Physiol Genomics 32: 16–27, 2007.
First published October 16, 2007; doi:10.1152/physiolgenomics.00197.2007.

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Functional and evolutionary relationships of troponin C

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Submitted 23 August 2007; accepted in final form 15 October 2007

Gillis TE, Marshall CR, Tibbits GF. Functional and evolutionary relationships of troponin C. *Physiol Genomics* 32: 16–27, 2007. First published October 16, 2007; doi:10.1152/physiolgenomics.00197.2007.—Striated muscle contraction is initiated when, following membrane depolarization, Ca^{2+} binds to the low-affinity Ca^{2+} binding sites of troponin C (TnC). The Ca^{2+} activation of this protein results in a rearrangement of the components (troponin I, troponin T, and tropomyosin) of the thin filament, resulting in increased interaction between actin and myosin and the formation of cross bridges. The functional properties of this protein are therefore critical in determining the active properties of striated muscle. To date there are 61 known TnCs that have been cloned from 41 vertebrate and invertebrate species. In vertebrate species there are also distinct fast skeletal muscle and cardiac TnC proteins. While there is relatively high conservation of the amino acid sequence of TnC homologs between species and tissue types, there is wide variation in the functional properties of these proteins. To date there has been extensive study of the structure and function of this protein and how differences in these translate into the functional properties of muscles. The purpose of this work is to integrate these studies of TnC with phylogenetic analysis to investigate how changes in the sequence and function of this protein, integrate with the evolution of striated muscle.

phylogenetic analysis; protein evolution; temperature; muscle

TROPONIN C (TnC), present in all striated muscle, is the Ca^{2+}-activated trigger that initiates myocyte contraction. The binding of Ca^{2+} to TnC initiates a cascade of conformational changes through the component proteins of the thin filament, leading to the formation of cross bridges (CBs) between actin and myosin and the generation of force by the myocyte. Therefore, the functional properties of TnC, including its ability to be activated by Ca^{2+}, have significant regulatory influence on the contractile reaction of the myocyte. Myocyte contractility is also influenced by the strength of interaction between actin and myosin, the rate of CB cycling, and the rate of ATP hydrolysis by myosin ATPase (24). There are two muscle-specific TnC proteins found in vertebrate striated muscle. The first, skeletal TnC (sTnC), is expressed in fast skeletal muscle and the second, cardiac TnC (cTnC), is expressed in cardiac and slow skeletal muscle. A critical difference between these two paralogs is that sTnC is activated by Ca^{2+} binding to two low-affinity sites on the NH_2 terminus of the protein (sites I and II), while cTnC is activated by Ca^{2+} binding to a single low-affinity site (site II). Site I is nonfunctional in cTnC due to sequence manipulations that have disrupted its ability to coordinate Ca^{2+} ion binding.

cTnC and sTnC have been cloned from a variety of vertebrate species across a range of phylogenic groups. The most ancient of these is the arctic lamprey, *Lampetra japonica,* a member of the earliest diverged vertebrate taxon. *L. japonica* sTnC and cTnC are 70 and 83% identical to human sTnC and cTnC, respectively. The presence of distinct cTnC and sTnC paralogs in *L. japonica* suggests that these proteins have played a key role in defining, as well as differentiating, the functional characteristics of skeletal and cardiac muscle since the beginning of vertebrate evolution. To date cTnC has been cloned from seven mammalian species, two bird species, one frog species, and 10 species of teleost fish, as well as two lamprey species. There is also one cTnC mutant that has been sequenced from the genomic DNA of a human patient with the pathology hypertrophic cardiomyopathy (HCM). Skeletal TnC orthologs have been cloned from an even larger range of animals including mammals, birds, fish, and lizards. TnC homologs have also been cloned from the skeletal muscle of invertebrates, a tunicate, an ascidian, and a variety of insects and mollusks.

The purpose of the following work is to consider the differences in the sequence, structure, and function of TnC in light of the evolutionary and functional constraints under which the different proteins have evolved. To date there have been a number of studies that have characterized the structural and functional differences between cTnC and sTnC as well as between a number of cTnC paralogs and mutants. This work includes the use of X-ray crystallography and three-dimensional NMR solution studies to solve protein structure and the use of fluorescent probes and two-dimensional NMR to characterize functional properties in solution. In addition, the replacement of native homologs, in both skeletal and cardiac tissues, with recombinant homologs has been used to characterize functional differences in vivo. To examine how the genes of TnC have evolved and to integrate gene evolution with the protein function data, we have completed phylogenetic analysis on the amino acid sequences of all known TnC homologs. Through such integration the following questions will be addressed: *1*) How do differences in TnC function relate to differences in muscle function? *2*) What is the relationship between TnC sequences and vertebrate evolution? *3*) Are there specific residues that are only common to orthologs with specific functional characteristics?

Article published online before print. See web site for date of publication (http://physiolgenomics.physiology.org).

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COMPARISON OF THE STRUCTURE AND FUNCTION OF sTnC AND cTnC

cTnC and sTnC are small (161 and 162 amino acids, respectively) dumbbell-shaped proteins composed of two Ca^{2+} binding domains separated by an α-helical linker (Fig. 1). Each Ca^{2+} binding domain contains two EF hands, common to other Ca^{2+} binding molecules, composed of a helix-loop-helix structural motif. The low-affinity NH_2-terminal domain contains Ca^{2+} binding sites I and II, while the high-affinity COOH-terminal domain contains Ca^{2+} binding sites III and IV. It has been suggested by Collins (8) that TnC, as well as calmodulin, myosin essential light chains, and myosin regulatory light chains evolved from a common ancestor containing four similar Ca^{2+} binding sites (EF hands) that arose by gene duplication and reduplication. While the functional properties of the Ca^{2+} binding sites of these different proteins have been altered through sequence manipulation, the tertiary structure of the proteins have been largely retained (8). Usually, each EF hand is able to bind one Ca^{2+} ion; however, in cTnC the first EF hand (site I) is nonfunctional due to sequence substitutions.

An EF hand binds Ca^{2+} through the coordination of the metal ion with six charged residues in the 12-residue loop region (37). This loop region is flanked by two α-helices. In cTnC and sTnC these are found between helices A and B, C and D, E and F, G and H for sites I, II, III, and IV, respectively (Fig. 1). When aligned with the Ca^{2+} ion the six residues approximate the axes of a three-dimensional Cartesian coordinating system. These residues form a pentagonal bipyramidal arrangement around the Ca^{2+} ion and are at positions x, y, z, *-y*, *-x*, *-z* (37). Please note, in this review each TnC protein is annotated with the paralog identified as "s" for skeletal or "c" for cardiac and each ortholog is indicated by the species from which it was cloned, for example, cTnC_Trout. Table 1 contains all of the TnCs discussed. At site I in all of cTnC orthologs except that from the trout (cTnC_Trout) a valine has been inserted preceding residue x, while x and y have been replaced with uncharged residues (Table 1). In cTnC_Trout there is an Ile instead of a Val, which is a conservative substitution. These sequence substitutions of site I have disrupted its ability to align with a Ca^{2+} ion. All known cTnCs from other mammalian species also contain a nonfunctioning site I. As mentioned earlier, the NH_2-terminal Ca^{2+} binding sites are low-affinity sites with the K_D being in the low μM range (34). These sites are activated when the intercellular Ca^{2+} concentration increases from ~100 nM following depolarization to initiate myocyte contraction. The high-affinity Ca^{2+} binding sites in the COOH-terminal domain (sites III and IV) are considered to have a structural function in cTnC and sTnC, helping to anchor the protein into the troponin complex, and are always saturated with either Ca^{2+} or Mg^{2+} under physiological conditions.

