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A functional comparison of cardiac troponin C from representatives of three vertebrate taxa: Linking phylogeny and protein function

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ABSTRACT

The Ca^{2+} affinity of cardiac troponin C (cTnC) from rainbow trout is significantly greater than that of cTnC from mammalian species. This high affinity is thought to enable cardiac function in trout at low physiological temperatures and is due to residues Asn^{2}, Ile^{28}, Gln^{29}, and Asp^{30} (Gillis et al., 2005, Physiol Genomics, 22, 1–7). Interestingly, the cTnC of the African clawed frog *Xenopus laevis* (frog cTnC) contains Gln^{29} and Asp^{30} but the residues at positions 2 and 28 are those found in all mammalian cTnC isoforms (Asp^{2} and Val^{28}). The purpose of this study was to determine the Ca^{2+} affinity of frog cTnC, and to determine how these three protein orthologs influence the function of complete troponin complexes. Measurements of Ca^{2+} affinity and the rate of Ca^{2+} dissociation from the cTnC isoforms and cTn complexes were made by monitoring the fluorescence of anilinonapthalenesulfote iodoacetamide (IAANS) engineered into the cTnC isoforms to report changes in protein conformation. The results demonstrate that the Ca^{2+} affinity of frog cTnC is greater than that of trout cTnC and human cTnC. We also found that replacing human cTnC with frog cTnC in a mammalian cTn complex increased the Ca^{2+} affinity of the complex by 5-fold, which is also greater than complexes containing trout cTnC. Together these results suggest that frog cTnC has the potential to increase the Ca^{2+} sensitivity of force generation by the mammalian heart.

1. Introduction

Myocyte contraction is initiated when Ca^{2+} binds to troponin C (TnC) and triggers a series of conformational changes through the component proteins of the thin filament that result in the formation of cross-bridges between actin and myosin. Troponin C along with troponin I (TnI) and troponin T (TnT) are the component proteins of the troponin (Tn) complex and it is changes in the interactions between these that lead to the activation of the contractile reaction. Manipulation of the functional characteristics of the troponin complex through either the phosphorylation of specific residues or by manipulation of the amino acid sequence of the component proteins can have a significant influence on the contractile function of striated muscle (Shaffer and Gillis, 2010). For example, the phosphorylation of cardiac TnI (cTnI) by protein kinase A (PKA) following β-adrenergic stimulation decreases the Ca^{2+} affinity of the Tn complex and this enables a faster rate of relaxation between beats (Dong et al., 2007; Fentzke et al., 1999). We have also demonstrated that changes to the amino acid sequence of cardiac TnC (cTnC) that increase its Ca^{2+} affinity, increase the Ca^{2+} sensitivity of force generation by chemically skinned cardiac myocytes (Gillis et al., 2005). More specifically, the Ca^{2+} affinity of rainbow trout cardiac TnC (trout cTnC) is approximately two-fold that of bovine cTnC and the residues responsible for this are Asn^{2}, Ile^{28}, Gln^{29}, and Asp^{30} (Gillis et al., 2005). When bovine cTnC was mutated to contain these four trout residues, its Ca^{2+} affinity was increased two-fold, and when native cTnC in rabbit cardiac myocytes was replaced with this mutant, the Ca^{2+} sensitivity of force generation was increased two-fold compared to controls (Gillis et al., 2005). The comparatively high Ca^{2+} affinity of trout cTnC is thought to be responsible, in part, for the comparatively high Ca^{2+} sensitivity of the trout heart (Gillis et al., 2000). This characteristic is proposed to help enable cardiac function in the trout at low physiological temperatures (Gillis and Tibbits, 2002).

Abbreviations: cTnC, cardiac troponin C; cTnI, cardiac troponin I; cTnT, cardiac troponin T; IAANS, anilinonapthalenesulfote iodoacetamide; k_{off}, rate of Ca^{2+} dissociation; k_{on}, rate of Ca^{2+} association; $cTnC^{T53C}$, cTnC mutant where all native cysteines have been replaced with serines and Thr^{53} has been mutated to a Cys; cTn, cardiac troponin; *n*H, Hill coefficient; $K_{F1/2}$, Ca^{2+} concentration at half-maximum Ca^{2+}-dependent fluorescence; $C_HI_KT_R$, cTn complex composed of human cTnC, rat cTnI and rat cTnT; $C_FI_RT_R$, cTn complex composed of frog cTnC, rat cTnI and rat cTnT; $C_TI_RT_R$, cTn complex composed of trout cTnC, rat cTnI and rat cTnT; $C_TI_TT_R$, cTn complex composed of trout cTnC, trout cTnI and rat cTnT.

