding properties and dynamics of the TnC$_{Phetrp}$-TnI$_{96-116}$ complex and located hydrophobic residues essential for high affinity binding of TnI$_{96-116}$. Furthermore, we have tested whether the TnC$_{Phetrp}$-TnI$_{96-116}$ complex is a better predictor than isolated TnC$_{Phetrp}$ for the Ca$^{2+}$ binding properties of the Tn complex in muscle and the potential for a particular TnC$_{Phetrp}$ mutant to support force production.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phenyl-Sepharose CL-4B and EGTA were purchased from Sigma. Quin-2 was purchased from Calbiochem (La Jolla, CA). All other chemicals were of analytical grade. The TnL$_{96-148}$ peptide was synthesized and purified by the Alberta Peptide Institute (Edmonton, Canada).

**Protein Mutagenesis and Purification**—The construction and expression of intact chicken skeletal TnC$_{Phetrp}$ in pET3a has been described (24). Chicken skeletal fast TnI was prepared as described for the rabbit protein (25). The TnC$_{Phetrp}$ mutants were constructed from the TnC$_{Phetrp}$ plasmid by primer based site-directed mutagenesis using a Stratagene QuickChange site-directed mutagenesis kit. The mutations were confirmed by DNA sequence analysis. The plasmids for TnC$_{Phetrp}$ and its mutants were transformed into E. coli BL21(DE3)pLysS cells (Novagen) and purified as described previously (6). Aliquots of TnC$_{Phetrp}$ and B29TnC$_{Phetrp}$ were labeled with the Cys-specific fluorescent probe 5-(iodoacetamido)-2-naphthalenesulfonyl chloride (IAEDANS) at position Cys$^{161}$ for the myofibril studies. Each TnC was reacted with 3–5-fold molar excess of IAEDANS for 6 h at room temperature with constant shaking in 50 mM Tris, 90 mM KCl, 1 mM EGTA, 6 mM urea, pH 7.5. The labeling reaction was stopped by the addition of 2 mM DTT, and the labeled proteins were exhaustively dialyzed against 10 mM MOPS, 90 mM KCl, pH 7.0, at 4 °C to remove unreacted label.

**Determination of Ca$^{2+}$ Affinities**—All state of fluorescence measurements were performed using a Perkin-Elmer LS5 Spectrofluorimeter at 15 °C. Trp fluorescence was excited at 275 nm and monitored at 345 nm as microliter amounts of CaCl$_2$ were added to 1 ml of each TnC$_{Phetrp}$ mutant (0.3 mM plus TnI$_{96-116}$ (3 mM) in 200 mM MOPS (to prevent pH changes upon the addition of metal), 90 mM KCl, 2 mM EGTA, 1 mM DTT, pH 7.0, at 15 °C. The [Ca$^{2+}$]$_{free}$ was calculated using the program ECGA02 developed by Robertson and Potter (26). The Ca$^{2+}$ affinities are reported as dissociation constants (K$_{d}$). Each K$_{d}$ represents the mean of 3–5 titrations fit with a logistic sigmoid function and is expressed mathematically equivalent to the Hill equation, as previously described (6).

**Determination of Tn$_{96-148}$ Peptide Affinities**—Trp fluorescence was monitored as described in the previous paragraph. Microliter amounts of TnL$_{96-148}$ were added to 1 ml of each TnC$_{Phetrp}$ mutant (0.6 mM) in 200 mM MOPS, 90 mM KCl, 2 mM EGTA, 1 mM [Ca$^{2+}$]$_{free}$, 1 mM DTT, pH 7.0, at 15 °C. Each peptide affinity, reported as a dissociation constant, represents the mean of three to five titrations fit to the root of a quadratic equation for binary complex formation as previously described (27).

**Calculation of Ca$^{2+}$ Association Rates**—The Ca$^{2+}$ association rates (k$_{on}$) were calculated using the simple relationship k$_{on}$ = k$_{off}$/(K$_{d}$), where k$_{off}$ represents the concerted release of two Ca$^{2+}$ ions, and K$_{d}$ represents the binding event of two Ca$^{2+}$ ions to the N-domain of TnC in the presence of TnI$_{96-148}$, as previously described (8).

**Muscle Fiber Experiments**—Single fibers were isolated the day of use from bundles of rabbit psoas muscle that had been stored in a glycerinating solution at -20 °C for no longer than 1 month. Solutions and the mechanical setup utilized for force measurements were as previously described (28). Briefly, a single fiber was soaked in relaxing solution containing 1% (v/v) Triton X-100 for 5 min to remove any residual sarclemma and sarcoplastic reticulum. The fiber was then tied down in troughs attached to a servo-controlled DC torque motor (Cambridge Technologies, Watertown, MA) and an isolating solution containing 1% (v/v) Triton X-100 for 5 min to remove any residual sarclemma and sarcoplastic reticulum. The [Ca$^{2+}$]$_{free}$ was also measured using the fluorescent Ca$^{2+}$-chelator Quin-2 (6, 8). Quin-2 was excited at 330 nm with its emission monitored through a 510-nm broad band pass interference filter (Oriel, Stratford, CT). The buffer used in all stopped flow experiments was 10 mM MOPS, 90 mM KCl, 1 mM DTT, pH 7.0.

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fibrils were prepared and stored as previously described (31). Endoge-
nous TnC was extracted from a sample of the stock myofibrils by first 
washing the myofibrils three times in a myofibril TnC extraction solu-
tion (10 mM MOPS, 90 mM KCl, 5 mM EDTA, 2 mM DTT, 0.02% Tween 
20, pH 8.0) to remove any residual glycerol. The myofibrils were then 
soaked in the TnC extraction solution for approximately 10 min at room 
temperature, pelleted, and resuspended in fresh TnC extraction solu-
tion an additional three times. The TnC extracted myofibrils were then 
extracted myofibrils in the 
the myofibrils was determined (31). 0.1 mg/ml aliquots of the TnC 

isolation but as part of the Tn complex, primarily interacting 
characterized (6). However, TnC does not function in muscle in 
residues that were individually mutated 
D). The 
the various helices (N 

FIG. 1. Cartoon representation of 
the regulatory domain of TnCF29W. 
The cartoon depicts the amino acids in the 
regulatory domain of TnCF29W that form 
the two Ca$^{2+}$-binding sites I and II) and 
the various helices (N–D). The black 
amino acids represent the hydrophobic 
residues that were individually mutated to Gln, excluding Trp$^{29}$.

spectra of the other TnC$^{29W}$ mutants were similar to TnC$^{29W}$ 
(data not shown) except for I37QTnC$^{29W}$. The addition of 
TnI$_{96-148}$ to apo I37QTnC$^{29W}$ increased the Trp fluorescence 
$\sim$1.3-fold, which subsequently decreased $\sim$1.3-fold upon the 
addition of 1 mM [Ca$^{2+}$]$_{free}$ at 345 nm with a similar blue shift in 
the maximum fluorescence as observed with TnC$^{29W}$. The 
reason for this atypical behavior of I37QTnC$^{29W}$ is currently 
unknown. Ile$^{37}$ is part of the first Ca$^{2+}$-binding loop located in 
the middle of the small $\beta$-sheet connecting the two N-terminal 
EF hands. Thus, Ile$^{37}$ may be critical for proper Ca$^{2+}$ 
binding and coordination of subsequent structural changes.

