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Sequence mutations in teleost cardiac troponin C that are permissive of high Ca^{2+} affinity of site II

Todd E. Gillis,[1,2] Chris D. Moyes,[3] and Glen F. Tibbits[2,4]
[1]*Department of Biological Sciences and* [2]*Cardiac Membrane Research Lab, Simon Fraser University,*
Burnaby, British Columbia V5A 1S6; [3]*Department of Biology, Queens University,*
Kingston, Ontario K7L 3N6; and [4]*Cardiovascular Sciences, British Columbia Research Institute*
for Children's and Women's Health, Vancouver, British Columbia, Canada V5Z 4H4

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Gillis, Todd E., Chris D. Moyes, and Glen F. Tibbits. Sequence mutations in teleost cardiac troponin C that are permissive of high Ca^{2+} affinity of site II. *Am J Physiol Cell Physiol* 284: C1176–C1184, 2003. First published January 8, 2003; 10.1152/ajpcell.00339.2002.—Cardiac myofibrils isolated from trout heart have been demonstrated to have a higher sensitivity for Ca^{2+} than mammalian cardiac myofibrils. Using cardiac troponin C (cTnC) cloned from trout and mammalian hearts, we have previously demonstrated that this comparatively high Ca^{2+} sensitivity is due, in part, to trout cTnC (ScTnC) having twice the Ca^{2+} affinity of mammalian cTnC (McTnC) over a broad range of temperatures. The amino acid sequence of ScTnC is 92% identical to McTnC. To determine the residues responsible for the high Ca^{2+} affinity, the function of a number of ScTnC and McTnC mutants was characterized by monitoring an intrinsic fluorescent reporter that monitors Ca^{2+} binding to site II (F27W). The removal of the COOH terminus (amino acids 90–161) from ScTnC and McTnC maintained the difference in Ca^{2+} affinity between the truncated cTnC isoforms (ScNTnC and McNTnC). The replacement of Gln^{29} and Asp^{30} in ScNTnC with the corresponding residues from McNTnC, Leu and Gly, respectively, reduced Ca^{2+} affinity to that of McNTnC. These results demonstrate that Gln^{29} and Asp^{30} in ScTnC are required for the high Ca^{2+} affinity of site II.

myocyte contractility; Antarctic icefish; salmonid

MUSCLE CONTRACTION IS INITIATED when membrane depolarization leads to an increase in cytosolic Ca^{2+}. The binding of Ca^{2+} to the low-affinity sites of troponin C (TnC) initiates a conformational change in the protein that is transmitted through the other components of the thin filament, allowing cross-bridge cycling between actin and myosin. Many of the characteristic responses of the contractile apparatus can be attributed to the Ca^{2+} binding properties of TnC. The differences between cardiac and skeletal myofibrils are due, in part, to the structural divergence in the TnC isoforms. While both fast skeletal muscle TnC (sTnC) and cardiac TnC (cTnC) are highly conserved within mammals and between mammals and the chicken, TnC from lower vertebrates exhibits less identity. Thus establishing the links between TnC structure and myofibrillar function should provide insight into both physiological responsiveness and evolutionary origins of contractile regulation.

TnC consists of two globular domains connected by an α-helical linker. Both the NH_2- and COOH-terminal domains contain two EF-hand Ca^{2+}-binding sites. The COOH-terminal domain possesses two high-affinity sites (III and IV) that are always bound with either Ca^{2+} or Mg^{2+}. These sites are considered to be structural sites, helping to anchor TnC into the troponin complex. The two isoforms of TnC that exist in vertebrate striated muscle differ in the number of active Ca^{2+}-binding sites in the NH_2-terminal domain. In the isoform of TnC found in fast skeletal muscle (sTnC), both sites I and II are functional, low-affinity Ca^{2+}-binding sites. In cTnC, which is expressed in both cardiac and slow skeletal muscle, site I is rendered nonfunctional by amino acid substitutions that disrupt its ability to form the pentagonal bipyramid necessary to coordinate Ca^{2+}. In both isoforms, Ca^{2+} binding to the NH_2-terminal site(s) is responsible for triggering contraction (30, 31). NMR structural studies have demonstrated that the Ca^{2+}-induced conformational change is greater in sTnC than in cTnC (35).

Many studies have attempted to assess the impact of the structural differences in TnC isoforms on the functional properties of striated muscle. Ca^{2+} affinity is very sensitive to pH in cardiac muscle but much less so in skeletal muscle. Studies using transgenic mice (24) and TnC replacement in skinned fibers (7, 25) have demonstrated that TnC isoforms influence the pH sensitivity of striated muscle. Cardiac muscle demonstrates important differences in the length-tension relationship, the basis of the Starling relationship, that have been attributed, at least in part, to cTnC (2, 13), though there is also compelling evidence to the contrary (22, 26). Differences in temperature dependence of cardiac and skeletal muscle contractility can also be attributed, in part, to the isoform of TnC that they contain. The Ca^{2+} affinity of skeletal myofilaments is relatively insensitive to temperature (11, 36), whereas

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Address for reprint requests and other correspondence: G. F. Tibbits, Cardiac Membrane Research Lab, Simon Fraser Univ., 8888 Univ. Drive, Burnaby, BC, Canada V5A 1S6 (E-mail: tibbits@sfu.ca).

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cardiac muscle becomes desensitized to Ca^{2+} at low temperatures (5, 6, 14, 16, 37). Replacement of native cTnC in skinned cardiac muscle with sTnC attenuates this desensitizing effect of temperature (15). This loss of Ca^{2+} sensitivity at low temperature has been attributed to a reduction in Ca^{2+} affinity of cTnC site II (10).

