The goal of this study was to examine the mechanism of magnesium binding to the regulatory domain of skeletal troponin C (TnC). The fluorescence of Trp^{29} immediately preceding the first calcium-binding loop in TnC^{F29W}, was unchanged by addition of magnesium, but increased upon calcium binding with an affinity of 3.3 μM. However, the calcium-dependent increase in TnC^{F29W} fluorescence could be reversed by addition of magnesium, with a calculated competitive magnesium affinity of 1.0 μM. When a Z acid pair was introduced into the first EF-hand of TnC^{F29W}, the fluorescence of G34DTnC^{F29W} increased upon addition of magnesium or calcium with affinities of 295 and 1.9 μM, respectively. Addition of 3 mM magnesium decreased the calcium sensitivity of TnC^{F29W} and G34DTnC^{F29W} by 2- and 6-fold, respectively. Exchange of G34DTnC^{F29W} into skinned psosas muscle fibers decreased fiber calcium sensitivity by 1.7-fold compared with TnC^{F29W} at 1 mM [magnesium]_t and ~3.2-fold at 3 mM [magnesium]_t. Thus, incorporation of a Z acid pair into the first EF-hand allows it to bind magnesium with high affinity. Further, the data suggest that the second EF-hand, but not the first, of TnC is responsible for the competitive magnesium binding to the regulatory domain.

The EF-hand is the most common Ca^{2+} binding motif found in nature (1). EF-hand proteins and their functions are numerous and diverse (for review see Ref. 2). In general, EF-hand domains can be classified functionally into two groups, those that regulate cellular activities through a reversible change in structure upon Ca^{2+} binding and release (such as the N-terminal regulatory domain of troponin C (TnC)) and both domains of calmodulin (CaM)) and those that simply buffer/transport Ca^{2+} (such as parvalbumin and calbindin D_{9k}) or anchor protein complexes (such as the C-terminal domain of TnC). Often the nonregulatory EF-hand domains bind Mg^{2+} competitively and display higher Ca^{2+} affinity than do the regulatory domains. However, it is becoming clear that regulatory EF-hand proteins, such as the N-terminal of CaM can also bind Mg^{2+} with a physiologically relevant affinity (3, 4). Thus, it is important to elucidate the mechanisms behind EF-hand Mg^{2+} binding.

The canonical EF-hand consists of 29 consecutive residues, with two helices flanking a 12-residue loop. The chelating loop residues in positions 1(+-x), 3(+-y), 5(+-z), 7 (-y), 9(-x), and 12 (-z) ligate Ca^{2+} through seven oxygen atoms arranged three dimensionally on the axes of a pentagonal bipyramid (for review see Refs. 5 and 6). Factors that control Ca^{2+} affinity are complex and involve residues within and outside of the Ca^{2+}-binding loop (7-11). The mechanisms utilized for EF-hand Mg^{2+} binding are less understood. The smaller Mg^{2+} cation is typically complexed by some of the same loop residues used for Ca^{2+} binding, although through six oxygen atoms arranged in an octahedral geometry (for review see Refs. 11 and 12). Interestingly, synthetic EF-hand peptides were observed to bind Mg^{2+} when their Ca^{2+}-binding loops contained a Z acid pair, chelating residues at loop positions +z and −z (13, 14).

The metal binding properties of TnC have been studied extensively using various biophysical and biochemical techniques (15-19). These studies have demonstrated that the two C-domain EF-hands have ~10-fold higher Ca^{2+} affinity and greater than 100-fold slower Ca^{2+} exchange rates than the two N-domain EF-hands (18, 20). In addition to Ca^{2+}, the C-domain sites also competitively bind Mg^{2+} with a physiologically relevant affinity. Because of their high Ca^{2+} and Mg^{2+} affinities and slow exchange rates, the C-domain sites are thought to play a structural role in muscle function by anchoring TnC into the Tn Complex.

The N-domain sites of TnC are considered to be Ca^{2+}-specific under physiological Mg^{2+} concentrations, and generally accepted to be directly involved in the Ca^{2+}-dependent regulation of muscle contraction (for review see Refs. 21 and 22). However, addition of Mg^{2+} has been shown to decrease the Ca^{2+} sensitivity of the regulatory domain of fluorescent TnC's in isolation (19, 23-27), in the Tn complex (23, 28), and in reconstituted muscle fibers (26, 29). Furthermore, several groups have dem-
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The F29W mutation in chicken skeletal TnC (TnC F29W) has been frequently used to study metal and ligand interactions with the regulatory N-domain sites of TnC (15, 23), whereas others hypothesized Mg$^{2+}$ as a direct Ca$^{2+}$ competitor for the N-terminal regulatory sites (19, 27, 32).

Because TnC regulates muscle contraction as a part of the troponin complex and not in isolation, it is important to understand the interactions of TnC with TnI. The binding of TnI to TnC increases the Ca$^{2+}$ sensitivity of Tn-regulated actomyosin ATPase and force development (26, 27, 30, 31). The question whether Mg$^{2+}$ competes with Ca$^{2+}$ for the N-domain sites remains unresolved and controversial. Some research groups have suggested the presence of auxiliary Mg$^{2+}$ binding sites in TnC (15, 23), whereas others hypothesized Mg$^{2+}$ as a direct Ca$^{2+}$ competitor for the N-terminal regulatory sites (19, 27, 32).

Because TnC regulates muscle contraction as a part of the troponin complex and not in isolation, it is important to understand the interactions of TnC with TnI. The binding of TnI to TnC increases the Ca$^{2+}$ sensitivity of the N- and C-domains of TnC—10-fold (15, 33). TnC and TnI interact in an antiparallel orientation (34, 35) such that the Ca$^{2+}$-dependent binding of the N-terminal regulatory domain of TnC to the C-domain of TnI is an early step in the generation of force in skeletal muscle (21). Previous studies demonstrated that region 96–116 of TnI interacts with actin and was largely responsible for the ability of TnI to inhibit the activity of the actomyosin ATPase that could be reversed upon TnC-Ca$^{2+}$ binding (36–38). Recent studies implicated additional residues within 116–148 of TnI as being important for the complete inhibitory activity and regulatory interactions with actin and TnC (39–42). 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.

The F29W mutation in chicken skeletal TnC (TnC F29W) has been frequently used to study metal and ligand interactions with the regulatory N-domain sites of TnC (9, 20, 43–46). TnC F29W is a physiologically active protein that produced maximal isometric tension with a Ca$^{2+}$ sensitivity indistinguishable from that of recombinant TnC, when reconstituted into skinned skeletal fibers (47). Interestingly, whereas Ca$^{2+}$ causes a large increase in TnCF29W fluorescence, Mg$^{2+}$ does not alter the fluorescence properties of TnC F29W (44, 45, 48). Previously, we have demonstrated that an endogenous Z acid pair was required for high affinity Mg$^{2+}$ binding to the first EF-hand of fluorescent CaM F29W (3). We wanted to test if Mg$^{2+}$ binding could be engineered into the first EF-hand of TnCF29W by substituting Gly in position 34 with Asp, thus introducing a Z acid pair (Fig. 1). We also wanted to determine how competitive Mg$^{2+}$ binding to the first EF-hand of TnC would affect the physiological properties of G34DTnC F29W reconstituted into skinned skeletal muscle fibers.

**Experimental Procedures**

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

**Protein Mutagenesis and Purification**—The construction and expression of intact chicken skeletal TnC F29W and isolated N-domain residues 1–90, TnC1–28, both in pET3a, have been described (44, 49–50). Chicken skeletal fast TnI was prepared as described for the rabbit protein (51). The G34DTnCF29W mutant was constructed from the TnCF29W plasmid by primer-based site-directed mutagenesis using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The mutation was confirmed by DNA sequence analysis. The plasmids for TnCF29W and G34DTnCF29W were transformed into Escherichia coli BL21DE3PlysS cells (Novagen) and purified as described previously (9).