Measurement of Ca$^{2+}$-Binding Affinities for the N-terminal 
Domains of TnC$^{29W}$ and Mutants in the Presence of TnI$_{96-148}$ 
at 15 °C—The Ca$^{2+}$-binding affinities ($K_d$) for TnC$^{29W}$ and 
each hydrophobic mutant were measured by following the 
Ca$^{2+}$-induced changes in Trp fluorescence in the presence of TnI$_{96-148}$. 
Examples of the Ca$^{2+}$-dependent increases in N-
terminal TnC$^{29W}$ and mutant TnC fluorescence are shown in 
Fig. 3 for L49QTnC$^{29W}$ (1), TnC$^{29W}$ (1), M81QTnC$^{29W}$ (1), 
I73QTnC$^{29W}$ (1), and F26QTnC$^{29W}$ (1). Table I summarizes 
the Ca$^{2+}$-binding data for these and the remaining TnC$^{29W}$ 
mutants. In the presence of TnI$_{96-148}$, TnC$^{29W}$ exhibited a 
half-maximal increase in its Trp fluorescence upon the addition of 
Ca$^{2+}$ at 267 ± 3 nM. The Ca$^{2+}$ affinities for the mutants 
ranged from 70 ± 1 nM for L49QTnC$^{29W}$ to 17 ± 3 μM for 
F26QTnC$^{29W}$. Therefore, substitution of hydrophobic residues 
with polar Gln produced N-domain TnC$^{29W}$ mutants that 
exhibited 243-fold variation in their Ca$^{2+}$ affinities in the presence 
of TnI$_{96-148}$. The Hill coefficients for all but two of the 
TnC$^{29W}$.TnI$_{96-148}$ mutant complexes (F26QTnC$^{29W}$ and 
I37QTnC$^{29W}$) were between 1.6 and 2.8 (see Table I), implying 
cooperative binding of Ca$^{2+}$ and TnI$_{96-148}$ to TnC$^{29W}$ and 
its mutants.

Similar to the binding of TnI to TnC, the binding of TnI$_{96-148}$ 
to TnC$^{29W}$ increases the Ca$^{2+}$ sensitivity of the regulatory 
do mains of TnC$^{29W}$—12-fold (Table I and Refs. 8 and 13–15). 
On average, the Ca$^{2+}$ sensitivity of the TnC$^{29W}$ mutants 
increased $\sim$11-fold (Table I). However, the Ca$^{2+}$ sensitivities of 
F26QTnC$^{29W}$, V80QTnC$^{29W}$, and M81QTnC$^{29W}$ increased 
$\geq$23-fold, whereas for F22QTnC$^{29W}$, L42QTnC$^{29W}$,
V45QTnCP29W, M46QTnCP29W, L49QTnCP29W, I61QTnCP29W, F78QTnCP29W, and M82QTnCP29W, the Ca$^{2+}$ sensitivities increased 5.5-fold when compared with the isolated TnCP29W mutant (Table I and Ref. 6). Thus, the hydrophobic to Gln mutations in TnCP29W not only affect the overall Ca$^{2+}$ sensitivity of the TnCP29W-TnI96–148 complex but also modulate the effectiveness of TnI96–148 to increase the Ca$^{2+}$ affinity of the regulatory domain of TnCP29W.

**Fig. 2. Effect of Ca$^{2+}$ on the fluorescence spectra of TnCP29W and I37QTnCP29W in the presence of TnI96–148.** Fluorescence emission spectra for TnCP29W (A) or I37QTnCP29W (B) are shown in the apo (solid lines), apo + TnI96–148 (dashed lines), and Ca$^{2+}$ + TnI96–148 (dotted lines) states. The Trp fluorescence spectra were recorded with an excitation wavelength of 275 nm in 200 mM MOPS, 90 mM KCl, 2 mM EGTA, 1 mM DTT, pH 7.0, at 15°C. The concentrations of the TnCP29W proteins, TnI96–148 peptide and [Ca$^{2+}$]free were 0.3 μM, 3 μM, and 1 mM, respectively.

Measurement of TnI96–148 Binding Affinities for the Ca$^{2+}$-saturated N-terminal Domains of TnCP29W and Mutants at 15°C—The binding of TnI96–148 to Ca$^{2+}$-saturated TnCP29W and its mutants caused on average a 26.6 ± 0.4% decrease in the Trp fluorescence (excluding I37QTnCP29W, which displayed a 23 ± 1% increase), which can be utilized to determine the peptide binding affinity (8, 20). Examples of the TnI96–148-dependent decrease in N-terminal TnCP29W and mutant Trp flu-
**Fig. 3.** Ca\(^{2+}\) binding to TnC\(^{\text{F29W}}\) and its mutants in the presence of TnI\(_{96-148}\). The Ca\(^{2+}\)-dependent increases in Trp fluorescence are shown for L49QTnC\(^{\text{F29W}}\) ( ), TnC\(^{\text{F29W}}\) ( ▲), M81QTnC\(^{\text{F29W}}\) ( □), I73QTnC\(^{\text{F29W}}\) ( △), and F26QTnC\(^{\text{F29W}}\) ( ○) as a function of \(-\log[\text{Ca}^{2+}]\). Microliter amounts of Ca\(^{2+}\) were added to 1 ml of each protein (0.3 μM) plus TnI\(_{96-148}\) (3 μM) in the same buffer and temperature as described in the legend of Fig. 2. Trp fluorescence emission was monitored at 345 nm with excitation at 275 nm. 0% Trp fluorescence corresponds to the apo state fluorescence, whereas 100% Trp fluorescence corresponds to the highest fluorescent state in the presence of Ca\(^{2+}\) for each individual TnC\(^{\text{F29W}}\) protein. Each data point represents the mean ± S.E. of three to five titrations fit with a logistic sigmoid equation.