The impact of temperature on myocardial Ca^{2+} sensitivity is particularly important in cold-blooded animals, such as fish, that experience low and fluctuating environmental temperatures. Functional comparison of cardiac myofibrils from mammals and rainbow trout suggests that trout cardiac muscle has a higher inherent Ca^{2+} affinity (6). It is thought that this higher affinity facilitates cardiac function at the low temperatures (0–20°C) normally experienced by this fish (6). We have demonstrated that the high Ca^{2+} sensitivity of trout myofibrils is due, in part, to the differences in the affinity of mammalian and trout cTnC for Ca^{2+} (10). Although the amino acid sequence of trout cTnC (ScTnC) is 91% identical to mammalian cTnC (McTnC) (27), and site II is completely conserved, ScTnC has 2.3-fold higher Ca^{2+} affinity than McTnC at 21°C (10). This difference in affinity was maintained when McTnC and ScTnC Ca^{2+} binding were compared at 7 and 37°C and over a range of pH values. The comparatively higher Ca^{2+} affinity of ScTnC would allow for force generation to be initiated at a lower intracellular $[Ca^{2+}]$ in the trout myocyte.

The purpose of the present study was to identify the residues responsible for interspecies differences in Ca^{2+} affinity of site II in cTnC. Although all known mammalian cTnC amino acid sequences exhibit 100% identity, there are differences between fish and mammals and within fish species. We created mutants to compare the impact of differences between salmonid (ScTnC) and McTnC. We also exploited naturally occurring differences between trout and Antarctic icefish (40). Ca^{2+} binding to site II was measured by titrating the F27W cTnC mutants with Ca^{2+} while measuring fluorescence. These results demonstrate that there is no difference in the Ca^{2+} affinity of ScTnC and icefish cTnC (IFcTnC), despite nine amino acid differences. The differences in Ca^{2+} affinity between mammals and fish can be attributed to amino acid substitutions in the NH_2-terminal domain, because the removal of the COOH terminus (amino acids 90–161) through the creation of cNTnC mutants maintained the difference in Ca^{2+} affinity. Gln^{29} and Asp^{30} were also demonstrated as being required for the higher Ca^{2+} affinity of ScNTnC.

METHODS

Construction of the IFcTnC F27W mutant. IFcTnC cDNA cloned into PCR2.1-TOPO was first subcloned into the pGex expression vector. To accomplish this, we engineered *Bam*HI and *Eco*RI restriction sites onto the 5′- and 3′-ends, respectively, of the IFcTnC cDNA, using *Accurase* DNA polymerase (DNmp, Farnborough Hants, UK). The sequences of the 5′ sense oligonucleotide primers used were as follows: *Bam*HI, TCAGGATCCATCGAAGGTCGTATGAACGACATCTACAAAGCA; *Eco*RI, GAGTTCATGAAAGGAGTAGAATAAGAATTCGCA. After PCR, the product was purified by gel electrophoresis and use of the QIAquick gel extraction kit (Qiagen, Mississauga, ON, Canada) and then digested with *Bam*HI and *Eco*RI (Pharmacia Biotech, Baie d'Urfé, QC, Canada). pGex plasmid was similarly digested and then ligated with the cassette using T4 DNA ligase (GIBCO BRL, Gaithersburg, MD), and the sequence of the insert was confirmed by sequencing at the Nucleic Acid/Protein Service Unit, University of British Columbia (UBC; Vancouver, BC, Canada) using Ampli*Taq* dye terminator cycle sequencing.

To substitute Trp for Phe at residue 27, we used the parental plasmid (pGex) containing the wild-type IFcTnC gene insert as a template for the extension of sense and antisense oligonucleotide primers containing a tryptophan point mutation as described in Gillis et al. (10). The sequence of the 5′ sense oligonucleotide primer was GCCGCCTTTGACATCTGGGTACCAGATGCCGAG. A cassette containing the mutation was made from the mutated plasmid by using the restriction enzymes *Bam*HI and *Sty*I (New England Biolabs, Mississauga, ON, Canada) and was ligated into the similarly digested parental plasmid containing the wild-type IFcTnC gene. The nucleotide sequence of the newly mutated insert was confirmed by sequencing. From this point on, IFcTnC will refer to F27W IFcTnC.

Construction of McNTnC and ScNTnC F27W mutants. To construct McNTnC and ScNTnC F27W mutants, we introduced a stop codon into McTnC and ScTnC cDNA after the Ser codon at residue 89 using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Both of these gene constructs had been previously mutated to contain the F27W mutation and then cloned into the pGex expression plasmid from Pharmacia Biotech as described in Gillis et al. (10). These existing parental cDNA inserts were used as templates for the extension of sense and antisense oligonucleotide primers containing the stop codon TAA. The sequences of the 5′ sense oligonucleotide primers were as follows: ScTnC, GGACGACAGCTAAGGGAAAACAGAGG; McTnC, GGTTCGGTGTATGAAAGATGACAGCTAAGGAAAAAC. Cassettes containing the mutation were then made using the restriction enzymes *Sty*I and *Bsm*I (New England Biolabs). Full-length F27W ScTnC and McTnC cDNA constructs subcloned into the pGex vector were similarly digested, and the cassette and plasmid were then purified as described above and ligated. The nucleotide sequences of the two newly mutated inserts were confirmed by sequencing. From this point on, McNTnC will refer to F27W McNTnC and ScNTnC will refer to F27W ScNTnC.