**Determination of Ca$^{2+}$ and Mg$^{2+}$ Affinities**—All steady-state fluorescence measurements were performed using a PerkinElmer Life Sciences LS5 spectrofluorimeter at 15 °C. Trp fluorescence was excited at 275 nm and monitored at 345 nm as microliter amounts of CaCl$_2$ or MgCl$_2$ were added to 1 ml of each TnCF29W mutant (0.3 mM) in 200 mM MOPS to prevent pH changes upon addition of metal; 90 mM KCl, 2 mM EGTA, 1 mM DTT, pH 7.0, at 15 °C. The Ca$^{2+}$ fluorescence was calculated using the computer program EGCA02 developed by Robertson and Potter (52). The Ca$^{2+}$ and Mg$^{2+}$ affinities are reported as dissociation constants $K_{d(Ca)}$ and $K_{d(Mg)}$, respectively. Each $K_d$ represents a mean of the coordinates of the N-terminal domain crystal structure of chicken skeletal TnC (Protein Data Bank number 1AVS) (74). These cartoons are not meant to represent the actual structures but are used simply to demonstrate the potential effect of the G34D mutation and to demonstrate the location of the F29W mutation adjacent to the first Ca$^{2+}$-binding loop. The spherical Ca$^{2+}$ ion is shown to be coordinated by residues at positions 30 ($+$x), 32 ($+$y), 34 ($+$z), 36 ($-$y), 38 ($-$x), and 41 ($-$z) that form the base and apexes of the pentagonal bipyramidal geometry.

![Fig. 1. Predicted structures of the first Ca$^{2+}$-binding loops of TnCF29W and G34DTnCF29W. Loop residues 29 through 41 are shown in a ribbon/stick configuration for TnCF29W and G34DTnCF29W, as rendered by RASMOl (72). The F29W and G34D mutations were predicted by the computer software program WHAT IF (73) and constructed from the coordinates of the N-terminal domain crystal structure of chicken skeletal TnC (Protein Data Bank number 1AVS) (74). These cartoons are not meant to represent the actual structures but are used simply to demonstrate the potential effect of the G34D mutation and to demonstrate the location of the F29W mutation adjacent to the first Ca$^{2+}$-binding loop. The spherical Ca$^{2+}$ ion is shown to be coordinated by residues at positions 30 ($+$x), 32 ($+$y), 34 ($+$z), 36 ($-$y), 38 ($-$x), and 41 ($-$z) that form the base and apexes of the pentagonal bipyramidal geometry.](image1.png)

**Fig. 2. Effect of Ca$^{2+}$ and Mg$^{2+}$ on the fluorescence spectra of TnCF29W and G34DTnCF29W.** Fluorescence emission spectra of TnCF29W (panel A) or G34DTnCF29W (panel B) in the apo, Ca$^{2+}$-saturated, or Mg$^{2+}$-saturated states. The spectra were recorded with an excitation wavelength of 275 nm. Protein concentration was 1 μM in 1 ml of 200 mM MOPS, 90 mM KCl, 2 mM EGTA, 1 mM DTT, pH 7.0, at 15 °C. Trp fluorescence spectra were recorded before the addition of metals (apo), after the addition of either 50 mM Mg$^{2+}$ (+Mg$^{2+}$) or 1 mM Ca$^{2+}$ ([Ca$^{2+}$]$_{free}$ +2Ca$^{2+}$).
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3–5 titrations fit with a logistic sigmoid function mathematically equivalent to the Hill equation, as previously described (8, 9).

Determination of Apparent TnI-(96–148) Peptide Affinities—TnI fluorescence was monitored as described in the previous paragraph. Micromolar amounts of TnI-(96–148) were added to 1 ml of each TnC<sup>F29W</sup> mutant (0.6 μM) in 200 mM MOPS, 90 mM KCl, 2 mM EGTA, 1 mM Ca<sup>2+</sup>–DTT, pH 7.0, at 15 °C. Each apparent peptide affinity represents a mean of 3 titrations fit with a logistic sigmoid function.

Determination of Ca<sup>2+</sup> and Mg<sup>2+</sup> Association and Dissociation Rates—Ca<sup>2+</sup>– and Mg<sup>2+</sup> association (k<sub>on(Ca)</sub> and k<sub>on(Mg)</sub>) and dissociation rates (k<sub>off(Ca)</sub> and k<sub>off(Mg)</sub>) were measured using an Applied Photophysics Ltd. (Leatherhead, UK) model SX.18 MV stopped-flow instrument with a dead time of 1.4 ms at 15 °C. The samples were excited using a 150-watt xenon arc source. Ca<sup>2+</sup>– and Mg<sup>2+</sup> binding kinetics of the N-terminal domain within intact TnC<sup>F29W</sup> and G34DTnC<sup>F29W</sup> were obtained utilizing TnI fluorescence changes excited at 275 nm with emission monitored through a UV transmitting black glass filter (UG1 from Oriel, Stratford, CT). Ca<sup>2+</sup>– dissociation rates in the absence of Mg<sup>2+</sup> were also measured using the fluorescent Ca<sup>2+</sup>– chelator Quin-2. Whereas the fluorescence of TnC<sup>F29W</sup> was selective for N-terminal Ca<sup>2+</sup>– dissociation, Quin-2 fluorescence reported Ca<sup>2+</sup>– dissociation from both the N- and C-domains within TnC<sup>F29W</sup>. However, the Ca<sup>2+</sup>– dissociation rate from the N-terminal domain within TnC<sup>F29W</sup> was easily distinguished from the rate of Ca<sup>2+</sup>– dissociation from the C-terminal domain because the latter rate was >95-fold slower in the absence and presence of TnI-(96–148) or intact TnI. Quin-2 fluorescence was excited at 390 nm, with its emission monitored through a 510-nm broad-band-pass interference filter (Oriel). Each data set represents an average of 10–15 traces, fit with a single exponential (variance 2 × 10<sup>−4</sup>). The curve fitting program (by P. J. King, Applied Photophysics Ltd.) uses the nonlinear Levenberg-Marquardt algorithm. The buffer used in all the Ca<sup>2+</sup>– stopped-flow experiments was 10 mM MOPS, 90 mM KCl, 1 mM DTT, pH 7.0.

Calculation of Ca<sup>2+</sup> and Mg<sup>2+</sup> Association Rates—The Ca<sup>2+</sup>– and Mg<sup>2+</sup> association rates were calculated using the equation k<sub>on</sub> = k<sub>off</sub>/(k<sub>diss</sub> + k<sub>aff</sub>), where k<sub>off</sub> represents the concerted release of two Ca<sup>2+</sup>– or single Mg<sup>2+</sup> ions and k<sub>aff</sub> represents the binding event of two Ca<sup>2+</sup>– or one Mg<sup>2+</sup> to the N-domain of TnC<sub>F29W</sub>, as previously described (3, 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 no longer than 1 month. Solutions and the mechanical setup utilized for force measurements were as previously described (53). Briefly, a single fiber was soaked in relaxing solution containing 1% (v/v) Triton X-100 for 5 min to remove any residual sarcolemma and sarcoplasmic reticulum. The fiber was then tied down with pens in troughs attached to a servo-controlled DC torque motor (Cambridge Technologies, Watertown, MA) and an isometric force transducer (model 403A, Cambridge Technologies) as previously described (54). Fiber sarcomere length, width, and depth were measured with a video image analysis system (Simple PCI, Compix Inc., Cranberry Township, PA). Resting sarcomere length was set between 2.50 and 2.60 μm. The fiber was then activated in a Ca<sup>2+</sup>– solution and rapidly slackened after isometric force reached plateau. The analogue output of the force transducer was digitized using a DaqBoard2000 and Dialog software (Iotech Inc., Cleveland, OH). The total force was measured between the plateau and baseline levels. The same procedure was utilized to obtain the resting force level of the fiber in a pCa 9.0 solution. The active force generated by the fiber in the various pCa solutions was calculated as the total force minus the resting force. Three active force measurements were performed in each pCa, as with the final activation taken as the maximal force generated by the native fiber (i.e. prior to extraction of the time course of the change in TnI fluorescence is shown as EDTA dissociates Mg<sup>2+</sup> from the N-terminal domains of G34DTnC<sup>F29W</sup> and TnC<sup>F29W</sup>. Each protein (2 μM) in the same buffer as Fig. 2 plus 10 mM Mg<sup>2+</sup> was rapidly mixed with an equal volume of 20 mM EDTA in the same buffer at 15 °C. TnI fluorescence was monitored through a UV-transmitting black glass filter (UG1 from Oriel) with excitation at 275 nm. The traces have been staggered for clarity. Each trace is an average of at least 15 traces, and the data were fit with a single exponential (excluding TnC<sup>F29W</sup>, which was flat, variance <2 × 10<sup>−4</sup>). The kinetic traces were triggered at time 0, the first ~2 ms of premixing time is shown (the apparent lag phase), and the average trace was fit after mixing was complete. Control experiments in which each protein (2 μM) in the same buffer as Fig. 2 plus 10 mM Mg<sup>2+</sup> was rapidly mixed with an equal volume of the same buffer were flat lines.
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**RESULTS**