**Table I**

| Mutant protein | Ca\(^{2+}\) | Hill coefficient | ΔCa\(^{2+}\) sensitivity | \(k_{d}\) | \(k_{a} \times 10^{7}\) | Peptide | K_d |
|---------------|------------|-----------------|--------------------------|--------|-----------------|--------|-----|
| TnC\(^{\text{F29W}}\) | 267 ± 3 | 2.76 ± 0.07 | 12.3 | 0.16 ± 0.02 | 4.3 | 146 ± 19 |
| M3QTnC\(^{\text{F29W}}\) | 312 ± 6 | 2.13 ± 0.07 | 9.6 | 11.57 ± 0.06 | 3.7 | 188 ± 12 |
| F13QTnC\(^{\text{F29W}}\) | 211 ± 4 | 2.21 ± 0.09 | 11.8 | 7.66 ± 0.05 | 3.6 | 133 ± 34 |
| L14QTnC\(^{\text{F29W}}\) | 885 ± 34 | 2.3 ± 0.02 | 6.7 | 14.5 ± 0.1 | 1.6 | 155 ± 25 |
| M18QTnC\(^{\text{F29W}}\) | 296 ± 10 | 2.3 ± 0.02 | 6.6 | 8.9 ± 0.3 | 3.0 | 189 ± 24 |
| I19QTnC\(^{\text{F29W}}\) | 417 ± 4 | 2.21 ± 0.09 | 10.6 | 14.2 ± 0.4 | 3.4 | 186 ± 15 |
| F22QTnC\(^{\text{F29W}}\) | 321 ± 6 | 2.38 ± 0.09 | 3.1 | 12.4 ± 0.1 | 3.9 | 443 ± 37 |
| F28QTnC\(^{\text{F29W}}\) | 17000 ± 3000 | 1.0 ± 0.2 | 23.2 | 169 ± 4 | 1.0 | 1473 ± 83 |
| M28QTnC\(^{\text{F29W}}\) | 327 ± 5 | 1.96 ± 0.05 | 11.6 | 15.2 ± 0.1 | 4.7 | 194 ± 22 |
| L37QTnC\(^{\text{F29W}}\) | 6600 ± 950 | 0.39 ± 0.03 | 12.2 | 98 ± 0.6 | 1.5 | 326 ± 48 |
| L42QTnC\(^{\text{F29W}}\) | 6284 ± 240 | 2.0 ± 0.1 | 1.0 | 15.9 ± 0.1 | 0.3 | 221 ± 19 |
| V45QTnC\(^{\text{F29W}}\) | 87 ± 1 | 2.16 ± 0.06 | 2.0 | 8.3 ± 0.2 | 9.5 | 259 ± 57 |
| M48QTnC\(^{\text{F29W}}\) | 288 ± 7 | 1.81 ± 0.07 | 3.1 | 12.6 ± 0.2 | 4.4 | 315 ± 11 |
| M49QTnC\(^{\text{F29W}}\) | 246 ± 7 | 2.4 ± 0.1 | 13.0 | 10.7 ± 0.1 | 4.3 | 183 ± 28 |
| L49QTnC\(^{\text{F29W}}\) | 70 ± 1 | 2.10 ± 0.07 | 2.4 | 8.01 ± 0.02 | 11.4 | 241 ± 10 |
| L58QTnC\(^{\text{F29W}}\) | 441 ± 15 | 2.0 ± 0.1 | 9.1 | 11.9 ± 0.1 | 2.7 | 204 ± 15 |
| I61QTnC\(^{\text{F29W}}\) | 495 ± 16 | 2.5 ± 0.2 | 5.2 | 22 ± 0.1 | 4.5 | 268 ± 13 |
| H62QTnC\(^{\text{F29W}}\) | 2628 ± 47 | 1.59 ± 0.04 | 14.2 | 102 ± 2 | 3.9 | 2075 ± 116 |
| V65QTnC\(^{\text{F29W}}\) | 592 ± 17 | 2.4 ± 0.2 | 13.5 | 10.2 ± 0.2 | 1.7 | 268 ± 23 |
| I73QTnC\(^{\text{F29W}}\) | 1824 ± 31 | 2.42 ± 0.09 | 12.5 | 29.3 ± 0.2 | 1.6 | 147 ± 10 |
| F75QTnC\(^{\text{F29W}}\) | 598 ± 16 | 1.61 ± 0.06 | 7.4 | 24.9 ± 0.5 | 4.2 | 230 ± 18 |
| F78QTnC\(^{\text{F29W}}\) | 5084 ± 97 | 2.5 ± 0.1 | 5.3 | 16.1 ± 0.1 | 0.3 | 128 ± 22 |
| L79QTnC\(^{\text{F29W}}\) | 6549 ± 20 | 2.4 ± 0.2 | 14.9 | 14.0 ± 0.1 | 2.1 | 234 ± 41 |
| V80QTnC\(^{\text{F29W}}\) | 788 ± 13 | 2.24 ± 0.08 | 27.9 | 16.1 ± 0.2 | 2.0 | 149 ± 20 |
| M81QTnC\(^{\text{F29W}}\) | 335 ± 10 | 2.4 ± 0.2 | 56.7 | 5.15 ± 0.02 | 1.5 | 256 ± 13 |
| M82QTnC\(^{\text{F29W}}\) | 149 ± 1 | 2.07 ± 0.04 | 4.6 | 5.8 ± 0.1 | 3.9 | 382 ± 17 |
| V83QTnC\(^{\text{F29W}}\) | 517 ± 19 | 2.4 ± 0.2 | 7.2 | 15.4 ± 0.3 | 3.0 | 210 ± 25 |
| M88QTnC\(^{\text{F29W}}\) | 420 ± 10 | 2.4 ± 0.1 | 6.2 | 13.4 ± 0.1 | 3.2 | 294 ± 34 |

\(\Delta\text{Ca}^{2+}\) sensitivity is the quotient of the Ca\(^{2+}\) affinity of the protein in isolation (6) divided by the Ca\(^{2+}\) affinity of the protein in the presence of TnI\(_{96-148}\).
dependent binding of TnC to Ca$^{2+}$-saturated TnCF$_{29W}$ and its mutants. The Tn$_{96,-148}$-dependent decreases in Trp fluorescence are shown for Ca$^{2+}$-saturated L49QTnC$_{29W}$ ( ), TnC$_{29W}$ ( ), M81QTnC$_{29W}$ ( ), I73QTNc$_{29W}$ ( ), and F26QTnC$_{29W}$ ( ) as a function of Tn$_{96,-148}$ concentration. Microtiter amounts of Tn$_{96,-148}$ were added to each TnC (0.6 μM) in the same buffer and temperature as in Fig. 2 in the presence of 1 mM [Ca$^{2+}$]$_{in}$ (or 10 mM [Ca$^{2+}$]$_{in}$ in the case of F26QTnC$_{29W}$). 100% Trp fluorescence corresponds to the Ca$^{2+}$-saturated state, whereas 0% represents the Ca$^{2+}$-Tn$_{96,-148}$-saturated state for each individual TnC$_{29W}$ protein. Each data point represents the mean ± S.E. of three to five titrations fit to the root of a quadratic equation for binary complex formation.