Construction of Q29L/D30G ScNTnC, Q29L ScNTnC, D30G ScNTnC, L29Q/G30D McNTnC, and D2N/L29Q/G30D McNTnC F27W mutants. The cDNA constructs in the pGex plasmid created to synthesize ScNTnC and McNTnC were used as templates for the extension of sense and antisense oligonucleotide primers containing codons to manipulate the residues at positions 29 and 30 using the QuickChange site-directed mutagenesis kit. The sequences of the 5′ sense oligonucleotide primers used were as follows: Q29L/D30G ScNTnC, GCCTTTGACATCTGGATCCTGGGGGCGGAGGACGGC; Q29L ScNTnC, GCCTTTGACATCTGGATCCTGGATGCGGAGGACGGC; D30G ScNTnC, GCCTTTGACATCTGGATCCAGGGGGCGGAGGACGGC; L29Q/G30D McNTnC, GCCTTCGACATCTGGGTGCAGGATGCAGAGGATGGCTGC. Cassettes containing the mutations were made using *Sma*I (GIBCO BRL) and *Bsm*I and were then ligated into the parental plasmids that had been similarly digested. The nucleotide sequences of the inserts were confirmed by sequencing. Once the sequence of L29Q/G30D McNTnC was confirmed, it was used as a template for the extension of sense and antisense oligonucleotide primers to make the D2N/L29Q/G30D McNTnC mutant. The sequence of the 5′ sense oligonucleotide primer used to replace the Asp at residue 2 with an Asn was CCATCGAAGGTCGTATGAATGACATCTATAAGGCGGC. A cassette containing the mutation was made using *Bsm*I and *Bam*HI and was then ligated into the parental plasmid that had been similarly digested. The nucleotide sequence of the insert was confirmed by sequencing.

Expression and purification of cTnC mutants. The pGex plasmids containing the ScNTnC, McNTnC, IFcTnC, Q29L/D30G ScNTnC, Q29L ScNTnC, D30G ScNTnC, L29Q/G30D McNTnC, and D2N/L29Q/G30D McNTnC inserts were transformed into the *Escherichia coli* strain BL21 for protein expression. The cTnC mutants were expressed as glutathione *S*-transferase fusion proteins that were then digested and purified as described previously (10). The identities of all cTnC mutants were confirmed by NH_2-terminal microsequencing and amino acid analysis completed at the Nucleic Acid/Protein Service Unit, UBC. The purities of the isolated proteins as well as their atomic masses were confirmed by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry completed at the UBC Mass Spectrometry Center. Collectively, these tests confirmed the identity of all cTnC mutants.

Solutions used in fluorescence studies. The solution used to measure fluorescence at 21°C, pH 7.0, was identical to that described in Gillis et al. (10) (in mM: 1.0 EGTA, 0.03 $CaCl_2$, 112.0 KCl, and 50 MOPS). A miniature Ca^{2+} electrode, made according to Baudet et al. (3), was used to confirm the purity and apparent Ca^{2+} affinity constant (K'_{Ca}) of the EGTA used in the solution under the experimental conditions. This electrode was also used to test the initial pCa of the solution and the change in pCa during Ca^{2+} titration. This electrode was calibrated by using Ca^{2+} standards (Orion Research, Boston, MA) and was sensitive to a pCa of 8.0. The EGTA had a calculated purity of 95.6% and a K'_{Ca} of 2.99×10^6 M^{-1}. It should be noted that Mg^{2+} was not included in the reaction buffers despite its physiological presence. The solution composition was carefully considered for simplicity of interpretation of the in vitro experiments as well as accuracy of pCa in the titrations, which were determined as described above.

Fluorescence studies. The fluorescence studies were carried out as previously described (10) using a Photon Technology International (PTI) model C-30 spectrofluorometer (London, ON, Canada) attached to a NesLab (Portsmouth, NJ) water bath to maintain the cuvette at 21.0 ± 0.1°C. Fluorescence was measured during Ca^{2+} titration of the cTnC mutants by using an excitation wavelength of 276 nm and an emission wavelength of 330 nm. The slit widths for both the excitation and fluorescence light pathways were adjusted to setting 4 on the PTI spectrofluorometer. The relative fluorescence was calculated as the ratio of the fluorescence of the protein and an internal rhodamine standard. The relative fluorescence was calculated as the ratio of the fluorescence of the protein and an internal rhodamine standard. Before the beginning of fluorescence measurements, the current gain setting of each photomultiplier tube (PMT) of the spectrofluorometer was adjusted so that the relative fluorescence of the Ca^{2+}-saturated cTnC mutants was equal to unity for each mutant.

Data manipulation and statistical analysis. The Ca^{2+}-dependent component of the fluorescence measurements from each titration was determined by subtracting the fluorescence at basal $[Ca^{2+}]$ from all measurements and then expressing the resultant values as percentages of the maximum fluorescence. Each data set was fitted by using the Hill equation with Origin 6.0. (Microcal Software, Northhampton, MA) as previously described (10). The χ^2, which was used as a goodness-of-fit index of the Hill equation to our data, ranged from 0.0017 ± 0.0006 (D30G ScNTnC) to 0.0003 ± 0.00008 (IFcTnC). The effect of the mutations on the $K_{1/2}$ values ($[Ca^{2+}]$ at half-maximal fluorescence) determined by the Hill equation curve fitting were analyzed statistically using a one-way repeated measures ANOVA followed by Bonferroni post hoc tests using the statistical software package SigmaStat. The values reported for $K_{1/2}$ are expressed as means ± SE in pCa units. Two means were considered to be significantly different when the P value was <0.05.

RESULTS

Ca^{2+} affinity of IFcTnC at 21°C, pH 7.0. Because of its location in the cTnC molecule, the fluorescent reporter monitors Ca^{2+} binding to site II. The $K_{1/2}$ values were used as a measure of the Ca^{2+} binding characteristics of site II. Visual comparison of the titration curves of IFcTnC and full-length ScTnC reveals that these curves are superimposable and are shifted to the left of McTnC (Fig. 1*A*). The $K_{1/2}$ of IFcTnC is not significantly different from that previously reported for full-length ScTnC (10) and is 2.4 times less than that of McTnC under the same conditions (Table 1). These results indicate that site II of IFcTnC has Ca^{2+} binding abilities similar to those of ScTnC.