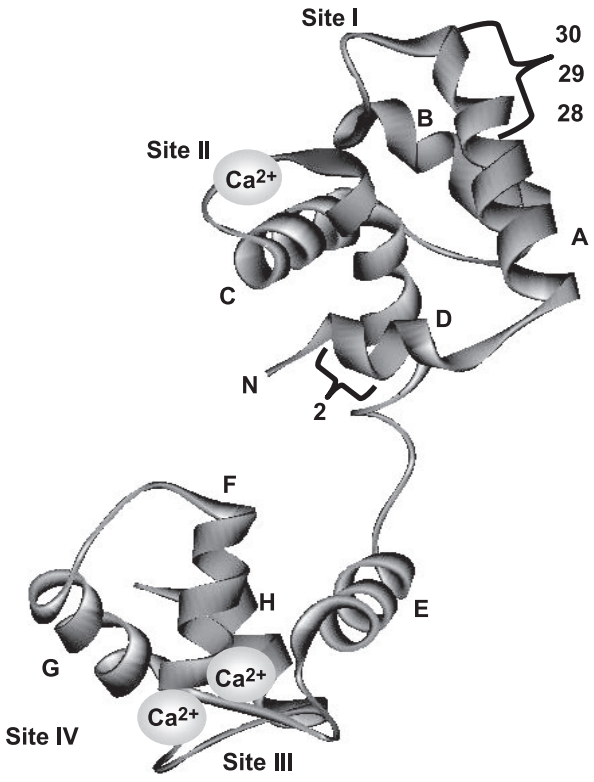

Fig. 1. Ribbon diagram of chicken cardiac troponin C (cTnC) based on NMR solution structure by Sia et al. (51). The relative positions of residues 2, 28, 29, and 30 are indicated on the figure. Ca^{2+} binding sites and helices are labeled.

Functional comparisons of the NH_2-terminal domains of human cTnC (cNTnC_Human) and chicken sTnC (sNTnC_Chicken) by one-dimensional 1H and two-dimensional {1H,15N}-HSQC NMR spectroscopy reveal that the Ca^{2+} affinity of chicken_sNTnC site II is 1.5-fold that of cNTnC_Human site II (34). In addition, Moreno-Gonzalez et al. (43), using stopped-flow kinetic analysis to characterize the rate of Ca^{2+} dissociation (k_{off}) from the NH_2 terminus of sTnC_Rabbit and cTnC_Rat, when these were each complexed with rabbit sTnI, and rabbit skeletal troponin T (sTnT), demonstrate that the k_{off} of cTnC_Rat is faster than that of sTnC_Rabbit. This illustrates that there are differences in the ability of these two TnC paralogs to be activated by Ca^{2+}. Moreno-Gonzalez et al. (43) have also demonstrated that skinned rabbit psoas fibers containing cTnC_Rat had a slower rate of Ca^{2+} activation (k_{tr}) and a lower maximum Ca^{2+}-activated force than those containing sTnC_Rabbit. Together these results demonstrate that the differences in the ability of sTnC_Rabbit and cTnC_Rat to be activated by Ca^{2+} have physiological consequences.

When a cardiac or fast skeletal myocyte is relaxed, sites III and IV of TnC are bound by divalent metals (either Ca^{2+} or Mg^{2+}). The near NH_2 terminus of TnI is bound to the COOH terminus of both TnC and TnT (31, 33). A section of the TnI molecule known as the inhibitory peptide is bound to two adjacent actin molecules (59, 60). The inhibitory peptide includes residues 136–147 in cardiac TnI (cTnI) and 105–115 in skeletal TnI (sTnI). It is the interaction between this peptide and actin that inhibits CB cycling between actin and myosin. As mentioned earlier, the activation of TnC by Ca^{2+} causes TnC to "open," exposing a hydrophobic patch on the surface of the NH_2-terminal domain. It is thought that this hydrophobic patch strengthens the interaction between TnC and a region of TnI known as the "switch" region (residues 147–163), which is adjacent to the inhibitory peptide. This increased interaction pulls TnI away from its inhibitory position on the actin filament (35). This change in the position of TnI allows for tropomyosin (TM) to "roll" across the surface of the actin filament moving from a position near the outer edge of the filament (24). In its initial position on the edge of the thin filament, TM is blocking

Table 1. *List of TnC sequences used in the analyses*

Name	Organism	Common Name	Taxonomy	Identifier	Database
TnC_Bee	Apis mellifera	honey bee	Invt.	GI: 58585246	GenBank
TnC_Silkworm	*Bombyx mori*	silkworm	Invt.	GI: 108744606	GenBank
TnC_Fruitfly	*Drosophila melanogaster*	fruit fly	Invt.	GI: 452388	GenBank
TnC_Barnicle 1	*Balanus nubilus*	giant acorn barnicle	Invt.	GI: 136030	GenBank
TnC_Barnicle 2	*Balanus nubilus*	giant acorn barnicle	Invt.	GI: 136033	GenBank
TnC_Crayfish 1	*Pontastacus leptodactylus*	narrow-fingered crayfish	Invt.	GI: 102757	GenBank
TnC_Crayfish 2	*Pontastacus leptodactylus*	narrow-fingered crayfish	Invt.	GI: 102758	GenBank
TnC_Waterbug	*Lethocerus indicus*	giant water bug	Invt.	GI: 29788123	GenBank
TnC_Beetle	*Tribolium castaneum*	red flour beetle	Invt.	GI: 91089849	GenBank
TnC-Sandworm	*Perinereis vancaurica tetradentata*	sand worm	Invt.	GI: 11596085	GenBank
TnC_Crab	*Tachypleus tridentatus*	horseshoe crab	Invt.	GI: 84675	GenBank
TnC_Squid	*Todarodes pacificus*	Japanese flying squid	Invt.	GI: 13537343	GenBank
TnC_Scallop	*Chlamys nipponensis akazara*	scallop	Invt.	GI: 1396048	GenBank
TnC_Scallop 1	*Patinopecten yessoensis*	Yessoscallop	Invt.	GI: 6451669	GenBank
TnC_Scallop 2	*Patinopecten yessoensis*	Yesso scallop	Invt.	GI: 6451674	GenBank
TnC_Amphioxus 1	*Branchiostoma belcheri*	amphioxus	Ceph.	GI: 46195428	GenBank
TnC_Amphioxus 2	*Branchiostoma lanceolatum*	amphioxus	Ceph.	GI: 1694951	GenBank
TnC_Tunacate 1	*Halocynthia roretzi*	sea squirt	Uro.	GI: 1722685	GenBank
TnC_Tunacate 2	*Halocynthia roretzi*	sea squirt	Uro.	GI: 74961920	GenBank
sTnC_Human	*Homo sapiens*	human	Mam.	GI: 4507617	GenBank
sTnC_Monkey	*Macaca mulatta*	Rhesus monkey	Mam.	GI: 109091943	GenBank
sTnC_Mouse	*Mus musculus*	mouse	Mam.	GI: 667837	GenBank
sTnC_Rabbit	*Oryctolagus cuniculus*	rabbit	Mam.	GI: 136047	GenBank
sTnC_Pig	*Scrofa domestica*	domestic pig	Mam.	GI: 71652	GenBank
sTnC_Chicken	*Gallus gallus*	chicken	Vt.	GI: 1311229	GenBank
sTnC_Turkey	*Meleagris gallopavo*	turkey	Vt.	GI: 136044	GenBank
sTnC_Alligator	*Alligator mississippiensis*	American alligator	Vt.	GI: 46092548	GenBank
sTnC_Lizard	*Sceloporus undulates*	prairie lizard	Vt.	GI: 46092546	GenBank
sTnC_Turtle	*Trachemys scripta elegans*	red-eared slider	Vt.	GI: 46092545	GenBank
sTnC_Edible Frog	*Rana Esculenta*	edible frog	Vt.	GI: 136048	GenBank
sTnC_Clawed Frog	*Xenopus laevis*	African clawed frog	Vt.	GI: 7441462	GenBank
sTnC_Lamprey	*Lampetra japonica*	Arctic lamprey	Vt.	GI: 2589013	GenBank
sTnC_Eel	*Anguilla anguilla*	European eel	Vt.	GI: 14285798	GenBank
sTnC_Zebrafish	*Danio rerio*	zebrafish	Vt.	GI: 18859495	GenBank
cTnC_Cow	*Bos Taurus*	cow	Mam.	GI: 77735654	GenBank
cTnC_Human	*Homo sapiens*	human	Mam.	GI: 136038	GenBank
cTnC_Monkey	*Macaca mulatta*	Rhesus monkey	Mam.	GI: 109039205	GenBank
cTnC_Mouse	*Mus musculus*	mouse	Mam.	GI: 6678369	GenBank
cTnC_Rabbit	*Oryctolagus cuniculus*	rabbit	Mam.	GI: 136040	GenBank
cTnC_Rat	*Rattus norvegicus*	Norway rat	Mam.	GI: 27667260	GenBank
cTnC_Pig	*Scrofa domestica*	domestic pig	Mam.	GI: 63100042	GenBank
cTnC_Squirrel	*Spermophilus beecheyi*	ground squirrel	Mam.		Ensemble
cTnC_Quail 1	*Coturnix coturnix*	common quail	Vt.	GI: 213622	GenBank
cTnC_Quail 2	*Coturnix japonica*	Japanese quail	Vt.	GI: 136037	GenBank
cTnC_Chicken	*Gallus gallus*	chicken	Vt.	GI: 136036	GenBank
cTnC_Clawed Frog	*Xenopus laevis*	African clawed frog	Vt.	GI: 1945537	GenBank
cTnC_Lamprey 1	*Lampetra japonica*	Arctic lamprey	Vt.	GI: 2589015	GenBank
cTnC_Lamprey 2	*Lethenteron japonicum*	Japanese lamprey	Vt.	GI: 2589016	GenBank
cTnC_Zebrafish	*Danio rerio*	zebrafish	Vt.	GI: 28822163	GenBank
cTnC_Mummichog	*Fundulus heteroclitus*	mummichog	Vt.		Ensemble
cTnC_Trout	*Oncorhynchus mykiss*	rainbow trout	Vt.	GI: 30721848	GenBank
cTnC_Bichir	*Polypterus senegalus*	gray bichir	Vt.	GI: 28822166	GenBank
cTnC_Puffer	*Tetraodon fluviatilis*	green puffer fish	Vt.		Ensemble
cTnC_Tuna	*Thunnus albacares*	yellow fin tuna	Vt.		1
cTnC_Sucker	*Catostomus commersonii*	white sucker	Vt.		1
cTnC_Goldfish	*Carassius auratus*	goldfish	Vt.		1
cTnC_Perch	*Perca flavescens*	yellow perch	Vt.		1
cTnC_Icefish	*Chaenocephalus aceratus*	ice fish	Vt.		1