M81QTnC$_{29W}$ (5 s$^{-1}$), L49QTnC$_{29W}$ (8 s$^{-1}$), TnC$_{29W}$ (11 s$^{-1}$), I73QTNc$_{29W}$ (29 s$^{-1}$), and F26QTnC$_{29W}$ (169 s$^{-1}$) complexed with Tn$_{96,-148}$. The rates of Ca$^{2+}$ dissociation for the remaining mutant complexes fell between that of M81QTnC$_{29W}$ and that of F26QTnC$_{29W}$ (Table I). Therefore, substitution of hydrophobic residues with polar Gln in the regulatory domain of TnC$_{29W}$ increased (~16-fold) and decreased (~2-fold) the Ca$^{2+}$ dissociation rate from the TnC$_{29W}$-Tn$_{96,-148}$ complex, creating an ~33-fold variation. To verify that the time course of the EGTA-induced Trp fluorescence decreases for the TnC$_{29W}$-Tn$_{96,-148}$ complexes followed Ca$^{2+}$ dissociation and not a slower structural change, Ca$^{2+}$ dissociation was also measured using the fluorescent Ca$^{2+}$ chelator Quin-2. Whereas the fluorescence of Trp was selective for the events of N-terminal Ca$^{2+}$ dissociation, Quin-2 fluorescence reported Ca$^{2+}$ dissociation from both the N- and C-domains of TnC$_{29W}$ and its mutants complexed with Tn$_{96,-148}$. However, the Ca$^{2+}$ dissociation rates from the N-terminal domain of TnC$_{29W}$ and its mutants were easily distinguished from the rates of Ca$^{2+}$ dissociation from the C-terminal domain (on average 0.159 ± 0.007 s$^{-1}$) because the latter rates were ~30-fold slower in the presence of Tn$_{96,-148}$ or intact TnC. Fig. 5B demonstrates that for all of the mutants, the Ca$^{2+}$ dissociation rate reported by Trp was in excellent agreement with the N-terminal rate determined by Quin-2. Therefore, the fluorescent Trp signal accurately reports Ca$^{2+}$ binding and dissociation from the TnC$_{29W}$-Tn$_{96,-148}$ mutant complexes.

To verify that Tn$_{96,-148}$ is a satisfactory model system for the regulatory domain binding of TnC to TnI, stopped flow studies were also conducted with intact chicken skeletal TnI. Fig. 5C shows the time course of the increases in Quin-2 fluorescence as Ca$^{2+}$ was dissociated from the N-terminal domains of M81QTnC$_{29W}$ (4 s$^{-1}$), L49QTnC$_{29W}$ (5 s$^{-1}$), TnC$_{29W}$ (9 s$^{-1}$), I73QTNc$_{29W}$ (22 s$^{-1}$), and F26QTnC$_{29W}$ (135 s$^{-1}$) complexed with intact TnI. The Ca$^{2+}$ dissociation rates measured from the regulatory domain of TnC$_{29W}$ and its mutants in the presence of Tn$_{96,-148}$ were similar to that measured in the presence of intact TnI. Therefore, the TnC$_{29W}$, Tn$_{96,-148}$ complex is a good model system to study the regulatory mechanisms of the Ca$^{2+}$-dependent binding of TnC to TnI.

Calculation of Ca$^{2+}$ Association Rates—The Ca$^{2+}$ association rates to TnC$_{29W}$ and its mutants in the presence of Tn$_{96,-148}$ were calculated using the Ca$^{2+}$ $K_a$ and $k_{off}$ values determined by Trp ($k_{on} = k_{off}/K_a$; Table I). The calculated $k_{on}$ for TnC$_{29W}$ in the presence of Tn$_{96,-148}$ was ~4.5 × 10$^{6}$ M$^{-1}$ s$^{-1}$, which was ~2.4-fold slower than the $k_{on}$ calculated or measured in the absence of the peptide (6, 8). In the presence of Tn$_{96,-148}$, $k_{on}$ varied ~38-fold between the TnC$_{29W}$ mutants with L49QTnC$_{29W}$ (~1.1 × 10$^{6}$ M$^{-1}$ s$^{-1}$) exhibiting the fastest $k_{on}$ and L42QTnC$_{29W}$ (~3 × 10$^{5}$ M$^{-1}$ s$^{-1}$) exhibiting the slowest $k_{on}$. Clearly, some of the hydrophobic mutations in the regulatory domain of TnC$_{29W}$ significantly alter the Ca$^{2+}$ association rate in the presence of Tn$_{96,-148}$.