Comparison of the Ca^{2+} affinities of ScNTnC and McNTnC at 21°C, pH 7.0. Comparison of the Ca^{2+} titration curves of ScNTnC and McNTnC reveals that the curve for ScNTnC is shifted to the left of McNTnC, indicating that less Ca^{2+} is required to saturate site II of ScNTnC (Fig. 1*B*). The $K_{1/2}$ of ScNTnC is ~0.30 pCa units greater than that of McNTnC, demonstrating that it has a higher Ca^{2+} affinity (Table 1). The removal of the COOH-terminal domain caused the $K_{1/2}$ values of both ScNTnC and McNTnC to be ~0.44 pCa units higher at 21°C, pH 7.0, than the respective full-length isoform (Table 1).

Ca^{2+} affinities of Q29L/D30G ScNTnC, Q29L ScNTnC, D30G ScNTnC, L29Q/G30D McNTnC, and D2N/L29Q/G30D McNTnC at 21°C, pH 7.0. The replacement of Gln^{29} and Asp^{30} in ScNTnC with Leu and Gly, respectively, caused site II of Q29L/D30G ScNTnC to have Ca^{2+} binding characteristics similar to those of McNTnC. The titration curves of Q29L/D30G ScNTnC and McNTnC are superimposable (Fig. 2*A*), and there were no differences in the $K_{1/2}$ values of the two proteins (Table 1). The Hill coefficient is typically used to describe the cooperativity of Ca^{2+} binding to a single molecule with multiple binding sites (sTnC) or of Ca^{2+}-activated tension in a functioning myofibril. However, this study determined that in the in vitro Ca^{2+} binding of cTnC, which has a single activation site, cooperativity is highly unlikely and is reflected in the fact the Hill coefficients were approximately unity for all cTnCs. In these experiments, the Hill coefficient is used only as a parameter of curve fitting, and a physiological interpretation of these relatively small differences has been avoided. The Hill coefficients for the Ca^{2+} binding curves of McNTnC and Q29L/D30G ScNTnC do not differ, reflecting the similarities in the shape of the Ca^{2+} binding curves (Table 2).

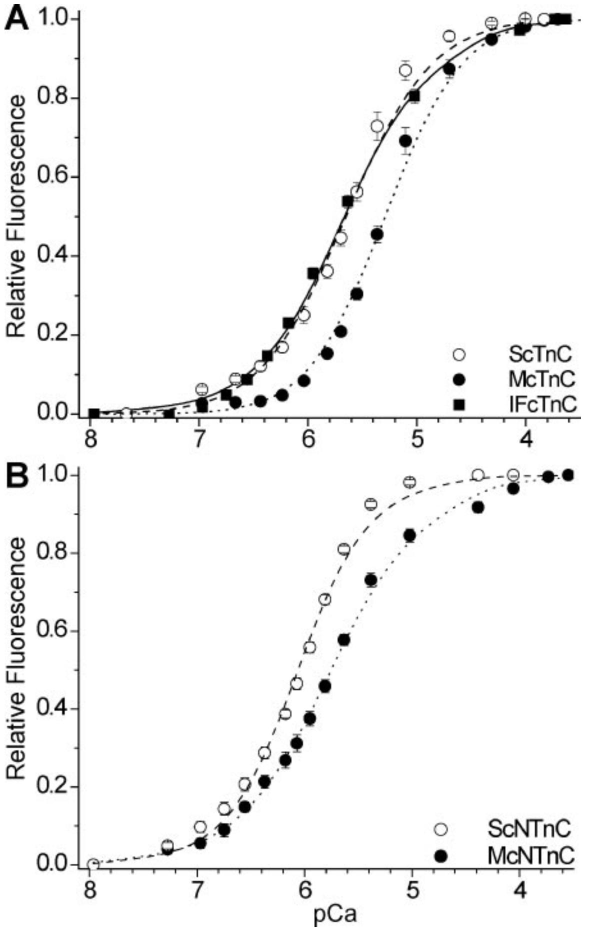

Fig. 1. Comparison of the Ca^{2+} titration curves of salmonid cardiac troponin C (ScTnC), mammalian cardiac troponin C (McTnC), icefish cardiac troponin C (IFcTnC), ScTnC NH_2-terminal domain (ScNTnC), and McTnC NH_2-terminal domain (McNTnC) at 21°C, pH 7.0. Data are normalized with respect to the maximal fluorescence of each Ca^{2+} titration and presented as means ± SE. The curves generated by fitting the data with the Hill equation have been added for comparison with the data points. *A*: titration of fluorescence of ScTnC ($n = 9$), McTnC ($n = 10$), and IFcTnC ($n = 8$). *B*: titration of fluorescence of ScNTnC ($n = 6$), 1–89 McNTnC ($n = 6$). In *A*, there is no difference between the $K_{1/2}$ values (pCa values at half-maximal fluorescence) of the curves for ScTnC and IFcTnC, whereas the $K_{1/2}$ of the curve for McTnC is significantly different from those of both ScTnC and IFcTnC. Data for ScTnC and McTnC are from Gillis et al. (9). In *B*, the $K_{1/2}$ of the curve for ScNTnC is significantly different from that for McNTnC.