Effect of Ca^{2+} and Mg^{2+} on the Fluorescence Spectra of the N-terminal Domains within TnC_{29W} and G43DTnC_{29W}—

TnC_{29W} undergoes an ∼2.4-fold increase in Trp fluorescence upon Ca^{2+} binding to its N-domain sites at 15 °C (Fig. 2A). No change in Trp fluorescence was observed upon addition of 50 mM Mg^{2+} to TnC_{29W} (Fig. 2A). We previously demonstrated that high Mg^{2+} binding to the first EF-hand of CaM required the presence of an endogenous Z acid pair (3). When a Z acid pair was introduced into the first EF-hand of TnC_{29W} (i.e., G34DTnC_{29W}), addition of Ca^{2+} led to an ∼2.2-fold increase in Trp fluorescence (Fig. 2B). Furthermore, G34DTnC_{29W} also underwent an ∼1.4-fold increase in its Trp fluorescence upon addition of Mg^{2+} (Fig. 2B). These results demonstrate that substitution of Gly-34 with Asp in the first EF-hand of TnCl led to Mg^{2+} binding to this EF-hand.

Measurement of the Mg^{2+} Binding Affinity for TnC_{29W} and the N-terminal Domains within TnC_{29W} and G34DTnC_{29W}—

Fig. 3A shows the Mg^{2+}-dependent increase in Trp fluorescence of G34DTnC_{29W} (▲), G34DTnC_{29W} exhibited a half-maximal increase in Trp fluorescence at 295 ± 10 μM. However, addition of Mg^{2+} (up to 50 mM) caused no change in the Trp fluorescence signal of TnC_{29W} (Fig. 3A, ▲). Thus, as expected, the G34D mutation in TnC_{29W} incorporates physiological Mg^{2+} binding to the first EF-hand of TnC_{29W}.

Fig. 3B shows that in the presence of 10 μM [Ca^{2+}]_{free} the Trp fluorescence signal of TnC_{29W} (●), TnC_{29W} (■), and G34DTnC_{29W} (▲) was greater than 90% saturated, while Mg^{2+} subsequently decreased in a concentration-dependent manner. Mg^{2+} half-maximally decreased the Trp fluorescence of TnC_{29W}, TnC_{29W} and G34DTnC_{29W} at 7.3 ± 0.8, 9 ± 1, and 1.9 ± 0.2 mM, respectively. Thus, the Ca^{2+}-dependent fluorescence of TnC_{29W}, TnC_{29W}, and G34DTnC_{29W} was reversed by Mg^{2+} binding, presumably because of the closing of the N-terminal hydrophobic pocket of TnC and not because of any type of interference from Mg^{2+} binding to the C-terminal domain. Knowing the $K_{d(Ca)}$ for each N-terminal domain protein (determined as described later) and assuming competitive Mg^{2+} binding, the $K_{d(Mg)}$, of TnC_{29W}, TnC_{29W}, and G34DTnC_{29W} was calculated to be 1.8 mM, 2.2 mM, and 303 μM, respectively. Thus, the calculated Mg^{2+} affinity for G34DTnC_{29W} was identical to that measured directly as described above. Furthermore, even though TnC_{29W} does not undergo a change in fluorescence upon Mg^{2+} binding (Fig. 3A, ■) its N-terminal domain appears to bind Mg^{2+} and displace Ca^{2+}.

Table I summarizes the data for Mg^{2+} binding to TnC_{29W} and G34DTnC_{29W}.

**Table I**

| TnC_{29W} | G34DTnC_{29W} | $K_d$ (Mg) (μM) | Competitive Mg^{2+} binding $K_{d(Mg)}$ (μM) |
|-----------|---------------|----------------|---------------------------------------------|
| G34DTnC_{29W} | 1.8 | 220 | 303 |
| G34DTnC_{29W} | 2.2 | 3800 | 590 |
| TnC_{29W} | 303 | 6200 | 714 |

* NA, because TnC_{29W} fluorescence was not affected by direct Mg^{2+} binding; these values were not available (NA).
were nearly identical at 457 ± 16 and 508 ± 9 nM, respectively. Thus, any differences in Ca2+ sensitivity observed between the two proteins in the presence of the peptide could not be because of differences in peptide affinities. The Ca2+ binding affinities for the TnC/29W/TnI-(96–148) and G34DTnC/29W/TnI-(96–148) complexes were measured following the Ca2+-induced increases in Trp fluorescence in the absence and presence of 3 mM Mg2+ at 15°C. Fig. 4C shows that in the absence of Mg2+, the Tn(C/29W/TnI-(96–148) (□) and G34DTnC(29W/TnI-(96–148) (△) complexes exhibited half-maximal Ca2+-dependent increases in Trp fluorescence at 267 ± 3 and 147 ± 2 nM Ca2+, respectively. Therefore, in the absence of Mg2+, the G34DTnC(29W/TnI-(96–148) complex exhibited an ~1.7-fold increase in its Ca2+ affinity, relative to that of the TnC(29W/TnI-(96–148) complex. Consistent with intact TnI binding to TnC, TnI-(96–148) enhanced the Ca2+ sensitivity of the regulatory domain of TnC(29W –12-fold (14, 32) and to G34DTnC(29W/TnI-(96–148) (■) and G34DTnC(29W/TnI-(96–148) (▲) complexes exhibited half-maximal increases in Trp fluorescence at 397 ± 13 and 765 ± 41 nM Ca2+, respectively. Therefore, in the presence of 3 mM Mg2+, the G34DTnC(29W/TnI-(96–148) complex exhibited ~1.9-fold decrease in Ca2+ sensitivity, relative to that of the TnC(29W/TnI-(96–148) complex. Furthermore, 3 mM Mg2+ shifts the Ca2+ sensitivity of the TnC(29W/TnI-(96–148) and G34DTnC(29W/TnI-(96–148) complexes ~1.5- and 5.2-fold, respectively. Again, assuming competitive Mg2+ binding, the Kd(Mg) of TnC(29W and G34DTnC(29W in complex with TnI-(96–148) was ~6.2 μM and 714 μM, respectively. Thus, unlike Ca2+ binding to the regulatory sites of TnC(29W and G34DTnC(29W, Mg2+ binding is not enhanced by TnI-(96–148) binding and may actually be slightly decreased (see Table I). Furthermore, in the presence of 3 mM Mg2+, TnI-(96–148) increased the Ca2+ sensitivity to the regulatory domains of both TnC(29W and G34DTnC(29W –15-fold. Thus, Mg2+ did not affect the enhancement of the Ca2+ affinity to the regulatory domain of the TnCs caused by TnI-(96–148) binding, consistent with Mg2+ acting only as a competitor for Ca2+ binding.