Naming of protein sequences: Troponin C (TnC) ortholog followed by the species. sTnC, fast skeletal TnC; cTnC, cardiac TnC Invt., invertebrate; Ceph., Cephalochrodate; Uro., Urochordate; Mam., mammal; Vt., lower vertebrate; [1]protein identifier was not available for the sequence included in the analysis (62).

weak and strong myosin binding sites. There are a number of weak binding sites exposed at low [Ca^{2+}], allowing for some weak interactions between actin and myosin, but there is no generation of force. The initial movement of TM, in response to Ca^{2+} binding to TnC, exposes additional weak binding sites allowing for myosin head attachment (24). As weak CBs form (zero force, rapid equilibrium), additional strong myosin binding sites are exposed when TM moves further across the thin filament. The formation of strong CBs leads to the generation of force by the contractile element (24).

There are differences between sTnC and cTnC in the size of conformational change caused by Ca^{2+} activating the N-do-

main. With sTnC, the hydrophobic patch exposed following Ca^{2+} activation is ~500 Å^2 in area on the surface of the NH_2 terminus (16). In contrast, the size of the hydrophobic patch exposed upon the Ca^{2+} activation of cTnC is 18 Å^2 (51). This hydrophobic patch increases to 162 Å^2 when the protein interacts with a cTnI peptide corresponding to the switch region (residues 147–163) (16, 35). These results demonstrate that sTnC is more "turned on" by Ca^{2+} than is cTnC and that the activation of cTnC is highly dependent on its interaction with cTnI. The differences in sTnC and cTnC response to Ca^{2+} binding to the N-domain are due to cTnC having only one functional Ca^{2+} binding site (site II). The binding of Ca^{2+} to site I causes a rearrangement of residue side chains that contributes to the enthalpy required to overcome the energy barrier of exposing the hydrophobic core (51).

The difference in the size of the hydrophobic patch exposed upon Ca^{2+} binding to the N-domain of cTnC and sTnC during Ca^{2+} activation influences the strength at which the Ca^{2+} signal is transferred through the thin filament. Li et al. (35), using NMR solution studies, have demonstrated that the strength of interaction between the hydrophobic patch of cNTnC (exposed upon Ca^{2+} activation) and a peptide encompassing the switch region is six times less than that between sNTnC and the corresponding sTnI peptide. As it is, as this interaction pulls TnI away from its inhibitory position on the actin thin filament, the lower strength of interaction between cTnC and cTnI during Ca^{2+} activation may make it more difficult for cTnC to "pull" cTnI away from its interaction with actin, enabling the exposure of myosin binding sites. Gillis et al. (21) have suggested that the lower strength of interaction between cTnC and cTnI is responsible, at least in part, for the cardiac contractile element being dependent on the formation of strong CBs, in addition to the Ca^{2+} activation of cTn, for full activation to occur. The increased dependence of cardiac muscle on strong CBs has been suggested to be partially responsible for the steeper length dependence of force generation in cardiac muscle compared with that in skeletal muscle (21). This is another illustration of how differences in the structure/function of cTnC and sTnC translate into differences in the physiological characteristics of the two different muscle types.

ROLE OF TnC HOMOLOG IN REGULATING THE EFFECT OF TEMPERATURE AND pH ON MUSCLE CONTRACTILITY

Cardiac function and skeletal muscle function have both been shown to be affected by changes in temperature. For example, as cardiac temperature lowers, the sensitivity of the contractile element to [Ca^{2+}] decreases and cardiac function becomes increasingly impaired as the maximum Ca^{2+}-activated force (C_{max}) decreases (5, 26, 28, 58). Figure 2 summarizes studies looking at the effect of temperature on the Ca^{2+} sensitivity of force generation by chemically skinned cardiac myofibrils dissected from trout, frog, rabbit, and rat hearts. In hearts from all species as temperatures decrease, the [Ca^{2+}] required to generate an equal amount of force increases. Comparatively, when mammalian skeletal muscle is cooled, Ca^{2+} sensitivity increases while C_{max} decreases (23, 56). The difference in the influence of temperature on the Ca^{2+} sensitivity of cardiac and skeletal muscle is due, in part, to the TnC paralog expressed, while the decrease in Ca^{2+} activated force

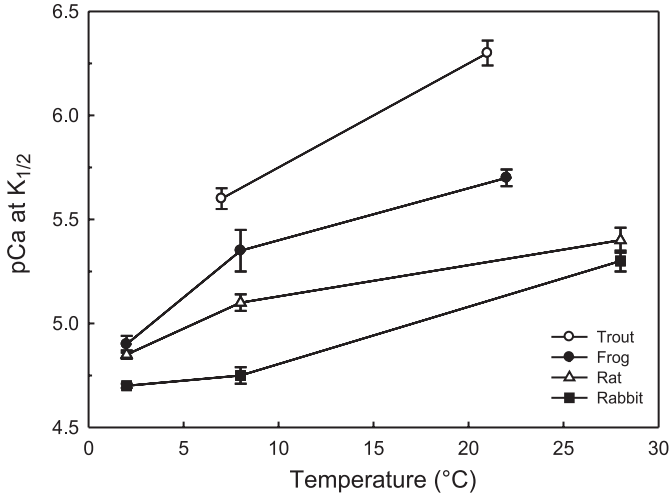

Fig. 2. Comparison of the Ca^{2+} sensitivity of force generation by skinned ventricular fibers isolated from hearts of: trout, frog, rat, and rabbit over a range of temperatures, while pH was maintained at 7.0. Ca^{2+} sensitivity was measured as the pCa at $K_{F1/2}$, which is the Ca^{2+} concentration required to generate half-maximum force. When compared at the same temperature, the trout preparations required 10-fold less Ca^{2+} to generate the same measure of force than those from the mammalian species. Figure is adapted with permission from Churcott et al. (7).

that occurs is due to a reduction in the Ca^{2+} affinity of cTnC (cardiac muscle) and a decrease in the maximal velocity (V_{max}) of actomyosin ATPase (cardiac and skeletal muscle). Harrison and Bers (27) demonstrated that the effect of low temperature on the Ca^{2+} sensitivity of skinned rat ventricular trabeculae was relieved when native cTnC was replaced with rabbit sTnC. This result implies that the activation of cTnC is impaired by either a reduced affinity for Ca^{2+} or changes in its interaction with cTnI required for the protein to fully activate. Recent studies have demonstrated that as temperature was reduced from 37°C to 21°C to 7°C the Ca^{2+} affinity of cTnC_Human decreased (20). Ca^{2+} affinity was measured by monitoring a fluorescent probe [Tyr inserted for Phe at residue 27 (F27W)] engineered into the protein that reports on conformational change as the protein is activated by Ca^{2+}. The results of the above study suggest, therefore, that the desensitizing effect of low temperature on cardiac function is due, in some measure, to the effect of temperature on the Ca^{2+} affinity of cTnC.