Replacement of Gln with Leu at residue 29 in ScNTnC (Q29L ScNTnC) decreased $K_{1/2}$ by 0.42 pCa units to 5.64 ± 0.31, a value significantly lower than the $K_{1/2}$ values of both ScNTnC and McNTnC (Table 1). The mutant D30G ScNTnC exhibited a decrease in the $K_{1/2}$ of 0.12 pCa units compared with ScNTnC. The $K_{1/2}$ of D30G ScNTnC, however, was significantly higher than that of McNTnC (Table 1). Comparison of the titration curve of D30G ScNTnC with those of ScNTnC and McNTnC reveals that at low $[Ca^{2+}]$ (pCa 8 to 7.5) this curve is shifted to the left of ScNTnC, whereas at higher concentrations the curve shifts to the right of ScNTnC to become more like McNTnC (Fig. 2*B*). It should be noted that site II of D30G ScNTnC never fully saturated with Ca^{2+}, because fluorescence continued to gradually increase at $[Ca^{2+}]$ greater than pCa 3.5. The fluorescence at pCa 3.5, however, was arbitrarily taken as maximum for fitting the data with the Hill equation. The difference in the shape of the Ca^{2+} binding curves of ScNTnC and D30G ScNTnC is reflected in the Hill coefficients with that for ScNTnC being greater, indicating a steeper slope (Table 2).

The replacement of Leu^{29} and Gly^{30} in McNTnC with Gln and Asp, respectively, decreased the $K_{1/2}$ by 0.09 pCa units (Table 1). The Ca^{2+} titration curve of the mutant protein, L29Q/G30D McNTnC, was shifted to the right of the Ca^{2+} titration curves for both McNTnC and ScNTnC (Fig. 2*C*). The replacement of Asp with Asn at position 2 in L29Q/G30D McNTnC, with the creation of D2N/L29Q/G30D McNTnC, decreased the $K_{1/2}$ by 0.16 pCa units relative to L29Q/G30D McNTnC (Table 1).

DISCUSSION

Heart rate is determined by many factors, but with each contraction cycle, Ca^{2+} binds and releases from TnC. If the Ca^{2+} affinity of TnC is too high, the rate of relaxation will be limited. Thus the cTnC-Ca^{2+} on- and off-rate constants must be compatible with cardiac performance. Despite the difference of nearly three orders of magnitude in heart rate across birds and mammals, there is very little variation in cTnC primary sequence. Within mammals and between mammals and the chicken, cTnC variation does not seem to be an important determinant of interspecies differences in myocardial contractility. When cTnC structure is compared more widely across vertebrates, a

Table 1. *$K_{1/2}$ of Ca^{2+} binding to McNTnC, ScNTnC, Q29L/D30G ScNTnC, Q29L ScNTnC, D30G ScNTnC, L29Q/G30D McNTnC, D2N/L29Q/G30D McNTnC, IFcTnC, McTnC, and ScTnC at 21.0°C, pH 7.0*

	IFcTnC	McTnC	ScTnC	Q29L/D30G ScTnC	Q29L ScTnC	D30G ScTnC	L29Q/G30D McTnC	D2N/L29Q/G30D McTnC
NH_2 domain		5.76 ± 0.27†	6.06 ± 0.22	5.75 ± 0.16†	5.64 ± 0.31*	5.94 ± 0.02	5.67 ± 0.02*	5.51 ± 0.03
n		6	6	7	6	8	7	8
Full-length	5.67 ± 0.03*	5.29 ± 0.04	5.65 ± 0.04*					
n	8	10	9					

Values are means ± SE; *n* = no. of experiments. Values indicated with the same symbol are not significantly different from each other ($P \geq 0.05$). IFcTnC, icefish cardiac troponin C; McTnC, mammalian cardiac troponin C; ScTnC, salmonid cardiac troponin C; ScNTnC, NH_2-domain ScTnC; McNTnC, NH_2-domain McTnC. Data for full-length ScTnC and McTnC are from Gillis et al. (10).