**Measurement of Ca2+ Dissociation Rates from the N-terminal Domains within TnC(29W and G34DTnC(29W Induced by EGTA, Quin-2, or Mg2+—Fluorescence stopped-flow measurements, utilizing the EGTA-induced decreases in Trp fluorescence, were conducted to determine the rates of Ca2+ dissociation from the N-terminal sites of TnC(29W and G34DTnC(29W.** Fig. 5A shows the time course of the EGTA-induced decreases in Trp fluorescence as Ca2+ was dissociated from TnC(29W and G34DTnC(29W (Tn EGTA traces). At 15°C, excess EGTA removed Ca2+ from TnC(29W and G34DTnC(29W at 342 ± 5 and 403 ± 8 s⁻¹, respectively. Under saturating Ca2+ conditions, the presence of 3 mM Mg2+ did not affect the rates of Ca2+ dissociation from TnC(29W or G34DTnC(29W (data not shown, Table II). These results were consistent with the Ca2+ dissociation rate from the regulatory sites of the fluorescent TnCDanx not being affected by Mg2+ (56).

To verify that the Trp signal changes were accurately reporting the true Ca2+ dissociation rates and not a slower or faster
structural change, Ca\(^{2+}\) dissociation rates in the absence of Mg\(^{2+}\) were also measured with the fluorescent Ca\(^{2+}\) chelator Quin-2. Fig. 5A also shows the time course of the Quin-2-induced increases in fluorescence as Ca\(^{2+}\) was dissociated from TnC\(^{29W}\) and G34DTnC\(^{29W}\) (Quin-2 traces). Nearly identical Ca\(^{2+}\) dissociation rates were measured using Quin-2 fluorescence for TnC\(^{29W}\) and G34DTnC\(^{29W}\) at 346 ± 3 and 397 ± 2 s\(^{-1}\), respectively, as were measured by the EGTA-induced Trp changes. Therefore, G34DTnC\(^{29W}\) exhibited an ~1.2-fold faster N-terminal Ca\(^{2+}\) dissociation rate, relative to that of TnC\(^{29W}\). Similarly, the C-terminal Ca\(^{2+}\) dissociation rates from TnC\(^{29W}\) and G34DTnC\(^{29W}\) were nearly identical at 0.48 ± 0.01 and 0.56 ± 0.01 s\(^{-1}\), respectively (data not shown).

Fig. 3B demonstrated that Mg\(^{2+}\) could reverse the Ca\(^{2+}\) induced decrease in Trp fluorescence for both TnC\(^{29W}\) and G34DTnC\(^{29W}\). If Mg\(^{2+}\) binding to the N-terminal domains of TnC\(^{29W}\) and G34DTnC\(^{29W}\) was truly competitive with Ca\(^{2+}\) binding, then it would be expected that Mg\(^{2+}\) could not bind these Ca\(^{2+}\)-saturated sites until Ca\(^{2+}\) dissociates, as has been demonstrated for the Ca\(^{2+}\) - and Mg\(^{2+}\)-binding protein parvalbumin (57, 58). Fig. 5B shows the time course of Mg\(^{2+}\) binding to Ca\(^{2+}\)-saturated TnC\(^{29W}\) and G34DTnC\(^{29W}\) (Trp \(Mg\]\(2^+\) traces) following the decreases in Trp fluorescence in a stopped-flow apparatus at 15 °C. Mg\(^{2+}\) was able to displace Ca\(^{2+}\) from TnC\(^{29W}\) and G34DTnC\(^{29W}\) at 348 ± 2 and 399 ± 6 s\(^{-1}\), respectively. Because these values are virtually identical to the Ca\(^{2+}\) dissociation rates in the absence of Mg\(^{2+}\), it is clear that Mg\(^{2+}\) binding to Ca\(^{2+}\)-saturated TnC\(^{29W}\) and G34DTnC\(^{29W}\) was limited by their respective Ca\(^{2+}\) dissociation rates, supporting the hypothesis that Mg\(^{2+}\) binds competitively with Ca\(^{2+}\).

### Measurement of Ca\(^{2+}\) Dissociation Rates from the N-terminal Domains within TnC\(^{29W}\) and G34DTnC\(^{29W}\) Induced by EGTA or Quin-2 in the Presence of TnI-(96–148) or Intact TnI—The rates of Ca\(^{2+}\) dissociation from TnC\(^{29W}\) and G34DTnC\(^{29W}\) induced by EGTA were also measured in the presence of TnI-(96–148) (Fig. 5C, Trp TnI-(96–148) EGTA traces). At 15 °C, EGTA removed Ca\(^{2+}\) from the TnC\(^{29W}\) and G34DTnC\(^{29W}\) at 10.6 ± 0.2 and 6.0 ± 0.3 s\(^{-1}\), respectively. Nearly identical rates were measured using Quin-2 to remove Ca\(^{2+}\) from the TnC\(^{29W}\) and G34DTnC\(^{29W}\) at 10.6 ± 0.2 and 6.3 ± 0.1 s\(^{-1}\), respectively (data not shown). Thus, the N-terminal Trp signal change for both proteins in the absence and presence of TnI-(96–148) accurately reports Ca\(^{2+}\) binding and dissociation.

To verify that TnI-(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 also shows the time course of the Quin-2-induced increases in fluorescence as Ca\(^{2+}\) was dissociated from the TnC\(^{29W}\)-TnI complex at 9.1 ± 0.8 s\(^{-1}\) and the G34DTnC\(^{29W}\)-TnI complex at 5.4 ± 0.5 s\(^{-1}\) (Quin-2 intact TnI traces). Thus, the Ca\(^{2+}\) dissociation rates from the TnC\(^{29W}\)-TnI and G34DTnC\(^{29W}\)-TnI complexes were similar to the Ca\(^{2+}\) dissociation rates when complexed with TnI-(96–148). Furthermore, G34DTnC\(^{29W}\) complexed with either TnI or TnI-(96–148) exhibited an ~1.7-fold slower Ca\(^{2+}\) dissociation rate, relative to that of the TnC\(^{29W}\) complexes. Thus, the binding of TnI to the regulatory domain of G34DTnC\(^{29W}\) slows Ca\(^{2+}\) dissociation ~73-fold, whereas this effect on TnC\(^{29W}\) was slowed only ~37-fold. However, both TnI-(96–148) and intact TnI similarly slowed the Ca\(^{2+}\) dissociation rate from the C-terminal domains within TnC\(^{29W}\) and G34DTnC\(^{29W}\) only ~4-fold (data not shown).

Following the Trp fluorescence signal, in the presence of TnI-(96–148), 3 mM Mg\(^{2+}\) did not affect the rate of Ca\(^{2+}\) dissociation from TnC\(^{29W}\) but increased the Ca\(^{2+}\) dissociation rate of the G34DTnC\(^{29W}\)TnI-(96–148) complex to that of the TnC\(^{29W}\),TnI-(96–148) complex (data not shown, Table II). The reason for this Mg\(^{2+}\) effect on the G34DTnC\(^{29W}\)TnI-(96–148) complex is currently unknown. One possibility is that the increased negative charge of Asp-34 decreased the rate of Ca\(^{2+}\) dissociation from the regulatory domain of G34DTnC\(^{29W}\) in the presence of TnI-(96–148), which becomes screened by the positive Mg\(^{2+}\) ions.