Under physiological conditions, when an animal's body temperature changes, there is a concurrent alteration in cellular and tissue pH (57). The purpose of this regulated change is to keep the relative alkalinity ([OH^-]/[H^+]) approximately constant. The relationship, called α-stat regulation, is −0.016 to −0.019 pH units/°C. It has been long established that a change in pH alters the Ca^{2+} sensitivity of cardiac tissue (11, 25, 29, 53), and cTnC has been shown to be partially responsible for this effect (1, 9, 20, 39, 40, 47, 48) Previous work has demonstrated that a 0.3-unit pH increase at 21°C causes a ~28% increase in the $K_{1/2}$ (Ca^{2+} concentration at half-saturation) of both cTnC_Trout and cTnC_Human (20). This means that under physiological conditions a decrease in temperature would decrease the Ca^{2+} affinity of cTnC while the concurrent increase in pH, due to α-stat regulation, would help compensate for this effect. Such compensation would therefore help cTnC to remain functional over a range of temperatures.

E-F Hand Site I

Ectothermic	cTnC_Trout	M-**N**DIYKAAVEQLTDEQKNEFKAAFDIF**IQD**AEDGCISTKELGKV...
	cTnC_Puffer	,-**N**,,,,,,,,,,E,,,,,,,,,,,,,**IQD**,,,,,,,,,,,,,,,...
	cTnC_Zebrafish	,-**N**,,,,,,,,,,,,,,,,,,R,,,,,,**VQD**,,,,,,,,,,,,,,,...
	cTnC_Mummichog	,-**N**,,,,,,,,,,,,,,,,,,,,,,,,,,**VQD**,,,,,,,,,,,,,,,...
	cTnC_Icefish	X-XXXXX,,,,,,,,D,,,,,,,,,,,,,,**VQD**,,,,,,,,,,,,,,,...
	cTnC_Sucker	X-XXXXX,,,,,,,,,,,,,,R,,,,,,**VQD**,,,,,,,,,,,,,,,...
	cTnC_Goldfish	X-XXXXX,,A,,,,,,,,,,R,,,,,,**VQD**,,,,,,,,,,,,,,,...
	cTnC_Tuna	X-XXXXX,,,,,,,,,,,,,,,,,,,,,,**VQD**,,,,,,,,,,,,,,,...
	cTnC_Bichir	X-XXXXX,,,,N,,E,,,,,,,,,,,,,**VQD**,,,,,,,,,,,,,,,...
	cTnC_Perch	X-XXXXX,,,,,,,E,,,,,,,,,,,,,**VQD**,,,,,,,,,,,,,,,...
	cTnC_Lamprey 1	,AE,VDR,,,,,,,E,,,K,,R,,,,,,**VQD**,,,,,,,,,,,,,,,...
	cTnC_Lamprey 2	,AE,VDR,,,,,,,E,,,K,,R,,,,,,**VQD**,,,,,,,,,,,,,,,...
	cTnC_Clawed Frog	,-D,,,,,,,,,,E,,,,,,R,,,,,,**VQD**,,,,,,,,,,,,,,,...
Endothermic	cTnC_Human	,-D,,,,,,,,,,E,,,,,,,,,,,,,VLG,,,,,,,,,,,,,,,...
	cTnC_Cow	,-D,,,,,,,,,,E,,,,,,,,,,,,,VLG,,,,,,,,,,,,,,,...
	cTnC_Pig	,-D,,,,,,,,,,E,,,,,,,,,,,,,VLG,,,,,,,,,,,,,,,...
	cTnC_Rabbit	,-D,,,,,,,,,,E,,,,,,,,,,,,,VLG,,,,,,,,,,,,,,,...
	cTnC_Monkey	,-D,,,,,,,,,,E,,,,,,,,,,,,,VLG,,,,,,,,,,,,,,,...
	cTnC_Cow	,-D,,,,,,,,,,E,,,,,,,,,,,,,VLG,,,,,,,,,,,,,,,...
	cTnC_Mouse	,-D,,,,,,,,,,E,,,,,,,,,,,,,VLG,,,,,,,,,,,,,,,...
	cTnC_Rat	,-D,,,,,,,,,,E,,,,,,,,,,,,,VLG,,,,,,,,,,,,,,,...
	cTnC_Squirrel	,-D,,,,,,,,,,E,,,,,,,,,,,,,VLG,,,,,,,,,,,,,,,...
	cTnC_Chicken	,-D,,,,,,,,,,E,,,,,,,,,,,,,VLG,,,,,,,,,,,,,,,...
	cTnC_Quail 1	,-D,,,,,,,,,,E,,,,,,,,,,,,,VLG,,,,,,,,,,,,,,,...
	cTnC_Quail 2	,-D,,,,,,,,,,E,,,,,,,,,,,,,VLG,,,,,,,,,,,,,,,...
	cTnC_HCM Mutant	,-D,,,,,,,,,,E,,,,,,,,,,,,,V**Q**G,,,,,,,,,,,,,,,...

Fig. 3. Comparison of the NH_2 terminus of all known cTnC homologs. Residues identified as being responsible for the comparatively high Ca^{2+} affinity of cTnC_Trout are indicated in bold. Sequences are organized to indicate if they were cloned from ectothermic or endothermic species. HCM Mutant, hypertrophic cardiomyopathy mutant found in a human patient. The X in place of an amino acid indicates that the residue is not known. This is due to the sequences being cloned from DNA using trout primers.

SPECIES-SPECIFIC cTnC HOMOLOGS REGULATE Ca^{2+} SENSITIVITY OF FORCE GENERATION

Living in temperate waters, ectothermic species such as the rainbow trout, *Oncorhynchus mykiss,* have variable body temperatures that, compared with mammalian species, are very low (5–15°C). In fact, these temperatures would be cardioplegic for a mammalian heart. One mechanism that allows for cardiac function in trout at low temperatures is that the Ca^{2+} sensitivity of the contractile element is significantly higher (>10-fold) than that of a mammalian heart when compared at the same temperature (7) (Fig. 2). This increased Ca^{2+} sensitivity is due, at least in part, to site II of cTnC_Trout having greater than twice the Ca^{2+} affinity than that of cTnC_Human, when measured at the same temperature (20). The difference in Ca^{2+} affinity between cTnC_Human and cTnC_Trout was demonstrated over a range of temperatures and pHs (7°C, pH 7.0; 7°C, pH 7.6; 21°C, pH 7.0; 21°C, pH 7.3, 37°C pH 7.0) (20). The higher Ca^{2+} affinity of cTnC_Trout allows for cardiac contraction to be initiated at lower Ca^{2+} concentrations in the trout heart compared with the mammalian heart. However, extrapolation of the data so that the Ca^{2+} sensitivity of the tissues could be compared at each of the animal's respective physiological temperature reveals that there is relative conservation of Ca^{2+} sensitivity. For example, the Ca^{2+} sensitivity of the trout heart at 7°C would be similar to the rat heart at 37°C. This idea integrates well with the concept of K_m conservation first suggested by Somero and coworkers (14, 15, 30, 54) through their extensive study of A_4-LDH from a variety of different ectothermic species with different physiological temperatures.

Through a series of Ca^{2+} binding studies using cTnC_Human mutants and cTnC_Trout mutants containing a Trp at residue 27, it was demonstrated that the residues responsible for the comparatively high Ca^{2+} affinity of cTnC_Trout are Asn^{2}, Ile^{28}, Gln^{29}, and Asp^{30} (NIQD) (19). These four residues in cTnC_Human, in order, are: Asp, Val, Lys, and Gly and represent four of the five sequence differences between cTnC_Trout and cTnC_Human in the NH_2-terminal domain (Fig. 3). The insertion of these residues into F27W cTnC_Human increased its Ca^{2+} affinity to that of F27W cTnC_Trout (twofold that of cTnC_Human) (Fig. 4) (19). None of the identified residues are located within site II of the protein so it is through an allosteric effect that the Ca^{2+} affinity of this