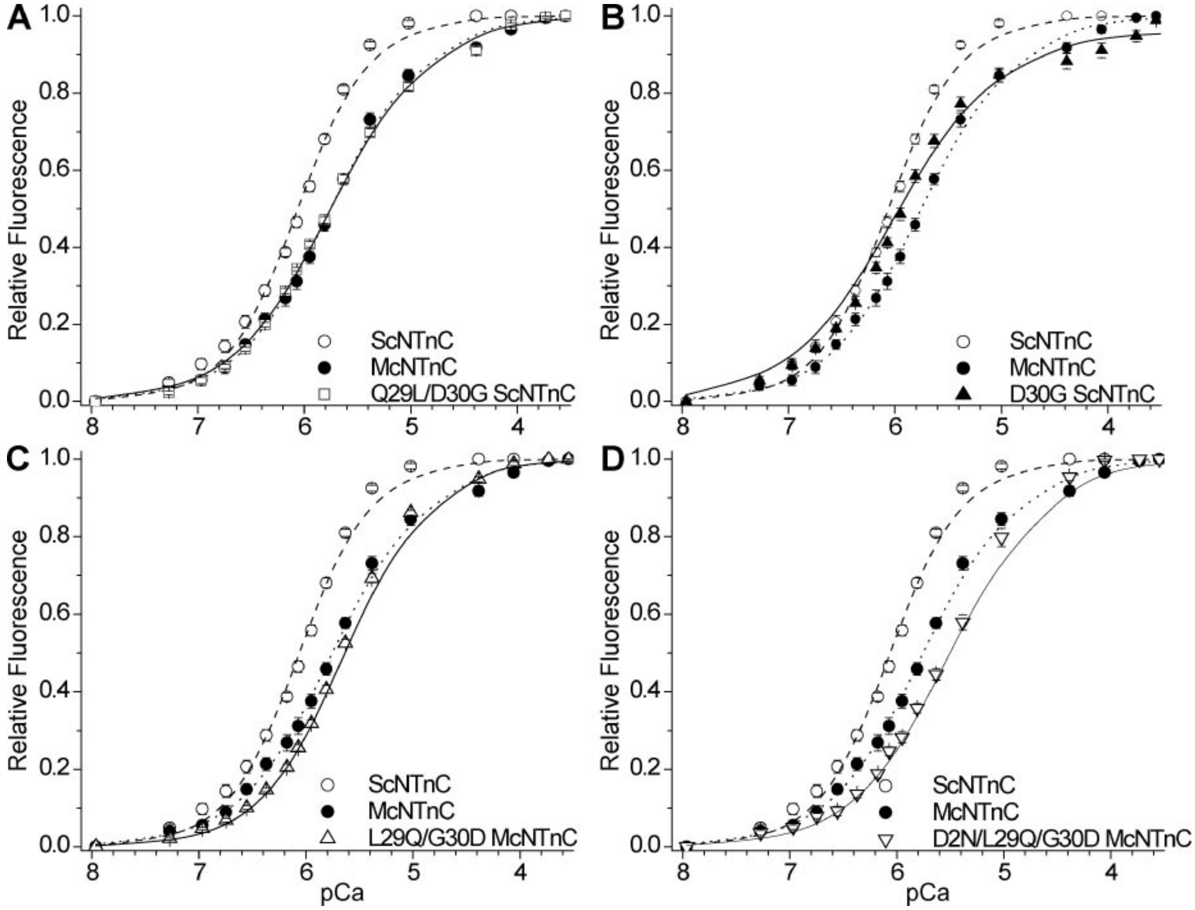

Fig. 2. Comparison of the Ca^{2+} titration curves of ScNTnC, McNTnC, Q29L/D30G ScNTnC, D30G ScNTnC, and L29Q/G30D McNTnC at 21°C, pH 7.0. Data are normalized with respect to the maximal fluorescence of each Ca^{2+} titration and presented as means ± SE. The curves generated by fitting the data with the Hill equation have been added for comparison with the data points. *A*: titration of fluorescence of ScNTnC ($n = 6$), 1–89 McNTnC ($n = 6$), and Q29L/D30G ScNTnC ($n = 7$). *B*: titration of fluorescence of ScNTnC, 1–89 McNTnC, and D30G ScNTnC ($n = 8$). *C*: titration of fluorescence of ScNTnC, 1–89 McNTnC, and L29Q/G30D McNTnC ($n = 7$). *D*: titration of fluorescence of ScNTnC, 1–89 McNTnC, and D2N/L29Q/G30D McNTnC ($n = 8$). In *A*, the $K_{1/2}$ of the curve for ScNTnC is significantly different from those for Q29D/L30G ScNTnC and McNTnC, and there is no difference between the $K_{1/2}$ values of Q29L/D30G ScNTnC and McNTnC. In *B*, the $K_{1/2}$ of the curve for D30G ScNTnC is significantly different from those of ScNTnC and McNTnC. In *C*, the $K_{1/2}$ of the curve for L29Q/G30D McNTnC is significantly different from those for ScNTnC and McNTnC. In *D*, the $K_{1/2}$ of the curve for D2N/L29Q/G30D McNTnC is significantly different from those for ScNTnC and McNTnC.

greater degree of variability is seen. Among the differences between fish and mammals and within fishes (40), it is not clear which structural variants have functional consequences.

Of the 13 sequence differences between McTnC and ScTnC, 5 occur in the NH_2-terminal domain (40). To determine the residues responsible for the higher Ca^{2+} affinity of ScTnC, we focused on this region of the protein. Previous studies have established that sequence manipulations within the NH_2-terminal domain can have significant effect on the ability of TnC to bind Ca^{2+} (12, 31). Examination of the cTnC isoform

Table 2. *Hill coefficients of Ca^{2+} binding to McNTnC, ScNTnC, Q29L/D30G ScNTnC, Q29L ScNTnC, D30G ScNTnC, L29Q/G30D McNTnC, D2N/L29Q/G30D McNTnC, IFcTnC, McTnC, and ScTnC at 21.0°C, pH 7.0*

	IFcTnC	McTnC	ScTnC	Q29L/D30G ScTnC	Q29L ScTnC	D30G ScTnC	L29Q/G30D McTnC	D2N/L29Q/G30D McTnC
NH_2 domain		1.00 ± 0.04*†	1.31 ± 0.04‡	0.95 ± 0.03†	1.09 ± 0.03*	0.99 ± 0.02†	1.14 ± 0.02	0.97 ± 0.04†
n	6	6	7	6	8	7	8	
Full length	1.08 ± 0.03*	1.47 ± 0.06	1.27 ± 0.06‡					
n	8	10	9					

Values are expressed as means ± SE; n = no. of experiments. Values indicated with the same symbol are not significantly different from each other ($P \geq 0.05$). Data for full-length ScTnC and McTnC are from Gillis et al. (10).

cloned from another fish, the Antarctic icefish, reveals that there are nine differences in sequence between icefish cTnC and ScTnC. However, of the five sequence differences between ScTnC and McTnC in the NH$_2$-terminal domain, three also occur between icefish cTnC and McTnC. These are Asn^2, Gln^{29}, and Asp^{30} (Fig. 3) (40). In McTnC, the residues at these positions are Asp, Leu, and Gly, respectively (Fig. 3). The titration curves of IFcTnC and ScTnC are superimposable, and the $K_{1/2}$ values of these curves are similar, demonstrating that the differences in sequence between IFcTnC and ScTnC do not appear to affect Ca^{2+} affinity. IFcTnC also contains Asn^2, Gln^{29}, and Asp^{30}, suggesting that one or a combination of these amino acids is responsible for the high Ca^{2+} affinity of ScTnC and IFcTnC.