#### Summary of Ca\(^{2+}\) binding and exchange with TnC\(^{29W}\) and G43DTnC\(^{29W}\) following the change in Trp fluorescence

| In isolation | +TnI-(96–148) |
|--------------|--------------|
| **K\(_d\)** (µM) | **K\(_d\)** (nM) |
| **n\(_{H}\)** | **n\(_{H}\)** |
| **k\(_{on}\)** (s\(^{-1}\)) | **k\(_{off}\)** (×10\(^{8}\) M\(^{-1}\) s\(^{-1}\)) |
| **k\(_{on}\)** (s\(^{-1}\)) | **k\(_{off}\)** (×10\(^{8}\) M\(^{-1}\) s\(^{-1}\)) |
| No Mg\(^{2+}\) | G34DTnC\(^{29W}\) | G34DTnC\(^{29W}\) |
| TnC\(^{29W}\) | 3.3 ± 0.1 | 3.3 ± 0.1 |
| G34DTnC\(^{29W}\) | 1.9 ± 0.1 | 1.9 ± 0.1 |
| 3 mM Mg\(^{2+}\) | TnC\(^{29W}\) | 5.9 ± 0.4 | 2.4 ± 0.1 |
| G34DTnC\(^{29W}\) | 11.6 ± 0.9 | 1.9 ± 0.1 |

*In the presence of 3 mM Mg\(^{2+}\), the Ca\(^{2+}\) association rate to G43DTnC\(^{29W}\) was first order at 440 ± 24 s\(^{-1}\) because Mg\(^{2+}\) must dissociate before Ca\(^{2+}\) can bind.*

### Table II

| **K\(_{a}\)** (µM) | **n\(_{H}\)** | **k\(_{on}\)** (s\(^{-1}\)) | **k\(_{off}\)** (×10\(^{8}\) M\(^{-1}\) s\(^{-1}\)) |
|-----------------|---------|-----------------|------------------|
| **K\(_{a}\)** (nM) | **n\(_{H}\)** | **k\(_{on}\)** (s\(^{-1}\)) | **k\(_{off}\)** (×10\(^{8}\) M\(^{-1}\) s\(^{-1}\)) |
| No Mg\(^{2+}\) | G34DTnC\(^{29W}\) | G34DTnC\(^{29W}\) | G34DTnC\(^{29W}\) |
| TnC\(^{29W}\) | 3.3 ± 0.1 | 3.3 ± 0.1 |
| G34DTnC\(^{29W}\) | 1.9 ± 0.1 | 1.9 ± 0.1 |
| 3 mM Mg\(^{2+}\) | TnC\(^{29W}\) | 5.9 ± 0.4 | 2.4 ± 0.1 |
| G34DTnC\(^{29W}\) | 11.6 ± 0.9 | 1.9 ± 0.1 |

*In the presence of 3 mM Mg\(^{2+}\), the Ca\(^{2+}\) association rate to G43DTnC\(^{29W}\) was first order at 440 ± 24 s\(^{-1}\) because Mg\(^{2+}\) must dissociate before Ca\(^{2+}\) can bind.*
At 3 mM Mg$^{2+}$, G34DTnC$^{F29W}$ was greater than 90% saturated with Mg$^{2+}$ (Fig. 3A, △). Fig. 6B shows the time course of the Ca$^{2+}$-induced increase in Trp fluorescence of G34DTnC$^{F29W}$ in the presence of 3 mM Mg$^{2+}$ as the [Ca$^{2+}$] was increased from 0 to 25 μM. As the [Ca$^{2+}$] increased, the rate of the reaction did not increase as expected for a second order reaction but remained fairly static at 440 ± 24 s$^{-1}$ (Fig. 6B and C, △). These static observed Ca$^{2+}$ association rates, close to the Mg$^{2+}$ dissociation rate from G34DTnC$^{F29W}$ (see Table 1), were consistent with the interpretation of competitive Ca$^{2+}$ and Mg$^{2+}$ binding to the N-terminal domain of G34DTnC$^{F29W}$. Thus, in the presence of saturating [Mg$^{2+}$], Ca$^{2+}$ cannot bind to the regulatory domain of G34DTnC$^{F29W}$ until Mg$^{2+}$ dissociates. Similar studies with TnC$^{F29W}$ yielded a Ca$^{2+}$ association rate in the presence of 3 mM Mg$^{2+}$ of 0.45 ± 0.03 × 10$^{-6}$ M$^{-1}$ s$^{-1}$ (r$^2$ = 0.999, Fig. 6C, △). Thus, at these [Mg$^{2+}$] and [Ca$^{2+}$] the Mg$^{2+}$ dissociation rate from the regulatory domain of TnC$^{F29W}$ was not rate-limiting for Ca$^{2+}$ binding, although it was slower than in the absence of Mg$^{2+}$. Mathematical modeling of the Ca$^{2+}$ association rate experiments with TnC$^{F29W}$ in the presence of 3 mM Mg$^{2+}$ predicted the Mg$^{2+}$ dissociation rate to be ~6000 s$^{-1}$ assuming a $k_{diss,Mg}$ of 2 × 10$^{-6}$ M$^{-1}$ s$^{-1}$ leading to a $K_{diss,Mg}$ of 3 mM (data not shown). Thus, 3 mM Mg$^{2+}$ slows the Ca$^{2+}$ association rate to TnC$^{F29W}$ only ~3.5-fold but drastically slows the Ca$^{2+}$ association rate to G34DTnC$^{F29W}$ by ~4.3 × 10$^5$-fold. Table II also compares the Ca$^{2+}$ binding properties of TnC$^{F29W}$ and G34DTnC$^{F29W}$ in the absence or presence of 3 mM Mg$^{2+}$, with or without TnI(96–148).

Over longer times (0–5 s), in the absence of Mg$^{2+}$, as the [Ca$^{2+}$] was increased from 0 to 5 μM slow decreases in the Trp fluorescence signal were observed (~1–2 s$^{-1}$) for both TnC$^{F29W}$ and G34DTnC$^{F29W}$ (less than 5% of the total Trp change, data not shown). The amplitudes of these slow decreases in Trp fluorescence decrease with increasing [Ca$^{2+}$] and were absent when the [Ca$^{2+}$] exceeded 5 μM. Computer modeling of these reactions predicted that these decreases in Trp fluorescence was associated with Ca$^{2+}$ removal from the N-domain sites of the TnCs by the high affinity C-domain sites that possess an ~100-fold slower Ca$^{2+}$ association rate and ~10-fold higher Ca$^{2+}$ affinity (20). Furthermore, the rapid increase in Trp fluorescence for both TnC$^{F29W}$ and G34DTnC$^{F29W}$ becomes too fast to observe as the [Ca$^{2+}$] exceeded 5 μM. Unexpectedly, as the [Ca$^{2+}$] was increased from 5 μM up to 1 mM another slow second order rate constant was observed for TnC$^{F29W}$ and Mg$^{2+}$ traces). Each protein (4 μM) in the same buffer as panel A plus 15 μM Ca$^{2+}$ was rapidly mixed with an equal volume of the same buffer plus 150 mM Mg$^{2+}$. Trp fluorescence was monitored as described in the legend to Fig. 3, panel C. The traces have been normalized and staggered for visual clarity. Panel C shows the rate of Ca$^{2+}$ dissociation from TnC$^{F29W}$ and G34DTnC$^{F29W}$ following the decreases in Trp fluorescence in the presence of TnI(96–148) or the increase in Quin-2 fluorescence in the presence of intact TnI. The time course of the decrease in Trp fluorescence shows that EGTA-dissociated Ca$^{2+}$ from the regulatory Ca$^{2+}$ binding sites of the G34DTnC$^{F29W}$TnI(96–148) and the TnC$^{F29W}$TnI(96–148) complexes (Fig. TnI$_{96–148}$, EGTA traces). Each TnC protein (6 μM) plus TnI(96–148) peptide (6 μM) in 10 mM MOPS, 90 mM KCl, 1 mM DTT, pH 7.0, plus 100 μM Ca$^{2+}$ was rapidly mixed with an equal volume of the same buffer plus 10 mM EGTA at 15 °C. TnI was rapidly mixed with an equal volume of the same buffer plus 10 mM EGTA at 15 °C. TnI fluorescence was monitored as described in the legend to Fig. 3, panel C. Panel C also shows the time course of the increase in Quin-2 fluorescence as Quin-2 dissociates Ca$^{2+}$ from the regulatory Ca$^{2+}$ binding sites of the G34DTnC$^{F29W}$TnI and the TnC$^{F29W}$TnI complexes (Quin-2 intact TnI traces). Each TnC (3 μM) plus intact TnI (30 μM) in 10 mM MOPS, 90 mM KCl, 1 mM DTT, pH 7.0, plus 10 μM Mg$^{2+}$ was rapidly mixed with an equal volume of the same buffer plus 150 μM Quin-2 at 15 °C. Quin-2 fluorescence was monitored as described in panel A. All the traces have been normalized and displaced vertically for clarity. All the traces in panels A–C are an average of at least 15 traces fit with a single exponential as described in the legend to Fig. 3, panel C (variance <2 × 10$^{-4}$).
G34DTnCF29W as increasing concentrations of Ca\textsuperscript{2+} shows the time course of the increases in Trp fluorescence of (except panel C).