Ca$^{2+}$ Binding to TnC$_{29W}$ in the Presence of Tn$_{96,-148}$ as a Predictor for the Ca$^{2+}$ Dependence of Force Development in Skeletal Muscle—Fig. 6A shows that V45QTnC$_{29W}$ and M46QTnC$_{29W}$ possess ~19- and 6.6-fold higher Ca$^{2+}$ affinity than TnC$_{29W}$ in the absence of Tn$_{96,-148}$, respectively (Table II and Ref. 6). On the other hand, M81QTnC$_{29W}$ and F78QTnC$_{29W}$ display ~6-fold higher Ca$^{2+}$ affinity than TnC$_{29W}$ in the absence of Tn$_{96,-148}$, respectively (Table II and Ref. 6). However, Fig. 6B shows that only the Ca$^{2+}$ sensitivities of V45QTnC$_{29W}$ and F78QTnC$_{29W}$ in the presence of the Ca$^{2+}$-saturated TnC$_{29W}$ mutants complexed with Tn$_{96,-148}$, were not the same. To test which TnC$_{29W}$ system (with or without Tn$_{96,-148}$) better represents the Ca$^{2+}$ sensitivity of force production in muscle, the endogenous TnC in psoas muscle fibers was extracted and then replaced with TnC$_{29W}$ or its mutants, and force versus pCa was measured. After TnC extraction, the average force generated by the single skinned muscle fibers was 2.3 ± 0.5% of the maximal force (data not shown). Subsequent reconstitution of the muscle fibers with V45QTnC$_{29W}$, M46QTnC$_{29W}$, TnC$_{29W}$, M81QTnC$_{29W}$, or F78QTnC$_{29W}$ recovered 82 ± 5, 73 ± 4, 90 ± 3, 65 ± 8, and 80 ± 2% of the maximal force at pCa 4, respectively. Fig. 6C demonstrates that the Ca$^{2+}$ dependence of force generation with TnC$_{29W}$ or its mutants followed qualitatively more closely to the Ca$^{2+}$ sensitivities of the mutant TnC$_{29W}$, Tn$_{96,-148}$ complexes and not to that of the isolated TnC$_{29W}$ proteins (see also Table II). Thus, the qualitative and quantitative changes in N-terminal Ca$^{2+}$ sensitivities for several of the TnC$_{29W}$ mutants compared with TnC$_{29W}$ in the presence of Tn$_{96,-148}$ were not the same.
Fig. 5. Rates of Ca\(^{2+}\) dissociation from TnC\(^{F29W}\) and its mutants in the presence of TnI\(_{96-148}\) or intact TnI. A shows the time course of decrease in Trp fluorescence as Ca\(^{2+}\) was removed by EGTA from the regulatory Ca\(^{2+}\)-binding sites of M81QTnC\(^{F29W}\), L49QTnC\(^{F29W}\), TnC\(^{F29W}\), I73QTnC\(^{F29W}\), and F26QTnC\(^{F29W}\) in the presence of TnI\(_{96-148}\). Each TnC\(^{F29W}\) protein (0.6 \(\mu\)M) plus TnI\(_{96-148}\) (6 \(\mu\)M) in 10 mm MOPS, 90 mm KCl, 1 mm DTT, pH 7.0, plus 100 \(\mu\)M Ca\(^{2+}\) was rapidly mixed with an equal volume of the same buffer plus 10 mm EGTA at 15 °C. Trp fluorescence was monitored through a UV-transmitting black glass filter (UG1 from Oriel) with excitation at 275 nm. B shows the time course of increase in Quin-2 fluorescence as Ca\(^{2+}\) was removed by Quin-2 from the regulatory Ca\(^{2+}\)-binding sites of M81QTnC\(^{F29W}\), L49QTnC\(^{F29W}\), TnC\(^{F29W}\), I73QTnC\(^{F29W}\), and F26QTnC\(^{F29W}\) in the presence of TnI\(_{96-148}\). Each TnC\(^{F29W}\) protein (3 \(\mu\)M) plus TnI\(_{96-148}\) (30 \(\mu\)M) in 10 mm MOPS, 90 mm KCl, 1 mm DTT, pH 7.0, plus 30 \(\mu\)M Ca\(^{2+}\) was rapidly mixed with an equal volume of the same buffer plus 150 \(\mu\)M Quin-2 at 15 °C. Quin-2 fluorescence was monitored through a 510-nm broad band pass interference filter with excitation at 330 nm. C shows the time course of increase in Quin-2 fluorescence as Ca\(^{2+}\) was removed by Quin-2 from the regulatory Ca\(^{2+}\)-binding sites of M81QTnC\(^{F29W}\), L49QTnC\(^{F29W}\), TnC\(^{F29W}\), I73QTnC\(^{F29W}\), and F26QTnC\(^{F29W}\) in the presence of intact TnI. Each TnC\(^{F29W}\) protein (3 \(\mu\)M) plus intact TnI (30 \(\mu\)M) in 10 mm MOPS, 90 mm KCl, 1 mm DTT, pH 7.0, plus 30 \(\mu\)M Ca\(^{2+}\) was rapidly mixed with an equal volume of the same buffer plus 150 \(\mu\)M Quin-2 at 15 °C. All of the traces have been staggered and normalized for clarity. Each trace is an average of at least 15 traces fit with a single exponential equation (variance < 2 \(\times\) 10\(^{-4}\)). The kinetic traces were triggered at time 0 with the first ~2 ms of premixing time shown (the apparent lag phase). The traces were fit after the mixing time was complete.
Fig. 6. Comparison of the Ca\(^{2+}\) binding properties of TnC and mutants in the absence and presence of TnI\(_{96-148}\) with their Ca\(^{2+}\) dependence of skeletal muscle force generation. A shows the Ca\(^{2+}\)-dependent increases in Trp fluorescence for V45Q TnC\(_{F29W}\) (●), M46Q TnC\(_{F29W}\) (■), TnC\(_{F29W}\) (▲), M81Q TnC\(_{F29W}\) (□), and F78Q TnC\(_{F29W}\) (○) as a function of -Log[Ca\(^{2+}\)]. The data have been adapted from Tikunova et al. (6). Because these data were collected under identical experimental conditions as were used here, we have for comparative purposes reproduced these data in this figure and Table II. Microliter amounts of Ca\(^{2+}\) were added to 1 ml of each protein (0.3 μM) in the same buffer and temperature as described in the legend to Fig. 2. Trp fluorescence was monitored as described in the legend to Fig. 3. The data sets were normalized individually for each mutant. B shows the Ca\(^{2+}\)-dependent increases in Trp fluorescence in the presence of TnI\(_{96-148}\) for V45Q TnC\(_{F29W}\) (●), M46Q TnC\(_{F29W}\) (■), TnC\(_{F29W}\) (▲), M81Q TnC\(_{F29W}\) (□), and F78Q TnC\(_{F29W}\) (○) as a function of -Log[Ca\(^{2+}\)]. The experimental details are identical to those described in the legend to Fig. 3. C shows the Ca\(^{2+}\) dependence of isometric force generation in single skinned psoas fibers reconstituted with V45Q TnC\(_{F29W}\) (●), M46Q TnC\(_{F29W}\) (■), TnC\(_{F29W}\) (▲), M81Q TnC\(_{F29W}\) (□), and F78Q TnC\(_{F29W}\) (○) as a function of -Log[Ca\(^{2+}\)]. The experimental conditions are described under “Experimental Procedures.” Each data point represents the mean ± S.E. from at least three separate fibers individually normalized and fit with a logistic sigmoid equation mathematically equivalent to the Hill equation.
Another effect observed in the reconstituted muscle, which could not be predicted by the Ca²⁺-binding properties of the isolated TnC, was the maximal amount of force recovered by a particular mutant. Fig. 7A shows the Ca²⁺-dependent increases in force recovered by TnCF<sub>29W</sub> (▲), L42QTnCF<sub>29W</sub>(●), I73QTnCF<sub>29W</sub>(●), and I62QTnCF<sub>29W</sub>(●). At pCa 4.0, TnCF<sub>29W</sub>, L42QTnCF<sub>29W</sub>, I73QTnCF<sub>29W</sub>, and I62QTnCF<sub>29W</sub> recovered 90 ± 3, 79 ± 3, 45 ± 6, and 12 ± 4% of the force generated by the endogenous TnC prior to extraction, respectively. Similar to I62QTnCF<sub>29W</sub>, I37QTnCF<sub>29W</sub> and F26QTnCF<sub>29W</sub> also recovered force poorly at 15 ± 1 and 13 ± 1% of the maximal amount of force generated by the fiber prior to endogenous TnC extraction, respectively (data not shown). Thus, the amount of maximal force sustained by the TnCF<sub>29W</sub> mutants was variable, with three of the mutants only marginally allowing any force production. As will be discussed, the binding of Tn<sub>96</sub> to the Ca²⁺-saturated TnCF<sub>29W</sub> mutants may offer clues as to why some of the mutants support little force.

To test whether I62QTnCF<sub>29W</sub> was actually binding to the thin filaments in the TnC-extracted muscle fibers, additional TnC exchange experiments were performed on the muscle. Fig. 7B at time 0 shows the maximal force recovered by TnCF<sub>29W</sub> at pCa 4.0 in a reconstituted muscle fiber. The fiber was then transferred to a relaxing solution containing 16.7 mM I62QTnCF<sub>29W</sub>, and the force generated at pCa 4.0 was measured at several time intervals. The amount of force production decreased with time, eventually reaching a value similar to that generated by fibers solely reconstituted with I62QTnCF<sub>29W</sub>. The data indicate that I62QTnCF<sub>29W</sub> was able to bind to the thin filaments and competitively displace TnCF<sub>29W</sub> from the Tn complex. Furthermore, when a muscle fiber was initially reconstituted with I62QTnCF<sub>29W</sub> (Fig. 7B, time 0, □) and then competitively displaced with 16.7 mM TnC in solution, maximal force increased with a time course similar to that at which I62QTnCF<sub>29W</sub> inhibited the force generated with TnCF<sub>29W</sub>. When the TnC-binding sites in the fiber were vacant (i.e. after endogenous TnC extraction), the addition of TnC at the concentration used for the competitive binding studies caused force to be maximal within 2 min (data not shown). Results similar to that of I62QTnCF<sub>29W</sub> were obtained in the displacement studies when I37QTnCF<sub>29W</sub> or F26QTnCF<sub>29W</sub> were tested (data not shown). Thus, the data supports the hypothesis that the TnCF<sub>29W</sub> mutants that minimally support force (<15%) bind to the thin filament and form the Tn complex.