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The studies described above suggest that manipulation of the sequence of cTnC is a mechanism by which evolutionary pressures have altered cardiac contractility. Interestingly, the amino acid sequence of cTnC from the clawed frog, *Xenopus laevis*, is 97% identical to rat cTnC but contains two of the Ca^{2+}-sensitizing residues (Gln^{29} and Asp^{30}) present in trout cTnC, while the other two (Asp^{2} and Val^{28}) are shared with mammalian isoforms of cTnC (Gillis et al., 2007a). Amphibians diverged from the vertebrate lineage after teleost fish but before eutherian mammals. Phylogenetic studies of cTnC from vertebrates demonstrate a similar pattern of divergence for fish cTnC's, frog cTnC and cTnC's from endothermic species (Gillis et al., 2007a). The amino acid sequence of frog cTnC may therefore represent an intermediate step in the evolution of cTnC in the vertebrate lineage. The purpose of this study is to characterize the function of frog cTnC and determine how it influences the functional properties of the cardiac Tn (cTn) complex. We specifically examined the Ca^{2+} binding properties of frog cTnC, trout cTnC and human cTnC in solution, and how this was affected when these proteins were complexed with cTnI and cTnT. The Ca^{2+} affinity of the isolated cTnC isoforms and cardiac troponin (cTn) complexes were determined using a steady state assay, while stopped flow kinetics was used to measure the rate of Ca^{2+} disassociation (k_{off}). Both of these assays relied on anilinonapthalenesulfote iodoacetamide (IAANS) engineered into each cTnC at a cysteine at residue 53. We also used circular dichroism measurements to see if the thermal stability of the proteins is reflective of physiological temperature.

2. Materials and methods

2.1. Cloning of frog cTnC

The University of Guelph Animal Care Committee approved all protocols and the adult, female *X. laevis* used in this study was obtained from Ward's Natural Science (St. Catherines, ON, Canada). The frog was pithed and the heart surgically removed and rinsed with ice-cold physiological saline. The ventricle was then quickly dissected, flash frozen using liquid nitrogen and stored at −80 °C until needed. Total RNA was extracted using TRIzol® Reagent (Invitrogen) according to manufacturer's directions. RNA was treated with DNase I, Amplification Grade (Sigma-Aldrich, Oakville, ON, Canada) to eliminate any genomic DNA contamination. After DNase treatment, the reverse transcription reaction was conducted using the First-Strand cDNA Synthesis Kit (Applied Biosystems Inc., Streetsville, ON, Canada) according to manufacturer's directions.

Primers were designed against *X. laevis* (frog) cTnC (GenBank Accession # BC053760.1) with Primer3 software (http://frodo.wi.mit.edu/primer3/) to include the *Nde* I (CA^TATG) or *Xho* I (C^TCGAG) restriction site in the 5′ and 3′ primers, respectively (Table 1). The PCR reactions with the *Xenopus* cDNA were performed using Platinum® *Taq* DNA polymerase (Invitrogen Life Technologies, Frederick, MD, USA) according to manufacturer's directions. The PCR products were subsequently purified and digested with *Nde*I and *Xho*I, then ligated into linearized pET-24a(+) plasmid using T4 DNA ligase (NEB, Ipswich, MA, USA), and transfected into NovaBlue competent cells (Novagen). Liquid cultures were grown overnight and isolated plasmids were sequenced to confirm the presence of the insert. From this point forward *Xenopus* cTnC will be referred to as frog cTnC.

Table 1
Sequence of primers utilized to engineer restriction sites for Nde1 and Xho1 into cDNA for frog cTnC and trout cTnC.