To confirm that sequence differences in the NH$_2$-terminal domain of ScTnC are responsible for its higher Ca^{2+} affinity, we removed the COOH terminus from both ScTnC and McTnC, creating cNTnC mutants. cNTnC mutants have been used in previous studies to examine the structure and Ca^{2+} activation of the NH$_2$-terminal domain (20, 34, 35). Spyracopoulos et al. (35) have demonstrated that the Ca^{2+}-saturated NMR solution structure of McNTnC is similar to that of the Ca^{2+}-saturated NH$_2$ domain of the full-length mutant (C35S/C84S) chicken cTnC (33). In the present study, ScNTnC and McNTnC had a higher Ca^{2+} affin-

```
                                             Site I
                                          ============
                       10        20        30        40
                       |         |         |         |
H-B-P         MDDIYKAAVEQLTEEQKNEFKAAFDIFVLGAEDGCISTKELGKVMRMLG
Salmon        ,N,,,,,,,,,,,D,,,,,,,,,,,,,IQD,,,,,,,,,,,,,,,,,,,
Icefish       ,N,,,,,,,,,,,DD,,,,,,,,,,,,,QD,,,,,,,,,,,,,,,,,,,
Frog          ,,,,,,,,,,,,,,,,,,,,R,,,,,,,QD,,,,,,,,,,,,,,,,,,,
                                          x y z-y-x -z
                              ~~~~~A~~~~~~        ~~~~~B~~~~~~

                                Site II
                             ============
              50        60        70        80        90
              |         |         |         |         |
H-B-P         QNPTPEELQEMIDEVDEDGSGTVDFDEFLVMMVRCMKDDSKG
Salmon        ,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,
Icefish       ,,,,,,,,,,,T,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,
Frog          ,,,,,,,,,,,,,,,,,,,M,,,,,,,,,,,,,,,,,,,,,,
                             x y z-y-x -z
                  ~~~~~C~~~~~~        ~~~~~D~~~~~~

                             Site III
                           ============
                      100       110       120
                      |         |         |
H-B-P         KSEEELSDLFRMFDKNADGYIDLEELKIMLQATGET
Salmon        ,T,,,,A,,,C,,,,,,,,,,,,Q,,,V,,E,,,,A
Icefish       ,T,,,,AE,,,,,,,,S,,,,,,D,,,M,,ES,,,A
Frog          ,,,,,,,,,,,,,,,,,,,,,,FD,,,M,,E,,,,,
                           x y z-y-x -z
                ~~~~~E~~~~~~         ~~~~~F~~~~~

                              Site IV
                           ============
                130       140       150       160
                |         |         |         |
H-B-P         ITEDDIEELMKDGDKNNDGRIDYDEFLEFMKGVE
Salmon        ,,,,,,,,,,,,,,,,,,,K,,,,,,,,,,,,,,
Icefish       ,,,,,,,,,,,,,,,,,,,K,,,,,,,,,,,,,,
Frog          ,,,,,,,,,,R,,,,,,,,,,,,,,,,,,,,,,,
                           x y z-y-x -z
                ~~~~~G~~~~~         ~~~~~H~~~~~~
```

Fig. 3. Comparison of the amino acid sequences of human/bovine/porcine (H-B-P) cTnC, salmonid (*Oncorhynchus mykiss*) cTnC (27), icefish (*Chaenocephalus aceratus*) cTnC (40), and African clawed frog (*Xenopus laevis)* cTnC (41). Regions of identity between H-B-P cTnC and the sequences of salmon, icefish, and frog cTnC are denoted by commas. Ca^{2+}-binding sites I–IV are based on analogous regions in the Herzberg-Moult-James (HMJ) model (17) for sTnC. Secondary structures are reported for comparison in the context of the HMJ model. Helices A–H are denoted by tilde (~) and coordinating residues by x, y, z, −y, −x, and −z.

ity than the respective full-length isoforms. The fact that this difference in affinity was similar for both McNTnC and ScNTnC suggests that any alteration in the ability of site II to bind Ca^{2+} caused by removal of the COOH-terminal domain was similar for both isoforms.

The difference in affinity between ScNTnC and McNTnC is consistent with that between the full-length ScTnC and McTnC (10) (Table 1). These data demonstrate that the removal of the COOH terminus of ScTnC and McTnC maintains the difference in affinity between isoforms while removing 8 of the 13 differences in amino acid sequence (Fig. 3). This result corroborates the IFcTnC data that suggested that NH_2-terminal domain amino substitutions are responsible for the differences in Ca^{2+} affinity.

To identify the specific residues responsible for the high Ca^{2+} affinity of ScTnC, we next focused on Gln^{29} and Asp^{30} because these nonconservative substitutions occur in series in a region of the protein, site I, that while unable to bind Ca^{2+} directly has significant influence on the Ca^{2+} binding characteristics of cTnC (12, 31). Replacement of Gln^{29} and Asp^{30} with the corresponding residues from McTnC, Leu and Gly, caused the new construct, Q29L/D30G ScNTnC, to behave almost identically to McNTnC. The $K_{1/2}$ of the mutant was not different from that of McNTnC, and the Ca^{2+} titration curves are virtually superimposed. Together, these results demonstrate that Gln^{29} and/or Asp^{30} is required for the higher affinity of ScNTnC.

The Ca^{2+} affinities of Q29L ScNTnC and D30G ScNTnC were measured to determine whether both Gln^{29} and Asp^{30} are necessary for the high Ca^{2+} affinity of ScNTnC. Both mutants demonstrated lower Ca^{2+} affinity than did ScNTnC. The replacement of Asp with Gly appears to make the Ca^{2+} affinity of the mutant intermediate between ScNTnC and McNTnC. The inability of D30G ScNTnC to become saturated at high $[Ca^{2+}]$, however, suggests that the protein, as a result of this mutation, has lost some functional integrity. We have previously observed a similar loss of functionality when the ability of ScTnC to bind Ca^{2+} was measured at 37°C (10). Because 37°C is well above the physiological temperature of the protein and lethal to the trout, this result was interpreted as a loss of thermal stability (10).