To directly visualize whether I62QTnCF<sub>29W</sub> was able to incorporate into the TnC-depleted muscle fiber, both TnCF<sub>29W</sub> and I62QTnCF<sub>29W</sub> were labeled with the extrinsic fluorescent probe IAE-DANS and reconstituted into psosas myofibrils. Fig. 7C shows representative phase contrast images of TnCF<sub>29W</sub>, IAE-DANS (top left panel) and I62QTnCF<sub>29W</sub>-IAE-DANS (top right panel) reconstituted myofibrils. As can be seen from the fluorescent images (Fig. 7C, bottom left panel for TnCF<sub>29W</sub>, IAE-DANS and bottom right panel for I62QTnCF<sub>29W</sub>-IAE-DANS), both IAE-DANS-labeled TnC proteins incorporate into the myofibril at the myosin-actin filament overlap and non-overlap space. Thus, as predicted from the physiological competition experiments, I62QTnCF<sub>29W</sub> binds to the thin filament at a similar location, as does TnCF<sub>29W</sub>, and forms the Tn complex, albeit in an inactive state.

**DISCUSSION**

The goal of the present study was to examine the effect of the hydrophobic mutations on the Ca²⁺- binding properties of the TnCF<sub>29W</sub>, Tn<sub>96</sub>-<sub>148</sub> complex and on the affinity of TnCF<sub>29W</sub> for Tn<sub>96</sub>-<sub>148</sub>. Furthermore, we wanted to examine whether the effect of hydrophobic mutations on the Ca²⁺ sensitivity of force development could be better predicted by the Ca²⁺ and Tn<sub>96</sub>-<sub>148</sub> binding properties of the TnCF<sub>29W</sub> mutants than by that of the isolated TnCF<sub>29W</sub>. Because the regulatory domain of chicken TnC is spectroscopically silent, the Phe<sup>29</sup> → Trp mutation was utilized to follow the structural changes in the N-domain of TnC induced by changes in Ca²⁺ concentration (6, 8, 9, 12, 24, 33–35). In our previous study, all 27 Phe, Ile, Leu, Val, and Met residues were individually mutated to polar Gln to examine the role of hydrophobic residues in Ca²⁺ binding and exchange with the regulatory domain of intact TnCF<sub>29W</sub> in isolation (6). The hydrophobic TnCF<sub>29W</sub> mutants exhibited ~340-fold variation in their Ca²⁺ binding affinities. Indicative of the Ca²⁺ affinity changes, the hydrophobic TnCF<sub>29W</sub> mutants also exhibited less than 70-fold and more than 45-fold variation in their Ca²⁺ association rates and dissociation rates, respectively (6). The data indicated that the local side chain interactions of the hydrophobic residues within the tertiary structures of the apo and Ca²⁺-bound regulatory domain of TnCF<sub>29W</sub> played an important role in dictating the Ca²⁺ binding properties of the protein.

The Ca²⁺ affinities of the mutant TnCF<sub>29W</sub>–Tn<sub>96</sub>-<sub>148</sub> complexes varied −243-fold. However, the variation in the Ca²⁺ sensitivity of the mutants in the absence of Tn<sub>96</sub>-<sub>148</sub> was an order of magnitude larger (6). It would appear that Ca²⁺ binding is optimized when the regulatory domain of TnC is in the open state (helices B and C swing away from helices N, A, and D) (6). The binding of TnI or C-terminal peptides of TnI to the regulatory domain of TnC helps to lock TnC into the open state and thus enhance the Ca²⁺ binding affinity of TnC−<sub>10−12</sub>-fold (8, 13–15). The high Ca²⁺ affinity mutants of TnCF<sub>29W</sub> (F22QTnCF<sub>29W</sub>, V45QTnCF<sub>29W</sub>, M46QTnCF<sub>29W</sub>, L49QTnCF<sub>29W</sub>, and M82QTnCF<sub>29W</sub>) may mimic TnI binding to TnC by shifting the decreased variation in the Ca²⁺ binding affinities of the TnCF<sub>29W</sub>, Tn<sub>96</sub>-<sub>148</sub> mutant complexes.

On the other hand, hydrophobic mutations to polar Gln in the regulatory domain of TnCF<sub>29W</sub> that may impede the forma-

| Protein                | In isolation | + Tn<sub>96</sub>-<sub>148</sub> | In muscle |
|------------------------|--------------|-------------------------------|------------|
|                       | Ca<sup>2+</sup> K<sub>d</sub> | ΔCa<sup>2+</sup> sensitivity | Ca<sup>2+</sup> K<sub>d</sub> | ΔCa<sup>2+</sup> sensitivity | Hill coefficient |
| V45QTnCF<sub>29W</sub> | 0.17 ± 0.02  | ↑ 19                          | 87 ± 1     | ↑ 3.1                       | 253 ± 31          | ↑ 2.3 | 1.9 ± 0.1 |
| M46QTnCF<sub>29W</sub> | 0.88 ± 0.06  | ↑ 7.6                         | 288 ± 7    | ↑ 11.1                      | 625 ± 50          | ↑ 1.1 | 2.5 ± 0.1 |
| TnCF<sub>29W</sub>    | 3.2 ± 0.2    | 1.0                           | 267 ± 3    | 1.0                        | 568 ± 40          | 1.0   | 2.4 ± 0.2 |
| M81QTnCF<sub>29W</sub> | 19 ± 4       | ↑ 5.9                         | 335 ± 10   | ↑ 1.3                       | 996 ± 52          | ↑ 1.8 | 2.5 ± 0.2 |
| F78QTnCF<sub>29W</sub> | 27 ± 4       | ↑ 8.4                         | 5084 ± 97  | ↑ 19                       | 2405 ± 190        | ↓ 4.2 | 2.6 ± 0.1 |