Panel A shows the time course of the increases in Trp fluorescence of (except panel C) described in the legend to Fig. 3, after mixing was complete. Trp fluorescence was monitored as described under “Methods.”

Results. To investigate the effects of increased competitive binding of Mg\textsuperscript{2+} to the regulatory domain of G34DTnCF29W compared with TnCP29W, both proteins were reconstituted into skinned psoas muscle fibers. Fig. 7A shows the Ca\textsuperscript{2+}-dependent increase in skinned psoas muscle force with TnCendogenous (○), TnCP29W (○), or G34DTnCF29W (△) in the presence of physiological 1 mM [Mg\textsuperscript{2+}]\textsubscript{free} (60). Half-maximal force occurred at 331 ± 7, 531 ± 3, and 1830 ± 460 nM Ca\textsuperscript{2+} for TnCendogenous, TnCP29W, and G34DTnCF29W, respectively. Thus, 1 mM [Mg\textsuperscript{2+}]\textsubscript{free} shifted the Ca\textsuperscript{2+} sensitivity of force development with G34DTnCF29W–3.4-fold compared with TnCP29W, consistent with physiological competitive Mg\textsuperscript{2+} binding to G34DTnCF29W.

Because the Mg\textsuperscript{2+} affinity for the regulatory domain of G34DTnCF29W is higher than that of TnCP29W, as the [Mg\textsuperscript{2+}]\textsubscript{free} increases there should be a larger shift in the Ca\textsuperscript{2+} sensitivity of force for muscle fibers containing G34DTnCF29W compared with those containing TnCP29W. Fig. 7B shows the Ca\textsuperscript{2+}-dependent increase in skinned psoas muscle force with TnCendogenous (○), TnCP29W (●), or G34DTnCF29W (△) in the presence of 3 mM [Mg\textsuperscript{2+}]\textsubscript{free}. Half-maximal force occurred at 540 ± 25, 770 ± 18, and 5770 ± 800 nM Ca\textsuperscript{2+} for TnCendogenous, TnCP29W, and G34DTnCF29W, respectively. Thus, in the presence of 3 mM [Mg\textsuperscript{2+}]\textsubscript{free} the Ca\textsuperscript{2+} sensitivity of force development was −7.5-fold lower for G34DTnCF29W compared with TnCP29W. Furthermore, as the [Mg\textsuperscript{2+}]\textsubscript{free} was increased from 1 to 3 mM, the Ca\textsuperscript{2+} sensitivity of force development with G34DTnCF29W was decreased −3.2-fold. However, under the same conditions the Ca\textsuperscript{2+} sensitivity of force development with TnCendogenous or TnCP29W was decreased only −1.5-fold. These results are consistent with competitive Mg\textsuperscript{2+} binding to the regulatory domains of all the TnC proteins, with the largest effect occurring with G34DTnCF29W because of its higher Mg\textsuperscript{2+} affinity. Table III summarizes the skinned muscle results.

After TnC was extracted, force was decreased to 5 ± 1% of the maximal force generated with TnCendogenous (data not shown). TnCP29W was capable of recovering maximal force to 93 ± 1%, whereas G34DTnCF29W recovered only 62 ± 5% of the maximal force. It has been demonstrated that partial extraction of TnC can reduce maximal force recovery and decrease the Ca\textsuperscript{2+} sensitivity of force development (61–64). We were concerned that the decreased Ca\textsuperscript{2+} sensitivity of force development generated by G34DTnCF29W could be explained by its incomplete force recovery and not by its increased Mg\textsuperscript{2+} affinity. Fig. 7C shows that upon partial extraction of TnCendogenous (○) to levels of 87, 69, 44, and 37%, maximal force recovery shifted the Ca\textsuperscript{2+} sensitivity of force development 2.1-, 2.5-, 4.4-, and 5.4-fold, respectively. This decrease in Ca\textsuperscript{2+} sensitivity of force development caused by partial extraction of TnCendogenous was greater ± S.E. of at least three determinants. The data were fit with a linear regression where the slope represents the calculated Ca\textsuperscript{2+} association rate, except for G34DTnCF29W in the presence of 3 mM Mg\textsuperscript{2+}, which demonstrated no Ca\textsuperscript{2+} dependence of its association rate as described under “Methods.”

Fig. 6. Measurement of the Ca\textsuperscript{2+} association rates to TnCP29W and G34DTnCF29W in the absence and presence of 3 mM Mg\textsuperscript{2+}.

Panel A shows the time course of the increases in Trp fluorescence of G34DTnCF29W as increasing concentrations of Ca\textsuperscript{2+} were rapidly mixed with the protein. 4 μM G34DTnCF29W in the same buffer as described in the legend to Fig. 5 was rapidly mixed with the same buffer in the mixing chamber immediately after mixing was complete for TnCP29W (●), G34DTnCF29W (△), TnC29W plus 3 mM Mg\textsuperscript{2+} (■), or G34DTnCF29W plus 3 mM Mg\textsuperscript{2+} (▲). Each point represents the average ± S.E. of at least three determinants. The data were fit with a linear regression where the slope represents the calculated Ca\textsuperscript{2+} association rate, except for G34DTnCF29W in the presence of 3 mM Mg\textsuperscript{2+}, which demonstrated no Ca\textsuperscript{2+} dependence of its association rate as described under “Methods.”
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Fig. 7. Ca\(^{2+}\) sensitivity of force development with unextracted and reconstituted psoas muscle fibers with TnC\(^{-}\)F29W and G34DTnC\(^{-}\)F29W in the presence of 1 or 3 mM [Mg\(^{2+}\)]\(_{\text{free}}\). Panel A shows the Ca\(^{2+}\) dependence of isometric force development in unextracted (TnC\(^{-}\)endogenous, ◦) and reconstituted psoas muscle fibers with TnC\(^{\text{C29W}}\) (□) or G34DTnC\(^{\text{C29W}}\) (△) in the presence of 1 mM [Mg\(^{2+}\)]\(_{\text{free}}\). Each trace represents the mean ± S.E. of at least three separate fibers fit with a logistic sigmoid curve mathematically equivalent to the Hill equation. The conditions for the experiments are as described under “Experimental Procedures.” Panel B shows the Ca\(^{2+}\) dependence of isometric force development in unextracted (TnC\(^{-}\)endogenous, ◦) and reconstituted psoas muscle fibers with TnC\(^{-}\)F29W (■) or G34DTnC\(^{-}\)F29W (▲) in the presence of 3 mM [Mg\(^{2+}\)]\(_{\text{free}}\). Panel C shows the Ca\(^{2+}\) dependence of isometric force development in unextracted (TnC\(^{-}\)endogenous, ◦) and reconstituted psoas muscle fibers with TnC\(^{\text{C29W}}\) (1 mM [Mg\(^{2+}\)]\(_{\text{free}}\) □) or 3 mM [Mg\(^{2+}\)]\(_{\text{free}}\) ■ or G34DTnC\(^{\text{C29W}}\) (in 1 mM [Mg\(^{2+}\)]\(_{\text{free}}\) □) or 3 mM [Mg\(^{2+}\)]\(_{\text{free}}\) ■ or G34DTnC\(^{\text{F29W}}\) (in 1 mM [Mg\(^{2+}\)]\(_{\text{free}}\) □) or 3 mM [Mg\(^{2+}\)]\(_{\text{free}}\) ■) versus percent of maximal force recovery compared with the mean [Ca\(^{2+}\)]\(_{\text{free}}\) of the unextracted

nearly identical to that determined by Brandt et al. (62). A linear regression fit to our data (r\(^2\) = 0.934) predicted that at the average force recovery observed with G34DTnC\(^{\text{F29W}}\) of 62%, the Ca\(^{2+}\) sensitivity of force development would be decreased −3.3-fold due only to incomplete force recovery. Fig. 7C also shows that the average decrease in Ca\(^{2+}\) sensitivity of force development for G34DTnC\(^{\text{F29W}}\) compared with TnC\(^{-}\)endogenous at 1 (□) and 3 mM [Mg\(^{2+}\)]\(_{\text{free}}\) was 5.5- and 10.7-fold, respectively. However, after taking into account incomplete force recovery, Mg\(^{2+}\) actually decreased the Ca\(^{2+}\) sensitivity of force generated by G34DTnC\(^{\text{F29W}}\) at 1 and 3 mM −1.7- and 3.2-fold, respectively, compared with TnC\(^{-}\)endogenous. Treating the data similarly for TnC\(^{\text{F29W}}\) predicted that the shift in Ca\(^{2+}\) sensitivity of force development with TnC\(^{\text{F29W}}\) compared with TnC\(^{-}\)endogenous could be entirely explained by incomplete force recovery. Therefore, in the presence of 3 mM [Mg\(^{2+}\)]\(_{\text{free}}\), the −3.2-fold shift in Ca\(^{2+}\) sensitivity of force development with G34DTnC\(^{\text{F29W}}\) was similar to the −2.0-fold shift in Ca\(^{2+}\) affinity of G34DTnC\(^{\text{F29W}}\) compared with TnC\(^{\text{F29W}}\) either in isolation or in the presence of TnI-(96–148).