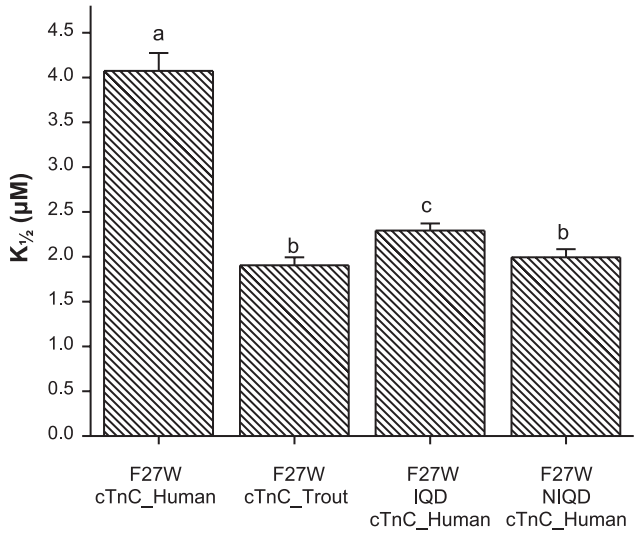

Fig. 4. Comparison of the Ca^{2+} affinity of the cTnC mutants F27W cTnC_Human (n = 10), F27W cTnC_Trout (n = 10), F27W IQD cTnC_Human (n = 10), and F27W NIQD cTnC_Human (n = 10). Ca^{2+} affinity is shown as the $K_{1/2}$, which is the Ca^{2+} concentration (in μM) required to half-saturate the molecule. All values are shown as means ± SE. Bars indicated with the same letter are not significantly different from each other ($P > 0.05$). Measurements were made at 21°C, pH 7.0. Figure is adapted with permission from Gillis et al. (19).

site is increased. In fact, there is complete sequence conservation of the residues in site II between cTnC_Trout and cTnC_Human (44).

To confirm that the comparatively high Ca^{2+} affinity of cTnC_Trout is partially responsible for the comparatively high Ca^{2+} sensitivity of trout cardiac myofibrils, we replaced native endogenous cTnC in single rabbit cardiac myocytes with recombinant WT cTnC_Human or NIQD cTnC_Human and then measured the Ca^{2+} sensitivity of force generation by the myocyte. NIQD cTnC_Human is the cTnC_Human mutant containing Asn^2, Ile^{28}, Gln^{29}, and Asp^{30} as in cTnC_Trout. Results demonstrate that the Ca^{2+} sensitivity of myocytes containing NIQD cTnC_Human were approximately two times that compared with myocytes containing either endogenous rabbit cTnC or WT cTnC_Human (Fig. 5). These results demonstrate that the effect of the NIQD mutation on Ca^{2+} affinity, seen in the solution studies, translates into higher Ca^{2+} sensitivity when the protein is functioning within the intact filament [cTnI, cardiac TnT (cTnT), TM], a significantly more "physiological" condition. This result also suggests that it is the high Ca^{2+} affinity of cTnC_Trout that is responsible, in part, for the high Ca^{2+} sensitivity of trout cardiac myocytes.

STRUCTURAL CONSEQUENCES OF THE NH_2-TERMINAL SEQUENCE DIFFERENCES BETWEEN TnC_TROUT AND cTnC_HUMAN

The residues identified as being responsible for the high Ca^{2+} affinity of cTnC_Trout, Asn^2, Ile^{28}, Gln^{29}, and Asp^{30} are located outside of Ca^{2+} binding site II (Figs. 1 and 3). We have previously suggested that these may be affecting both the ability of the molecule to bind Ca^{2+} as well as changes in protein conformation once activated by Ca^{2+}. The solution structure of the NH_2 terminus of cTnC_Trout (cNTnC-Trout) with site II saturated with Ca^{2+} (cNTnC_Trout·Ca^{2+}) has been solved by Blumenschein et al. (4) at 7 and 30°C. As in cNTnC_Human, the saturation of site II in cNTnC_Trout does not cause the protein to assume a fully "open" conformation. However, comparison of cNTnC_Trout and cNTnC_Human at 30°C reveals that there are subtle differences in the fold of the molecules that cause the positions of Ca^{2+} binding site I and II to be slightly rotated in the molecule (4). Residue positions 28, 29, and 30 in cTnC occur in the sequence in the first loop (loop A) of Ca^{2+} binding site I. Through interpretation of $^3J_{HNH\alpha}$ coupling constants and backbone ^{15}N-relaxation measurements of cNTnC_Human, Spyracopoulos et al. (55) suggest that sites I and II are structurally linked. It not surprising, therefore, that sequence differences in site I are altering the structure/position of site II in the molecule. This change in position of site II within the molecule may alter the ability of the site to bind Ca^{2+}.

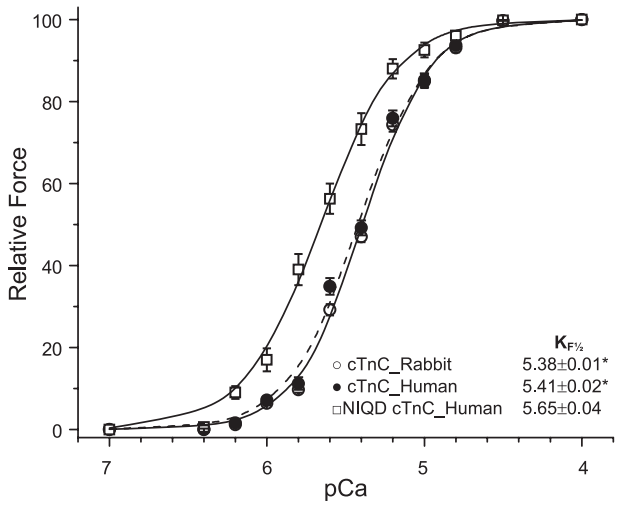

Fig. 5. Ca^{2+} titration of steady-state force generation in single, skinned, rabbit cardiac myocytes containing either endogenous (pre-extracted) RcTnC (n = 19), WT cTnC_Human (n = 12), or NIQD cTnC_Human (n = 7) at 15°C, pH 7.0. Data are normalized with respect to the maximal force generated during each Ca^{2+} titration and presented as means ± SE. The curves generated by fitting the data with the Hill equation have been added to the figures for comparison against the data points. The Ca^{2+} concentration required to generate half-maximum force ($K_{F1/2}$) are shown as means ± SE. Means indicated with the same superscript are not significantly different from each other ($P > 0.05$). Measurements were made at 21°C, pH 7.0. Figure is adapted with permission from Gillis et al. (19).

By monitoring [N^{15}]-labeled F27W cNTnC_Trout during Ca^{2+} titration using 2-D{^1H,^{15}N}-HSQC NMR, investigators have demonstrated that the residues within site I and II undergo a large positional change within the molecule during Ca^{2+} activation (4, 18, 19). A similar change occurs in the same residues in cNTnC_Human during Ca^{2+} activation. Interestingly, Ile^{28}, Gln^{29}, and Asp^{30} are located in site I of cTnC_Trout, and Gln^{29} in cTnC_Trout is a Leu in cTnC_Human. Gln is a hydrophilic residue, while Leu is hydrophobic. Tikunova and Davis (61) have demonstrated that the replacement of hydrophobic residues at positions 20, 44, 45, 48, and 81 with Gln in McTnC increased the Ca^{2+} affinity of all mutants. These authors suggest that the insertion of Gln decreased internal hydrophobic interactions, facilitating the opening of the molecule during activation. However, as demonstrated by Blumenschein et al. (4), residue 29 is on the surface of the molecule and, therefore, exposed to solvent. The presence of a hydrophilic residue (Gln) in cTnC_Trout at residue 29 instead of a hydrophobic residue (Leu), will not, therefore, affect the stability of the core of the molecule. It is possible, however, that the L29Q substitution could increase the interaction between the protein and its solvent, thereby decreasing local protein stability. Previous studies have suggested that such a phenomenon enables cold-adapted trypsin, α-amylase, and subtilisin to function at low temperatures as a result of an increase in surface hydrophilicity, compared with warm-adapted orthologs (17, 52). By increasing the surface hydrophilicity of cTnC_Trout, the presence of Gln at position 29 may act to destabilize this region of the protein that undergoes a large positional change during Ca^{2+} activation (19). Compared with cTnC_Human this difference could alter the free energy landscape associated with activation and help decrease the energy barrier (ΔG) needed to be overcome for a conformational change to occur. This could, therefore, make it easier for the molecule to be activated once Ca^{2+} binds to site II.