**TABLE II**

Comparison of the Ca²⁺ binding properties of TnCF<sub>29W</sub> and mutants in the absence and presence of Tn<sub>96</sub>-<sub>148</sub> with their Ca²⁺ dependence of skeletal muscle force generation.
Fig. 7. Comparison of TnC$^{F29W}$ and mutants with varied maximal force recoveries in TnC reconstituted muscle fibers and myofibrils. A shows the Ca$^{2+}$ dependence of isometric force generation in single skinned psoas fibers reconstituted with TnC$^{F29W}$ ( ), L42QTnC$^{F29W}$ ( ), I62QTnC$^{F29W}$ ( ), 173QTnC$^{F29W}$ ( ), and 162QTnC$^{F29W}$ ( ) as a function of $-\log$([Ca$^{2+}$]). The experimental conditions are described under “Experimental Procedures.” Each data point represents the mean ± S.E. from at least three separate fibers fit with a logistic sigmoid equation mathematically equivalent to the Hill equation. Information regarding the parameters of the fit for TnC$^{F29W}$ can be found in Table II. L42QTnC$^{F29W}$ displayed half-maximal isometric force at 1.3 ± 0.2 μM Ca$^{2+}$ with a Hill coefficient of 2.1 ± 0.2. L42QTnC$^{F29W}$ displayed half-maximal isometric force at 3.2 ± 0.4 μM Ca$^{2+}$ with a Hill coefficient of 1.0 ± 0.1. 162QTnC$^{F29W}$ displayed half-maximal isometric force at 1.6 ± 0.1 μM Ca$^{2+}$ with a Hill coefficient of 1.2 ± 0.1. B, the open squares show the time course decay of maximal isometric force as 162QTnC$^{F29W}$ displaces TnC$^{F29W}$ from single skinned psoas fibers (t$_{1/2}$ = 3.3 ± 0.4 min). Time 0 represents the maximal isometric force generated in the pCa 4.0 solution for TnC$^{F29W}$ reconstituted muscle fibers. Subsequently the fibers were soaked in a resting solution containing 16.7 μM 162QTnC$^{F29W}$ and periodically contracted in a pCa 4.0 solution every 3 min for the first 12 min then every 5 min thereafter. The closed squares show the time course increase of maximal isometric force as TnC displaces 162QTnC$^{F29W}$ from single skinned psoas fibers (t$_{1/2}$ = 2.2 ± 0.1 min). Time 0 represents the maximal isometric force generated in the pCa 4.0 solution for 162QTnC$^{F29W}$ reconstituted muscle fibers. Subsequently the fibers were soaked in a resting solution containing 16.7 μM TnC and periodically contracted in a pCa 4.0 solution every 3 min for the first 12 min then every 5 min thereafter. Each data point represents the mean ± S.E. from three separate fibers fit with a single exponential equation. C shows the phase contrast (top panels) and IAE-DANS fluorescence (bottom panels) images obtained from TnC extracted rabbit psoas myofibrils reconstituted with TnC$^{F29W}$, IAE-DANS (left panels) or 162QTnC$^{F29W}$, IAE-DANS (right panels). The vertical line designates the location of the Z lines, and the horizontal lines designate the locations of the A bands (actin-myosin filament overlap).
side chain interaction. Consistent with this idea, analysis of the NMR structure of the Ca\textsuperscript{2+}/H\textsubscript{11001}-TnC-TnI\textsubscript{115–131} complex (36) or a modeled structure of TnC-TnI (37) indicates that there are nine different hydrophobic residue side chains within the N-domain of TnC that come within 4 Å of six different hydrophobic side chains within TnI\textsubscript{115–131}. All of the high Ca\textsuperscript{2+} affinity mutant hydrophobic residue side chains (Phe\textsubscript{22}, Val\textsubscript{45}, Met\textsubscript{46}, Leu\textsubscript{49}, and Met\textsubscript{82}) come in close contact to TnI\textsubscript{115–131} and modestly decrease TnI\textsubscript{96–148} binding \textsuperscript{1.7–3}-fold. However, as mentioned above, neither Phe\textsubscript{26} nor Ile\textsubscript{62} come in close contact with TnI but interact with the Ca\textsuperscript{2+}-binding loop \beta-sheet residues Ile\textsubscript{37} and Ile\textsubscript{62} (Fig. 8). Thus, Phe\textsubscript{26} and Ile\textsubscript{62} may help maintain the open state in such a way as to allow high affinity TnI\textsubscript{96–148} binding to the regulatory domain of TnC F\textsubscript{29W}. Consistent with this idea, inhibiting the Ca\textsuperscript{2+}-dependent opening of the regulatory domain of TnC by the introduction of a disulfide bond between the NAD and BC units decreased the affinity of TnI binding \textsuperscript{−15}-fold (38).

The \textsuperscript{−12}-fold increase in TnC\textsuperscript{F\textsubscript{29W}} Ca\textsuperscript{2+} affinity upon binding of TnI or TnI\textsubscript{96–148} is primarily reflected by an \textsuperscript{−30}-fold slower rate of Ca\textsuperscript{2+} dissociation from the TnC\textsuperscript{F\textsubscript{29W}}-TnI\textsubscript{96–148} complex (8). Hydrophobic residue substitutions to polar Gln in the regulatory domain of TnC\textsuperscript{F\textsubscript{29W}} varied the Ca\textsuperscript{2+} dissociation rates from the TnC\textsuperscript{F\textsubscript{29W}}-TnI\textsubscript{96–148} complex \textsuperscript{33}-fold. This broad range in Ca\textsuperscript{2+} dissociation rates is primarily reflected by the ability of some of the hydrophobic mutations to speed the rate of Ca\textsuperscript{2+} dissociation, up to \textsuperscript{−15}-fold compared with the TnC\textsuperscript{F\textsubscript{29W}}-TnI\textsubscript{96–148} complex. Consistent with the inability of TnI\textsubscript{96–148} to enhance the Ca\textsuperscript{2+} sensitivity of the TnC\textsuperscript{F\textsubscript{29W}} mutants with increased Ca\textsuperscript{2+} affinity, the Ca\textsuperscript{2+} dissociation rate from the mutant TnC\textsuperscript{F\textsubscript{29W}}-TnI\textsubscript{96–148} complexes could only be slowed \textsuperscript{−2}-fold.