Primer	Sequence
frog cTnC forward (NdeI)	GCGTGCCATATGGATGATATTTACAAAGCAGCGGTTG
frog cTnC reverse (XhoI)	TCGCGCTCGAGTTATTCAACTCCCTTCATGAATTCC
trout cTnC forward (NdeI)	GCGTGCCATATGAACGACATCTACAAAGCA
trout cTnC reverse (XhoI)	TCGGCCTCGAGTTATTCTACTCCTTTCATGAACTC

2.2. Mutation of the cDNA for frog cTnC and trout cTnC

Previous studies have measured Ca^{2+} binding to the N-terminus of cTnC when it is in complex with cTnI and cTnT through the use of the fluorescent probe IAANS. To attach the IAANS, the cDNA for these proteins were mutated to replace native cysteine residues at positions 35 and 84 with serine residues and to replace the threonine at residue 53 with a cysteine. IAANS is then attached to Cys^{53} via a disulphide bond. This mutation and subsequent labeling of the cTnC molecules has been demonstrated to not affect the Ca^{2+} binding properties of mammalian isoforms of cTnC in isolation or when complexed with cTnI and cTnT (Hazard et al., 1998; Liu et al., 2012). While the amino acid sequences of the frog cTnC and trout cTnC differ from that of mammalian cTnCs, it is not expected that IAANS will alter the function of these proteins as it is attached in a region of the protein that does not influence the Ca^{2+} activation of the molecule or its interaction with cTnI or cTnT (Davis et al., 2007; Kirkpatrick et al., 2011). This method was therefore followed in the current study for frog cTnC and trout cTnC. The cDNA for trout cTnC was a gift from Dr. Glen Tibbits (Simon Fraser University). There is an additional cysteine in trout cTnC at position 103. This was also, therefore, mutated to serine. This sequence manipulation is not expected to influence the Ca^{2+} binding properties of the molecule as position 103, in the C-terminal domain, does not interact with the N-terminus of the protein (Takeda et al., 2003) nor do changes to the C-terminus affect Ca^{2+} binding to site II (Gillis et al., 2005; Gillis et al., 2003b). The primers utilized to complete the above mutations to frog cTnC and trout cTnC are listed in Table 2. These mutations were performed using the QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's directions, and subsequently sequence-verified. The two mutant proteins will be denoted as frog cTnC and trout cTnC.

2.3. Expression of component proteins

Frog $cTnC^{T53C}$, trout $cTnC^{T53C}$, human cTnC (human $cTnC^{T53C}$), rat cTnI, rat cTnT, and trout cTnI were expressed as previously described using BL21 Gold (DE3) competent *E. coli* cells (Novagen, San Diego, CA) (Kirkpatrick et al., 2011). The expressed proteins were then purified using an AKTA FPLC (GE Healthcare) as previously described (Kirkpatrick et al., 2011). The purity of all proteins was confirmed using SDS-PAGE. Each cTn component protein was then lyophilized and stored at −20 °C.

2.4. IAANS labeling of the $cTnC^{T53C}$ mutants

Purified and lyophilized human $cTnC^{T53C}$, trout $cTnC^{T53C}$, and frog $cTnC^{T53C}$ were labeled with the fluorescent probe IAANS as previously described (Kirkpatrick et al., 2011). In brief, the three proteins were dialyzed into (in mmol l^{-1}): 50 Tris, 6000 urea, 90 KCl, 1 EGTA, pH 7.5. Protein concentration was determined using a Bradford assay and then a 5-fold molar excess of IAANS was added to the solution. The labeling reaction proceeded for 8 h at 5 °C in the dark, and was then stopped with 2 mmol l^{-1} DTT. The IAANS labeled $cTnC^{T53C}$ isoforms

Table 2
Sequence of primers used to mutate the sequences of frog cardiac troponin C (cTnC) and trout cTnC to enable the attachment of IAANS to Cys^{53}. The sequence of the forward primer of each primer set is shown.

Primer	Sequence
frog cTnC C35S	ACGCTGAAGATGGCAGCATTAGCACCAAG
frog cTnC T53C	GGGGCAGAATCCCTGTCCTGAGGAGTTA
frog cTnC C84S	GGTTATGATGGTCCGCAGCATGAAAGACGACAG
trout cTnC C35S	ATGCGGAGGACGGCAGCATCAGTACCAAG
trout cTnC T53C	GGGGCAGAACCCCTGCCCCGAGGAGCTG
trout cTnC C84S	GGTGATGATGGTGAGGAGCATGAAGGACGACAG
trout cTnC C102S	GAACTGGCAGATCTCTTCAGCATGTTTGACAAGAATG