The specific mechanism by which the presence of Gln and Asp at residues 29 and 30, respectively, increases the affinity of site II in ScTnC is not known; however, we suggest that it is due to an allosteric effect on the ability of site II to bind Ca^{2+}. Additionally, through interpretation of $^3J_{HNH\alpha}$ coupling constants and data from ^{15}N relaxation measurements obtained from NMR studies of apo- and Ca^{2+}-saturated McNTnC, Spyracopoulos et al. (35) have suggested that sites I and II are structurally linked. It is not unreasonable, therefore, to propose that the manipulation of the sequence in this area of the protein could allosterically affect the ability of site II to bind Ca^{2+}. The replacement of a hydrophobic residue (Leu) with Gln and the addition of a negative charge through the insertion of Asp are likely to have an effect on protein tertiary structure, consistent with NMR solution observations (4). Additionally, titration of ^{15}N-labeled ScNTnC while monitoring two-dimensional ^1H,^{15}N-heteronuclear single quantum correlation NMR spectra has demonstrated that site I behaves differently in ScNTnC than in McNTnC as it continues to remain responsive to Ca^{2+} following the saturation of site II (9). Together, these results demonstrate that site I in ScNTnC differs both structurally and functionally from McNTnC.

To determine whether only Gln^{29} and Asp^{30} are responsible for the high Ca^{2+} affinity of ScNTnC, we mutated the sequence of McNTnC to insert these residues at positions 29 and 30 in place of Leu and Gly, respectively. The Ca^{2+} affinity of this new protein was significantly less than that of both ScTnC and McTnC. This result suggests that one or more residues in combination with Gln^{29} and Asp^{30} is/are responsible for the high Ca^{2+} affinity of ScTnC. The three remaining possibilities are Asn^2, Asp^{14}, and Val^{28}, which are in place of Asp, Glu, and Ile, respectively, in McNTnC. The D14E and V28I substitutions are fairly conservative. Additionally, IFcTnC, which has the same Ca^{2+} affinity as ScTnC, also contains Asn at position 2 (Fig. 3). The sequence of cTnC cloned from the frog, *Xenopus laevis*, another ectothermic species, contains Asp at position 2 as in McTnC (41) (Fig. 3). This suggests that the presence of Asn at residue 2 may be unique only to cTnC cloned from fish and is not a common genotype of ectothermic hearts. To determine whether Asn^2 is required, in addition to Gln^{29} and Asp^{30}, for the high Ca^{2+} affinity of ScNTnC, we replaced the Asp^2 in L29Q/G30D McNTnC with an Asn. The Ca^{2+} affinity of the resultant mutant was lower than that of L29Q/G30D McNTnC. This suggests that either Asn^2 is not involved in the high Ca^{2+} affinity of site II in ScTnC or that other residues in addition to Asn^2, Gln^{29}, and Asp^{30} are required. This result also demonstrates that the manipulation of the amino acid sequence far outside of site II affects Ca^{2+} affinity. This finding is supported by earlier work by Gulati et al. (12), who demonstrated that the NH_2-terminal α-helical overhang of cTnC, where residue 2 is located, plays a role in regulating the Ca^{2+} binding characteristics of site II in cTnC.

Fluorescent probes engineered into TnC through Phe to Trp mutation in site I have been used previously to study the Ca^{2+} binding dynamics of this molecule (8, 19, 21, 27, 29, 32, 38). We have demonstrated the effectiveness of Trp at residue 27 in reporting Ca^{2+} binding to site II in McTnC and ScTnC without significantly affecting the α-helical content of the protein, which reflects the general structure of the molecule, using far UV circular dichroism spectra (27). Additionally, the $K_{1/2}$ values of Ca^{2+} binding to the F27W cNTnC mutants and full-length IFcTnC in the present study are within the range previously reported for that of Ca^{2+}-triggered tension generation in cardiac myocytes under similar conditions (16, 18, 23, 24, 28, 39). We therefore maintain that the F27W mutation effectively reports on the Ca^{2+}-induced conformational response of the cTnC isoforms without significantly altering the functional characteristics of the proteins.

We do not feel that the absence of Mg^{2+} confounds the results, because Allen et al. (1) demonstrated that Mg^{2+} (1–8 mM) had no effect on the relationship between [Ca^{2+}] and activated force in rat ventricular skinned fibers or on the relationship between pCa and ATPase activity in skinned cardiac cells. These results therefore indicate that Mg^{2+} has no measurable influence on the ability of Ca^{2+} to activate cTnC.

In summary, this study has demonstrated that it is the sequence differences between ScTnC and McTnC in the NH_2-terminal domain that are responsible for the high Ca^{2+} affinity of ScTnC. Additionally, Gln^{29} and Asp^{30} were demonstrated as being required for the comparatively high Ca^{2+} affinity of ScTnC. Because the replacements of these two residues into McNTnC did not increase its Ca^{2+} affinity, it is clear that other residues in addition to Gln^{29} and Asp^{30} are required. The affinity of L29Q/G30D McNTnC was not increased through the replacement of Asp at residue 2 with an Asn, illustrating that other residues in place of, or in addition to, Asn^{2}, along with Gln^{29} and Asp^{30}, are required for the high Ca^{2+} affinity of ScTnC. The identification of these residues represents the focus of future experiments because they represent logical targets for pharmacological intervention to increase the Ca^{2+} affinity of McTnC to enhance the Ca^{2+} sensitivity of cardiac tissue. Such an agent would be useful in end-stage heart failure, allowing for increased inotropism and improved stroke volume.

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