DISCUSSION

Whether Mg\(^{2+}\) competes with Ca\(^{2+}\) for the N-terminal regulatory domain of TnC is unresolved (15, 19, 23, 27, 32). Several groups demonstrated that as the [Mg\(^{2+}\)] increased, the Ca\(^{2+}\) sensitivity of TnC, Tn-activated actomyosin ATPase, and force development decreased (19, 23–31). These results suggested that Mg\(^{2+}\) binding to the regulatory domain of TnC, competitive or otherwise, affected its biochemical and physiological properties. We studied Mg\(^{2+}\) binding and exchange with the regulatory domain of TnC and its mutant, utilizing the Trp fluorescence of TnC\(^{\text{C29W}}\), which possesses a Phe → Trp mutation immediately preceding the first Ca\(^{2+}\)-binding loop.

We have previously demonstrated that an endogenous Z acid pair in the first EF-hand of CaM\(^{\text{F19W}}\) was required for physiologically relevant Mg\(^{2+}\) binding to this EF-hand (3). Because the first EF-hand of TnC has a Gly at the +z position, we hypothesized that substitution of Gly with Asp should enable the first EF-hand of TnC to bind Mg\(^{2+}\). Indeed, when Gly-34 at the +z position was substituted with Asp, the Trp fluorescence of G34DTnC\(^{\text{F29W}}\) increased −1.4-fold upon addition of Mg\(^{2+}\), with a K\(_{d,Mg}\) ∼ 295 μM. This same mutation in the absence of Mg\(^{2+}\) led to an −1.8-fold increase in Ca\(^{2+}\) affinity of the regulatory domain. However, in the presence of 3 mM Mg\(^{2+}\), the Ca\(^{2+}\) sensitivity of G34DTnC\(^{\text{F29W}}\) decreased −6-fold. From the K\(_{d,(Ca)}\) obtained in the absence and presence of Mg\(^{2+}\), we calculated the K\(_{d,(Mg)}\) to be ∼588 μM for G34DTnC\(^{\text{F29W}}\). Thus, the measured K\(_{d,(Mg)}\) was in good agreement with the calculated K\(_{d,(Mg)}\).

These experiments demonstrate that introduction of an Asp residue at the +z position in the first EF-hand of TnC\(^{\text{F29W}}\) enabled this EF-hand to competitively bind Mg\(^{2+}\) with a physiologically relevant affinity. Consistent with our results, introduction of a Z acid pair into the CD site of oncomodulin led to an −12- and 52-fold increase in Ca\(^{2+}\) and Mg\(^{2+}\) affinity to this site, respectively (65). Similarly, in CaM\(^{\text{F19W}}\), replacement of Asp with Asn at the +z position of the first EF-hand led to an −5- and 58-fold decrease in Ca\(^{2+}\) and Mg\(^{2+}\) affinity to the N-terminal sites, respectively (3, 8). Thus, it would appear that
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Table III
Summary of skinned fiber results

| Concentration of Mg²⁺ | pCa₅₀ | pCa₅₀ corrected | nH |
|----------------------|-------|-----------------|----|
| 1 mM Mg²⁺           | 6.48 ± 0.01 | 6.48 | 2.7 ± 0.1 |
| TnCendog            | 6.27 ± 0.05 | 6.43 | 2.6 ± 0.2 |
| G34DTnC²⁺²⁹W        | 5.7 ± 0.1 | 6.29 | 1.2 ± 0.2 |
| 3 mM Mg²⁺           | 6.26 ± 0.02 | 6.26 | 4.8 ± 0.9 |
| TnCendog            | 6.11 ± 0.01 | 6.27 | 3.0 ± 0.1 |
| G34DTnC²⁺²⁹W        | 5.2 ± 0.2 | 5.77 | 1.0 ± 0.1 |

*pCa₅₀ was corrected for incomplete force recovery caused by TnC²⁺²⁹W and G34DTnC²⁺²⁹W. First, the -fold decrease in [Ca²⁺]₅₀ caused by incomplete force recovery was calculated for each mutant using the linear fit to the partial TnC extraction data in Fig. 7C (-fold decrease in [Ca²⁺]₅₀ = -0.07 (percent maximal force) + 7.5, with 93% and 62% maximal force for TnC²⁺²⁹W and G34DTnC²⁺²⁹W, respectively). Second, the experimentally measured -fold decrease in [Ca²⁺]₅₀ was divided by the -fold decrease in [Ca²⁺]₅₀ caused by incomplete force recovery to obtain the corrected [Ca²⁺]₅₀, which was then converted to pCa₅₀.

Modification of the +z position in some EF-hand proteins modulates the Mg²⁺ affinity more so than the Ca²⁺ affinity.

Interestingly, the fluorescence of TnC²⁺²⁹W did not change upon binding Mg²⁺ but increased upon binding Ca²⁺ to the regulatory sites (43, 44, 48). However, we have demonstrated that Mg²⁺ can competitively bind and displace Ca²⁺ from TnC²⁺²⁹W at the Ca²⁺ dissociation rate of ~350 s⁻¹, with a Kₐ (Mg) in the range of 2.2–3.8 mM at 15 °C. This low Mg²⁺ affinity of TnC²⁺²⁹W was consistent with previously calculated values of 0.8–5 mM (averaging to 3.7 ± 0.6 mM) using other fluorescent TnCs in which competitive Mg²⁺ binding was assumed (23–26, 28, 29, 56). We propose that Mg²⁺ binds to the second regulatory Ca²⁺ binding site of TnC²⁺²⁹W because the Trp residue adjacent to the first Ca²⁺ binding site was unquenched by the direct Mg²⁺ binding studies. Recent structures of the Ca²⁺/Mg²⁺/Na⁺-binding proteins calbindin D₉k and CaM have demonstrated that Mg²⁺ binding primarily affects only the local structural environment of the cation-binding loop with the effects in CaM primarily occurring in the N-terminal portion of the loop (4, 66, 67). Thus, if Mg²⁺ bound to the first EF-hand of TnC²⁺²⁹W Trp₂⁹ should have been affected, as we have demonstrated for G34DTnC²⁺²⁹W. Therefore, Mg²⁺ must be binding to the second N-domain EF-hand of TnC²⁺²⁹W. In support of this conclusion, preliminary experiments in our laboratory utilizing the Trp fluorescence of cardiac TnC²⁺²⁹W (eTnC²⁺²⁹W) have demonstrated that direct Mg²⁺ titrations did not affect the fluorescence of this protein, whereas Ca²⁺ binding enhances the fluorescence as previously reported (68). However, as was the case with TnC²⁺²⁹W, addition of increasing [Mg²⁺] to Ca²⁺-saturated eTnC²⁺²⁹W reversed the Ca²⁺-induced increase in the Trp fluorescence with a Kₐ (Mg) of ~3.6 mM. For eTnC²⁺²⁹W only the second N-terminal EF-hand is functional and appears to bind both Ca²⁺ and Mg²⁺. In any regard, the regulatory domain of TnC under physiological [Mg²⁺] of ~1 mM and resting [Ca²⁺] of ~100 mM (60) would be ~20–30% saturated (whereas G34DTnC²⁺²⁹W would be 60–70% saturated) and thus is not Ca²⁺ specific as the current dogma claims. Thus, researchers should consider that interaction of Mg²⁺ with the regulatory domain of TnC may affect the outcome of experimental studies. The reason for the low affinity and competitive Mg²⁺ binding to the regulatory domain of TnC might be to ensure that at resting [Ca²⁺], or slight fluctuations thereof, the muscle would not be activated until neurally stimulated.