A reduction in temperature from 30 to 7°C causes a change in the structure of cNTnC_Trout as a number of the helices shift their relative position. This change in the fold of cNTnC_Trout makes it more similar to cNTnC_Human at 30°C. In fact these two structures (cNTnC_Trout at 7°C and cNTnC_Human at 30°C) are more similar than the structures of cNTnC_Trout at 7 and 30°C. Seven degrees Celsius is within the temperature range at which cTnC_Trout normally functions, while 30°C is close to

the physiological temperature of a mammalian heart. Therefore, when compared at their respective physiological temperatures, the structures of cNTnC_Trout and cNTnC_Human are very similar. In addition, functional data support this idea as the pCa_{50} (Ca^{2+} concentration at half-maximal fluorescence), used as a measure of affinity, of cTnC_Human site II at 37°C was 5.42 ± 0.02, while this same value for cTnC_Trout at 7°C was 5.23 ± 0.03 (20). This difference in affinity is less than when the two proteins are measured at the same temperature and is comparatively minor, considering that the temperatures at which they were measured differ by 30°C. Together these results suggest that the differences in sequence between cNTnC_Trout and cNTnC_Human help to counteract the effect of temperature on protein structure and therefore allow what could be considered a similar "functional" conformation at two different temperatures.

COMPARISON OF TnC SEQUENCES ACROSS TAXONOMIC GROUPS

As discussed earlier, cTnC and sTnC have different functional properties, and these differences in protein function translate into a number of functional differences between cardiac and skeletal muscle. To examine how the genes of TnC have evolved and to frame this evolution in a functional context, phylogenetic analysis on the amino acid sequences of all known TnC homologs has been completed. Ota and Saitou (45) have previously constructed phylogenetic trees using TnC as well as five other muscle protein genes cloned from a variety of species. However, the present work benefits from the increased number of TnC genes now sequenced, compared with that in the Ota and Saitou study (61 vs. 26), from a more diverse range of species (41 vs. 16) including lamprey, amphioxus, and sea squirt (Table 1). The current analysis has been completed using sTnC orthologs from 16 vertebrate species, cTnC orthologs from 25 vertebrate species, and TnC orthologs from 20 invertebrate species. Sequence and phylogenetic analyses were performed essentially as described (38). All sequences used in this study were obtained from either the National Center for Biotechnology Information (Bethesda, MD) nonredundant (nr) protein database, the Ensembl Genome Browser (Wellcome Trust Genome Campus, Hinxton, Cambridge, UK) (3), or from Ref. 62. We ensured that other members of the EF-hand Ca^{2+}-binding family (e.g., calmodulin) were identified in our search, providing further confidence that no TnC sequences were missed. A complete list of all the TnC sequences used in this study is shown in Table 1 (see Supplement for FASTA amino acid sequences).[1]

Phylogenetic analyses were performed using ClustalX (primarily for initial tree constructions and manipulations) and the PHYLIP package (data not shown) for verification of the initially derived trees (version 3.6b; Joe Felsenstein, Department of Genome Sciences, University of Washington, Seattle, WA) (13). Included in the alignment was the out-group calmodulin 3 sequenced from the rat, *Rattus norvegicus* (GenBank GI number 6978591).

Neighbor-joining trees (50) were generated using ClustalX, followed by tree evaluation with bootstrap resampling (1,000 replicates). Further phylogenetic analyses were performed using the PHYLIP package, where trees were also constructed using maximum parsimony and maximum likelihood methods (data not shown). The program TREEVIEW (version 1.6.6) (46) was used to examine and display all trees.

Alignment of all TnC, cTnC, and sTnC sequences used in the analysis reveals that TnC is conserved across a phylogenetically diverse group of organisms. A subset of these sequences, to be used as representative sequences, is displayed in Fig. 6. The high sequence identity attests to the importance of TnC in muscle contraction in both invertebrate and vertebrate species. As previously reported by Ota and Saitou (45), the tree topology reveals a clear delineation between invertebrate and vertebrate TnC homologs (Fig. 7). Invertebrate TnC homologs group together, but the lengths of the branches that link these are relatively long, representing the higher variability of the sequences within this group. Vertebrate TnC sequences are distinctly grouped into two clades representing cTnC and sTnC paralogs. Based on our analysis, the vertebrate TnC paralogs are the result of a gene duplication event specific to the vertebrate lineage. This duplication occurred sometime after the divergence of *Urochordata* (e.g., sea squirt) but before the divergence of *Agnatha* (e.g., lamprey) as lampreys have both cTnC and sTnC paralogs. It is hypothesized that such whole genome duplication occurred at some point between 500 and 600 million years ago (MYA) just before the Cambrian explosion (41). Based on their analysis of TnC, Ota and Saitou (45) suggested that fast skeletal muscle and cardiac/slow muscle became distinct after a gene duplication that occurred before the frog/mammal divergence ~350 MYA. As mentioned above, one difference between the current analysis and that of Ota and Saitou (45) is that our analysis contained sequences from representative Urochordates (e.g., sea squirt) and Agnathans (e.g., lamprey). These sequences, in particular, have provided additional strength to our analysis as it is commonly thought that the vertebrate heart, as a distinct organ, first appeared in the Urochordates. Through evolution the heart has developed from a muscular tube, found in Urochordates, into the four chambered organ with coronary circulation found in higher vertebrates. For a complete review of this area please see Burggren et al. (6). One group of animals that would provide insight into timing of the evolution of sTnC and cTnC are the hagfishes, as these animals evolved after the Urochordates but before the lamprey and jawed vertebrates. It is not known if this group has either: sTnC and cTnC genes, or only one TnC. Additionally, teleost fish are thought to have undergone a second gene duplication event during the Devonian ~440 MYA (41), and the salmonid fishes are thought to have undergone a third duplication 25–100 MYA (42). This would have resulted in three paralogs of cTnC and sTnC. If there were selective pressure to keep these duplicates they should be retained in the genome. However, only one cTnC_Trout gene has been identified, while no attempts have been made to clone sTnC from trout. There are currently no whole genome projects for any salmonid.

SEQUENCE DIFFERENCES INFLUENCING PROTEIN FUNCTION

The sequence analysis has identified a number of regions and residues that are highly conserved between tissue-specific paralogs and/or across all TnC proteins. This discussion will

[1] The online version of this article contains supplemental material.