The TnC\textsuperscript{F\textsubscript{29W}}-TnI\textsubscript{96–148} complex may be a good model system to study how different Tn complexes respond to changes in Ca\textsuperscript{2+} concentration in muscle. Interestingly, the rate of Ca\textsuperscript{2+} dissociation from the TnC\textsuperscript{F\textsubscript{29W}}-TnI\textsubscript{96–148} complex, and not isolated TnC\textsuperscript{F\textsubscript{29W}}, is similar to the rate of fast twitch skeletal muscle relaxation (Fig. 9). Previous experiments with TnC mutants in which the Ca\textsuperscript{2+} sensitivity of the regulatory domain in solution was increased or decreased demonstrated similar
qualitative shifts in the force-pCa relationship upon reconstitution in skeletal muscle fibers (8, 12, 34, 35, 39, 40). This may be the case only in those circumstances when the Ca$^{2+}$ sensitivity of the isolated TnC and the TnC-TnI complex are shifted in a similar direction. To test this hypothesis we reconstituted skinned rabbit psoas fibers with TnC F29W mutants that displayed the same qualitative changes in Ca$^{2+}$ sensitivities in the absence or presence of TnI,6–148 (V45Q TnC F29W and F78Q TnC F29W) and two that did not (M46Q TnC F29W and M81Q TnC F29W). Comparison of the effects of these mutations on the force-pCa relationship suggests that the TnC F29W-TnI,6–148 complex is a better predictor than isolated TnC F29W for how changes in Ca$^{2+}$ binding to TnC modulate the Ca$^{2+}$ sensitivity of force production. For instance, even though both Val$^{16}$ → Gln and Met$^{46}$ → Gln mutations increase the Ca$^{2+}$ affinity of isolated TnC F29W, only the Val$^{16}$ → Gln mutation increased the Ca$^{2+}$ sensitivity of force development. These results are consistent with the fact that only Val$^{15}$ → Gln, but not Met$^{46}$ → Gln increases the Ca$^{2+}$ affinity of the TnC F29W-TnI,6–148 complex.

Furthermore, the Ca$^{2+}$ sensitivity of force development generated with F78Q TnC F29W was dramatically lower than that generated with M81Q TnC F29W or TnC F29W, even though both Phe$^{78}$ → Gln and Met$^{81}$ → Gln mutations in isolated TnC F29W decreased the Ca$^{2+}$ sensitivity of the regulatory domain to a similar extent (6). These results are consistent with the fact that the Phe$^{78}$ → Gln but not Met$^{81}$ → Gln mutation leads to a dramatic decrease in Ca$^{2+}$ affinity of the TnC F29W-TnI,6–148 complex. However, the Phe$^{78}$ → Gln mutation appears to have a larger effect on the Ca$^{2+}$ affinity of the TnC F29W-TnI,6–148 complex than on the Ca$^{2+}$ sensitivity of force development. Thus, the Ca$^{2+}$ binding properties of the TnC-TnI complex are not the only determinants of Ca$^{2+}$ sensitivity of force development. There is evidence that skeletal troponin T, tropomyosin, and actomyosin can modulate the Ca$^{2+}$ sensitivity of muscle mechanics either directly through TnC or through mechanisms yet to be explained (for review see Ref. 3).

A striking result observed with I62Q TnC F29W was the dramatic reduction of force production generated by muscle fibers reconstituted with this mutant, even though the data shows it is able to bind to the thin filament. The near lack of force production cannot be explained by the low Ca$^{2+}$ sensitivity of the I62Q TnC F29W-TnI,6–148 complex because the F78Q TnC F29W-TnI,6–148 complex has a 2-fold lower Ca$^{2+}$ sensitivity but is able to produce ~80% of maximal force. However, Ca$^{2+}$-saturated I62Q TnC F29W has an ~14-fold decreased affinity for TnI,6–148 as compared with TnC F29W. The large decrease in TnI,6–148 binding affinity for I62Q TnC F29W is the likely reason why this mutant is unable to support force. It appears that Ca$^{2+}$-I62Q TnC F29W in the fiber might not effectively compete with actin binding to the regulatory domain of TnI, thus keeping the muscle fiber in a state of inactivation. Consistent with this interpretation, F26Q TnC F29W, which had an ~10-fold lower affinity for TnI,6–148, also produced only ~13% of the maximal force upon reconstitution in the muscle fibers (data not shown). The exact opposite effect occurred when the regulatory regions of TnC and TnI were cross-linked, causing a regulated thin filament system to be permanently activated even in the absence of Ca$^{2+}$ (41). However, Ca$^{2+}$-saturated I37Q TnC F29W (a β-sheet mutant) bound TnI,6–148 with only an ~2-fold lower affinity than TnC F29W but still only produced ~15% maximal force upon reconstitution in the muscle fibers (data not shown). Furthermore, Ca$^{2+}$-saturated I73Q TnC F29W, another β-sheet mutant, bound TnI,6–148 with an affinity nearly identical to that of TnC F29W but only produced ~45% maximal force. Again, this points out that additional events besides Ca$^{2+}$ binding and subsequent TnI binding are involved in the signal pathway of force production.

Thus, the Ca$^{2+}$-saturated TnC with a decreased skeletal troponin T affinity (but similar affinity for TnI) has been implicated in a loss of reconstituted thin filament ATPase activity (27). However, another mutant TnC with apparently normal Ca$^{2+}$, TnI, and skeletal troponin T binding also displayed a diminished reconstituted thin filament ATPase activity through an unidentified mechanism apparently important for the Ca$^{2+}$-dependent regulation of signal transduction (42).

In summary, we utilized TnC F29W to study Ca$^{2+}$ binding and exchange with a series of hydrophobic N-domain TnC mutants in the presence of TnI,6–148 and intact TnI. The TnC F29W-TnI,6–148 mutant complexes exhibited ~243-fold variation in their Ca$^{2+}$ binding affinities, ~38-fold variation in their Ca$^{2+}$ association rates, and ~33-fold variation in their Ca$^{2+}$ dissociation rates. The regulatory peptide of TnI, TnI,6–148 was an accurate mimic of intact TnI for measuring Ca$^{2+}$ dissociation rates from the TnC-TnI complexes. Furthermore, the effect of hydrophobic mutations on the Ca$^{2+}$ sensitivity of force development could be better predicted from the Ca$^{2+}$ affinities of the TnC F29W-TnI,6–148 mutant complexes than from that of the isolated TnC F29W mutants. Interestingly, TnC F29W mutants with >10-fold lower TnI,6–148 affinities in the presence of saturating Ca$^{2+}$, compared with that of TnC F29W, were able to bind to the thin filaments but led to dramatic reduction of force recovery in reconstituted muscle fibers. Thus, not just Ca$^{2+}$-binding to TnC but the changes in the interactions with other regulatory proteins are critical in the pathway of signal transduction of force development. In conclusion, elucidating the determinants of Ca$^{2+}$ binding and exchange with TnC in the presence of its target protein TnI may provide a deeper understanding of how TnC and other closely related EF hand proteins respond to Ca$^{2+}$ and control signal transduction.

Acknowledgments—We thank Dr. Lawrence Smillie for the generous gifts of the chicken fast twitch skeletal muscle TnC F29W plasmid and TnI protein and for help in obtaining the TnI,6–148 peptide. Dr. Peter Reiser for the expert advice and training pertaining to the physiological muscle fiber experiments as well as use of his equipment, Dr. Darl Swartz for the expert advice and training pertaining to the myofibril imaging experiments as well as use of his equipment, and Zenhui Yang for help and training in preparing the myofibril samples.

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Mutations of Hydrophobic Residues in the N-terminal Domain of Troponin C Affect Calcium Binding and Exchange with the Troponin C-Troponin I96–148 Complex and Muscle Force Production

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J. Biol. Chem. 2004, 279:17348-17360.
doi: 10.1074/jbc.M314095200 originally published online February 16, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M314095200

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