Because TnC regulates muscle contraction as a part of the Tn complex, we have also studied Ca²⁺ and Mg²⁺ binding to TnC²⁺²⁹W and G34DTnC²⁺²⁹W in the presence of the TnI(96–148) peptide. The addition of the TnI(96–148) peptide to TnC²⁺²⁹W and G34DTnC²⁺²⁹W produced ~12- and 14-fold increases in their N-domain Ca²⁺ binding affinities, respectively, as has been previously reported for intact TnI (15, 33). In the absence of Mg²⁺, the Ca²⁺ affinity of the G34DTnC²⁺²⁹W, TnI(96–148) was 1.7-fold higher than that of the TnC²⁺²⁹W-TnI(96–148) complex. However, in the presence of 3 mM Mg²⁺ the Ca²⁺ affinity of the TnC²⁺²⁹W-TnI(96–148) and G34DTnC²⁺²⁹W,TnI(96–148) complexes decreased ~1.5- and 5-fold, respectively. From the Kₐ of TnC²⁺²⁹W, we estimated Kₐ of Mg²⁺ to be ~6 and 0.7 mM, for the TnC²⁺²⁹W,TnI(96–148) and G34DTnC²⁺²⁹W,TnI(96–148) complexes, respectively. These results suggest that TnI(96–148) peptide binding increased the Ca²⁺ affinity, but not Mg²⁺ affinities of the TnI sites of TnC²⁺²⁹W and G34DTnC²⁺²⁹W. A similar result had been used previously to argue that Mg²⁺ must bind at an auxiliary N-terminal site(s) of TnC²⁺²⁹W because the Mg²⁺ affinity was not enhanced in the presence of TnI (23). However, Mg²⁺ binding to the N-domain of TnC²⁺²⁹W, or G34DTnC²⁺²⁹W, appears not to open the N-terminal hydrophobic pocket of TnC required for TnI binding that subsequently increases the Ca²⁺ affinity. Furthermore, it would appear that competitive Mg²⁺ binding and displacement of Ca²⁺ from the regulatory domain of TnC²⁺²⁹W and G34DTnC²⁺²⁹W actually closes the hydrophobic pocket.

Functionally, Mg²⁺ did not cause force development in reconstituted skeletal muscle fibers at 1 or 3 mM [Mg²⁺] free with G34DTnC²⁺²⁹W or TnC²⁺²⁹W, consistent with Mg²⁺ binding not inducing the same structural changes as does Ca²⁺. In the absence of Mg²⁺, the Ca²⁺ affinity of G34DTnC²⁺²⁹W was ~2-fold higher than that of TnC²⁺²⁹W yet the Ca²⁺ sensitivity of force development in the presence of 1 and 3 mM [Mg²⁺] free with G34DTnC²⁺²⁹W was decreased compared with TnC²⁺²⁹W, consistent with competitive Mg²⁺ binding to G34DTnC²⁺²⁹W. Furthermore, the Ca²⁺ sensitivity of force was decreased only ~1.5-fold with TnC²⁺²⁹W, and TnC²⁺²⁹W, but was decreased ~3.2-fold with G34DTnC²⁺²⁹W as the [Mg²⁺] free was increased from 1 to 3 mM. These results were also consistent with competitive Mg²⁺ binding to the regulatory domains of TnC²⁺²⁹W, and TnC²⁺²⁹W. After taking incomplete force recovery into consideration, in the presence of 3 mM Mg²⁺, G34DTnC²⁺²⁹W was ~3.2-fold less sensitive to Ca²⁺ compared with TnC²⁺²⁹W or TnC²⁺²⁹W. However, the magnitude of the shift in Ca²⁺ sensitivity could vary depending upon the cause of the reduced force activity could vary depending upon the cause of the reduced force recovery (partial TnC extraction versus substitution by a mutant). In any case, the force produced by G34DTnC²⁺²⁹W was less sensitive to Ca²⁺ than that of TnC²⁺²⁹W, consistent with G34DTnC²⁺²⁹W possessing a higher Mg²⁺ affinity.

Unexpectedly, G34DTnC²⁺²⁹W recovered only ~62% of maximal force. Our results demonstrated that reduced affinity of the regulatory domain within G34DTnC²⁺²⁹W for the inhibitory region of TnI was not the cause for the decreased force recovery. However, we cannot rule out the possibility that the conformation of the G34DTnC²⁺²⁹W,TnI complex was somehow different from that of the TnC²⁺²⁹W,TnI complex, leading to reduced force. The extent of force recovery by G34DTnC²⁺²⁹W
was similar to that of posa muscle fibers reconstituted with cTnC (69, 70). The G34D mutation is in the first EF-hand of TnC, which is the same EF-hand in cTnC that does not bind Ca$^{2+}$. It may be that the mechanism(s) that underlie the decreased force recovery of fast twitch skeletal muscle reconstituted with cTnC are the same for G34DTCNF29W. It has been speculated that the decreased force recovery with cTnC may be due in part to its decreased actomyosin-ATPase rate in reconstituted skeletal muscle systems and lack of Ca$^{2+}$ binding cooperativity because of its single regulatory Ca$^{2+}$ binding site (71). There does appear to be decreased cooperativity of force development with G34DTCNF29W, with the Gly34DTnCF29W could be used as a molecular tool to test whether the EF-hand of G34DTnCF29W, exchange of this protein into the skinned skeletal psoas muscle fibers led to lower Ca$^{2+}$ at near maximal force in reconstituted psoas muscle (47). Thus, the reason for the lack of maximal force recovery for G34DTCNF29W remains unresolved.

A major consequence of the high Mg$^{2+}$ affinity and relatively slow Mg$^{2+}$ dissociation rate of G34DTCNF29W (at a rate comparable with its Ca$^{2+}$ dissociation rate) would be to drastically slow the Ca$^{2+}$ association rate in the presence of physiological Mg$^{2+}$ by a factor of $\sim 4 \times 10^5$. Our laboratory is interested in delineating the influence of the Ca$^{2+}$ -kinetics of the regulatory domain of TnC has on striated muscle contraction and relaxation. G34DTCNF29W could be used as a molecular tool to test whether slower Ca$^{2+}$ association rates to the regulatory domain of TnC could slow Ca$^{2+}$-induced rates of muscle contraction.

In conclusion, the data suggests that the second EF-hand, but not the first, of TnC NF29W was able to competitively bind Mg$^{2+}$. Physiologically relevant Mg$^{2+}$ binding could be engineered into the first EF-hand of TnC NF29W by substituting Gly34 with Asp creating a Z acid pair. In the absence of Mg$^{2+}$, the Gly34 $\rightarrow$ Asp mutation also increased the Ca$^{2+}$ affinity of TnC NF29W (Tnl(96–148) complex). However, in the presence of Mg$^{2+}$, the Gly34 $\rightarrow$ Asp mutation decreased the Ca$^{2+}$ affinity of TnC NF29W and the TnC NF29W,Tnl(96–148) complex. Consistent with competitive Mg$^{2+}$ binding to the first EF-hand of G34DTCNF29W, exchange of this protein into skinned skeletal posa muscle fibers led to lower Ca$^{2+}$ sensitivity of force development.

Acknowledgments—We thank Warren Erdahl and Clifford Chapman for technical assistance with atomic absorption measurements of our Ca$^{2+}$ stocks, Catalina Allione for help in preparation of the manuscript, and Dr. Ruth Autschl for helpful discussion of the data.

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