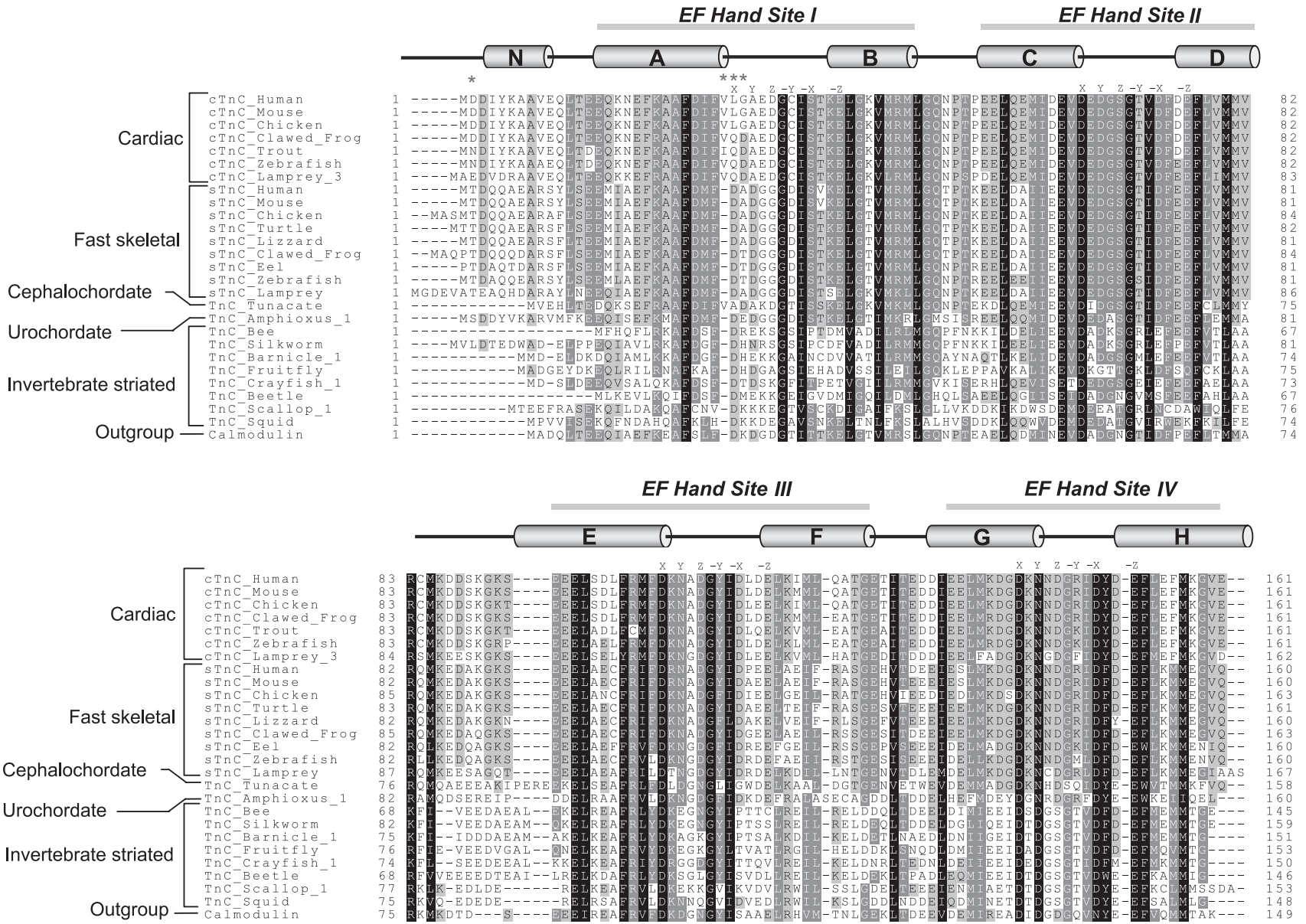

Fig. 6. Sequence comparison of representative TnC sequences. Sequences are organized into cardiac homologs, skeletal homologs, and invertebrate striated muscle homologs with representative species being listed for each. Shading indicates 4 levels of sequence conservation with black representing 90%, dark gray 70%, and light gray 50% identity. The Ca^{2+}-coordinating positions in each EF-hand site are shown above the sequences, and the helices are labeled. Calmodulin 3 from rat is included as it was used as the out-group to root the tree. *Position of 4 residues determined to be responsible for the high Ca^{2+} affinity of cTnC_Trout.

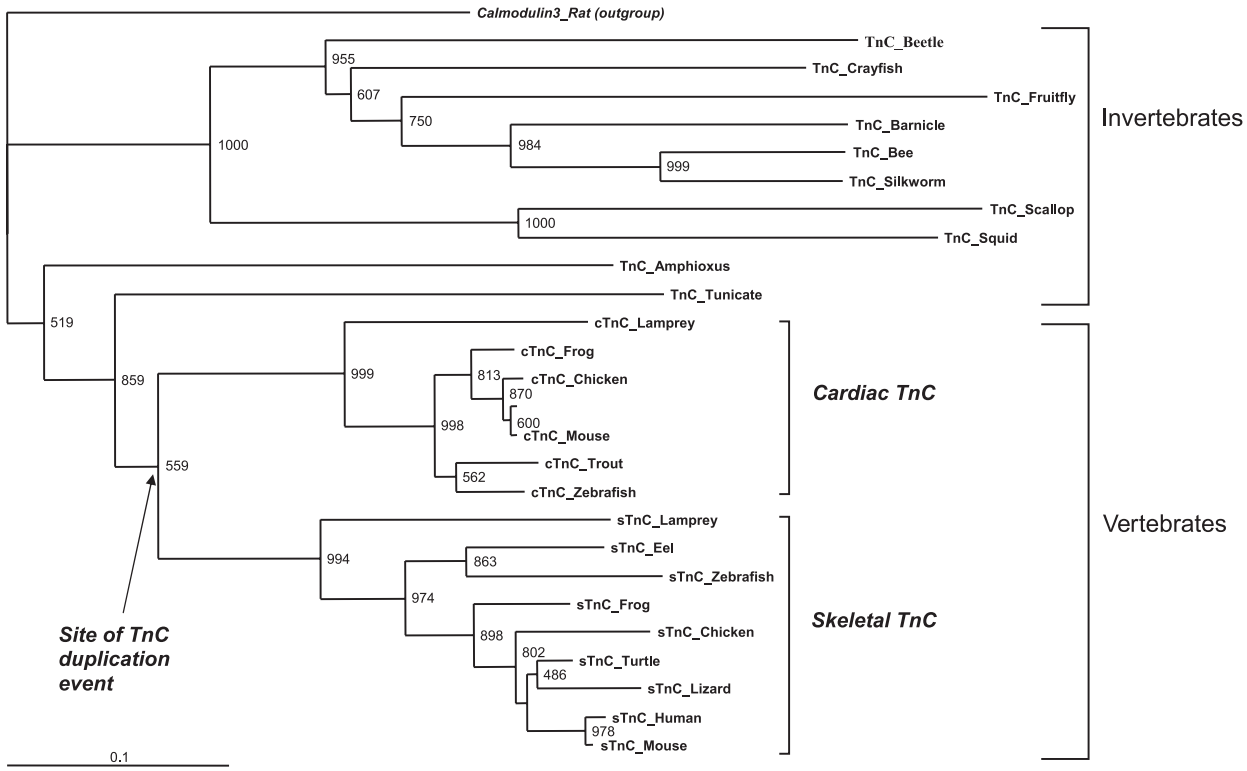

Fig. 7. A maximum parsimony (MP) consensus tree of the TnC family. We included 27 representative sequences in this analysis. The MP tree was constructed by resampling 100 datasets (bootstrap) with randomized input order of 10 jumbles using Seqboot, Protpars, and Consense programs in the PHYLIP software. The tree is rooted with an out-group of calmodulin 3 (*Rattus norvegicus*) that is related to the TnC family. The amphioxus and sea squirt sequences fall according to their evolutionary position between invertebrate and vertebrate species; they are not considered to be orthologous to any of the vertebrate paralogs. Clear splits are observed between invertebrate striated muscle TnC and TnC paralogs (sTnC and cTnC) from vertebrate species, as well as between sTnC and cTnC at the site of TnC duplication in vertebrates, and this is indicated on the figure. Numbers indicate bootstrap values and represent the number of times out of 1,000 replicates that the same result was achieved for the respective partition.

primarily focus on the NH_2-terminal domain, as it is sequence differences in this region of TnC that have been found to dramatically alter the Ca^{2+} activation of the protein and, as a result, the active properties of striated muscle. One region of TnC that demonstrates high sequence conservation is site II. Within this site, except for conservative substitutions at two residues there is complete sequence conservation of all vertebrate cTnCs and sTnCs (Fig. 6). The fact that residues within site II have maintained identity for >500 million yr is a testament to the importance of this motif to TnC function.

Compared to site II, the sequence of site I of cTnC paralogs display lower identity and there is significant variation in the residues that compose the Ca^{2+} binding moiety of this site (Fig. 6). For example, it is the insertion of Val^{28} and the replacement of Asp^{29} and Asp^{31} in all cTnCs that have disrupted the ability of site I to bind Ca^{2+} (55). In addition, there is an Asp at residue 33, the "z" residue in site I, in all cTnCs, as well as in TnC cloned from the tunicate but not in any sTnCs at this position (Fig. 6). The presence of the Asp in TnC_Tunicate, as is found in cTnC, is interesting because the gene duplication that likely gave rise to a cardiac-specific TnC is thought to have occurred after the evolution of tunicates.

The differences in amino acid sequence between cTnC orthologs in site I not only alter the function of site I but also the Ca^{2+} binding characteristics of site II. For example, it has been previously established that Asn^2, Ile^{28} Gln^{29}, Asp^{30} in cTnC_Trout are responsible for the comparatively high Ca^{2+} affinity of site II. These residues are found, to some degree, in all known cTnCs cloned from ectothermic species. The equivalent residues in cTnC_Human, Asp^2, Val^{28}, Leu^{29}, and Gly^{30}, are present in all cTnCs cloned from endothermic species. The only exception is a cTnC_Human mutant cloned from a human heart exhibiting the pathology familial hypertrophic cardiomyopathy (FHC). FHC is a disease that can be caused by mutations to a number (>10) of genes that encode for sarcomeric proteins (49). The common phenotype of this disease is an asymmetrical enlargement of the left and/or right ventricle as well as fibrosis (49). There is no known hemodynamic etiology for this pathology. One theory is that the mutations to the different sarcomeric proteins alter the ability of the heart to be activated by Ca^{2+}, thus inducing compensatory changes (49).

In the HCM cTnC_Human mutant, as in cTnC_Trout, the residue at position 29 is a Gln instead of a Leu (L29Q) (Fig. 3) (2). Liang et al. (36) have demonstrated that the Ca^{2+} affinity of the L29Q cTnC_Human mutant is intermediate between cTnC_Trout and cTnC_Human and that, when incorporated into cardiac myocytes using cTnC extraction/replacement methods, the Ca^{2+} sensitivity of force generation by the mouse cardiac myocytes is also intermediate to one containing native cTnC and a cTnC_Human mutant with the same Ca^{2+} affinity of cTnC_Trout. Using artificial Ca^{2+} transients to estimate the rate of Ca^{2+} association (k_{on}) to site II of F27W mutants of cTnC_Trout, L29Q cTnC_Human, NIQD cTnC_Human, and

cTnC_Human, these authors suggest that L29Q and NIQD significantly increase the Ca^{2+} association rates relative to cTnC_Human when in solution (36). The authors suggest that the faster k_{on} is caused by the mutation destabilizing the unbound or "apo" structures on the NH_2-terminal domain, thereby making it easier for the helices to move upon Ca^{2+} activation. These results support our interpretation of data from experiments in which the structure and Ca^{2+} activated structural transition of cNTnC_Trout and cNTnC_Human were characterized by one-dimensional 1H and two-dimensional {1H,15N}-HSQC NMR spectroscopy (4, 18, 19).

A second potentially interesting residue within site I of TnC is at position 38. In all mammalian sTnCs there is a Val at this position, while in all other vertebrate sTnCs, all cTnCs as well as calmodulin, there is a Thr. The insertion of a nonpolar residue (Val) for a polar residue (Thr) may have significant effects on the functional characteristics of this site that actively binds Ca^{2+} in vertebrate sTnC. To date there have been no studies that have examined the functional consequences of this sequence difference; however, on the basis of sequence comparisons, a resulting phenotypic difference could be predicted.

There is much less sequence conservation within site I when invertebrate TnC is examined (Fig. 6). This is not too surprising as previous studies suggest that there is variation between orthologs in which of the four Ca^{2+} binding sites are functional. For example, sites I and II do not bind Ca^{2+} in TnC cloned from scallop, *Chlamys nipponensis akazara,* and squid, *Todarodes pacificus*, (10), while sites I and III are nonfunctional in TnC cloned from crayfish, *Astacus leptodatylus*, and barnacle, *Balanus nubili*, (8, 32). This demonstrates that there is much more functional and, therefore, sequence variation between these TnC orthologs.

THERMAL STRATEGIES INFLUENCING cTnC SEQUENCE

Within the cTnC clade, lamprey cTnC is separated from teleost cTnCs, and these are distinctly separated from mammalian and avian cTnCs (Fig. 7). Comparison of cTnC from mammalian species reveals that there is almost complete sequence identity among homologs (Fig. 6). cTnC_Bovine, cTnC_Porcine, and cTnC_Rabbit are identical in sequence, while these proteins are ~99.4% identical to cTnC_Human and cTnC_Mouse. This conservation of sequence is higher than that for either cTnI or cTnT cloned from these same animal species as sequence identity between these homologs averages ~92% for cTnI and 84% for cTnT. The high sequence identity between mammalian cTnCs suggests that there are rigid structure-functional requirements for cTnC operating within the mammalian heart. Similar requirements also appear to exist in the avian heart as cTnCs cloned from the chicken, *Gallus gallus*, and the common quail, *Coturnix coturnix,* have a high degree of sequence identity to cTnC_Human (96.8–99.4%). The low sequence variability between cTnCs from mammalian and avian hearts suggests that there has been a relatively high selective pressure on this protein isoform. The high sequence identity between cTnCs from birds and mammals is remarkable considering that these two groups of animals have had separate evolutionary histories since the Carboniferous (~340 MYA) (12). The negative aspect of such adaptation is that any change in these conditions, such as a decrease in temperature, could have a serious affect on function.

When cTnCs from endothermic species are compared with those from ectothermic species sequence identity between homologs begins to decrease, and this is reflected in the increased length of the branches between these two groups (Fig. 7). For example, comparison of cTnC_Human to cTnC_Trout reveals 92% identity. There is also higher variability between cTnCs from fish and frog species compared with between mammalian homologs. For example cTnC_Trout is 94% identical to cTnC cloned from the Antarctic icefish, *Chaenocephalus aceratus*. This higher sequence variation likely reflects the different physiological conditions of each of the different ectothermic species from which the protein has been cloned.

Residue 30 is a Gly in all mammalian and avian cTnCs but is an Asp in all known ectothermic cTnCs (teleosts, amphibians). As Gly is a small, nonpolar residue and Asp is a hydrophilic residue, this represents a nonconservative substitution. In a previous study we replaced Asp^{30} in F27W cNTnC_Trout with a Gly and found that the pCa_{50} of the protein was decreased by 0.12 pCa units equal to a 32% increase in the Ca^{2+} concentration required to half saturate the molecule (22). This demonstrates that this substitution alters the Ca^{2+} activation of the protein. A second sequence manipulation, unique to cTnCs from endothermic hearts is the presence of the hydrophobic residue, Leu at residue 29. The replacement of a hydrophilic residue (Asp) with a nonpolar residue (Gly) at position 30 and the presence of a hydrophobic residue at position 29 may represent an adaptation to cTnC functioning at the relatively high (~37°C) core temperature in endothermic species. These two residue replacements may act to decrease the interaction of this region of the protein with the surrounding solvent, thereby stabilizing the protein. As mentioned above, this region (site I) of the protein undergoes a significant conformational change during Ca^{2+} activation. It is likely that during vertebrate evolution, Leu^{29} and Gly^{30} appeared in cTnC in a common ancestor to birds and mammals and have been maintained over this evolutionary time period (340 million yr). A second, but unlikely, hypothesis is that Leu^{29} and Gly^{30} have arisen multiple times in endothermic species as convergent evolution. However, it is not possible to test this hypothesis as currently there are no known cTnC sequences from any reptiles that are more closely related to birds than mammals, but are ectothermic.

All cTnCs from ectothermic species, including the clawed frog, *Xenopus laevis,* contain at least two of the four residues identified as being responsible for the high Ca^{2+} affinity of cTnC_Trout (Fig. 3). These two are Gln^{29} and Asp^{30}. Additionally, in cTnC from the green puffer, *Tetraodon fluviatilis*, there is complete sequence identity at all four positions (Asn^2, Ile^{28} Gln^{29}, and Asp^{30}) (Fig. 6), while cTnC from the zebrafish, *Danio rerio*, birchir, *Polypterus senegalus,* and *Fundulus heteroclitus* contain three of the four residues (Fig. 3). As these identified residues, especially Gln^{29} and Asp^{30}, are common in all ectothermic species, it is likely that their presence is not due to drift but has been selected for and therefore they may be considered to be "modifier" amino acids.

GREATER SEQUENCE VARIABILITY IN sTnC ORTHOLOGS MAY REFLECT GREATER ADAPTIVE CHANGE IN SKELETAL MUSCLE

Compared to vertebrate cTnCs, there is less sequence identity in vertebrate sTnC orthologs when compared across the same range of animal species (Fig. 6). For example, there are no two mammalian homologs that have the same sequence, and there is higher variability between mammalian and avian sTnCs (90% identity = 14 sequence differences) compared with cTnCs (96.8%–99.4% = 1–6 sequence differences). The branches within the vertebrate cTnC clade are shorter than those in the vertebrate sTnC clade, indicating that the cardiac isoform has not undergone sequence divergence to the extent that the skeletal isoform has. Additionally, as mentioned earlier, comparison of human sTnC to that from *E. japonicus* demonstrates 70% identity compared with 83% for cTnC from these same species, equal to 48 and 31 sequence differences, respectively. The higher sequence variability in sTnC orthologs likely suggests that skeletal muscle has undergone more adaptive change than cardiac muscle. This could, as result, reflect a greater range of physiological capabilities or physiological conditions of the skeletal muscles from two species compared with the hearts of the same species.

CONCLUSIONS

By considering how differences in cTnC and sTnC function relate to differences in tissue function and to the evolution of the TnC gene, we hope to provide some useful insight into how the evolution of a single protein can influence the functional capability of a tissue. By comparing the amino acid sequences of all known TnCs, we have also identified a number of residues in sTnC and cTnC that may represent interesting targets for functional studies. It is also hoped that this discussion helps to illustrate the power of a comparative approach when studying relationships between protein structure and function. Such an approach can provided unique insight into the mechanisms by which evolution has driven protein design and how these may be exploited to create novel proteins.

GRANTS

T. E. Gillis is supported by a National Sciences and Engineering Research Council (NSERC) Discovery grant. G. F. Tibbits holds operating grants from NSERC and the Heart and Stroke Foundation of BC and Yukon. G. F. Tibbits is also the recipient of a Tier I Canada Research Chair.

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