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Cloning and tissue expression of eleven troponin-C isoforms in the American lobster, *Homarus americanus*

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ABSTRACT

Troponin-C is the Ca^{2+}-binding subunit of the troponin regulatory complex in striated muscles. As TnC isoforms can influence the Ca^{2+}-activation properties of fiber phenotypes, the diversity and tissue distribution of TnC cDNAs were assessed in the American lobster, *Homarus americanus*. We cloned ten full-length cDNAs and one partial cDNA coding for distinct TnC isoforms. Five were sequenced from expressed sequence tag clones and were designated Ha-$TnC_{2b'}$ (2094 nt, 141 aa and 15.9 kDa), -$C_{4'}$ (1667 nt, 155 aa and 17.3 kDa), -C_5 (2884 nt, 149 aa and 17.3 kDa), -C_6 (2439 nt, 155 nt and 17.4 kDa) and -C_{6x} (2171 nt, 154 aa and 16.9 kDa). The remainder were cloned using a combination of reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends: five full-length cDNAs, designated Ha-TnC_1 (814 nt, 150 aa and 17.1 kDa), -C_{2a} (639 nt, 152 aa and 17.2 kDa), -$C_{2b''}$ (2136 nt, 155 aa and 17.5 kDa), -C_3 (1046 nt, 150 aa and 16.9 kDa), -$C_{4''}$ (842 nt, 108 aa and 12.1 kDa) and one partial (3′) cDNA, designated Ha-$TnC_{4'''}$ (563 nt and 57 aa). Ha-TnC_1, -C_{2a}, and -$C_{2b'}$ corresponded to lobster TnC sequences in the GenBank protein database (Ha-TnC_1, -C_{2a}, and -C_{2b}). Alternative splicing appeared responsible for $TnC_{2b'}$ and -$C_{2b''}$; $TnC_{4'}$, -$C_{4''}$ and -$C_{4'''}$; and TnC_6 and -C_{6x}. The deduced amino acid sequences differed primarily in the terminal regions and EF-hands I and III. Ha-TnC_{6x} had a highly divergent 76 aa proline-rich N-terminal sequence. Tissue expression of the Ha-TnC isoforms was analyzed qualitatively by endpoint PCR. Ha-TnC_1, -C_{2a}, -$C_{2b'}$, -$C_{2b''}$ and -C_3 were expressed primarily in skeletal muscles; Ha-TnC_5 was expressed in heart; and Ha-TnC_4 and -C_6 variants were expressed in muscles and other tissues. The number and diversity of TnC sequences suggest the potential for varying the Ca^{2+}-activated properties of the troponin–tropomyosin regulatory complex through differential expression of TnC isoforms.

1. Introduction

Skeletal muscles in lobsters are composed of three major fiber types: fast, slow-phasic (S_1) and slow-tonic (S_2) (Mykles, 1985b; Silverman et al., 1987), the latter two representing extremes of a continuum of slow fibers (Medler et al., 2004). The three fiber types differ in their contractile properties, myofibrillar protein isoform composition, and anatomical location (Hooper and Thuma, 2005; Mykles, 1985a,b, 1988, 1997; Mykles et al., 2002; Silverman et al., 1987; Sohn et al., 2000). Fast fibers are located in the deep abdominal flexor and extensor muscles and comprise approximately 70% of the cutter claw closer muscle. S_1 fibers are located in the superficial abdominal flexor and extensor muscles, the crusher claw closer muscle and in the ventral region of the cutter claw closer muscle. S_2 fibers are located in the distal region of the cutter claw closer and the superficial abdominal flexor and extensor muscles (Mykles, 1985a,b).

Muscles of the American lobster, *Homarus americanus*, display a complex diversity of myofibrillar protein isoforms. cDNAs have been cloned encoding three myosin heavy chain (MHC) isoforms (fast, S_1, and S_2; Cotton and Mykles, 1993; Medler and Mykles, 2003; Medler et al., 2004), three tropomyosin (Tm) isoforms (fast/S_1 splice variants and S_2; Mykles et al., 1998; Medler et al., 2004), fast fiber-specific P75 (Medler and Mykles, 2003) and, surprisingly, twelve actin isoforms, eight of which are expressed in skeletal muscle (Kim et al., 2009). SDS-PAGE also reveals two isoforms of paramyosin, five of troponin-I (TnI), three of troponin-C (TnC), and three of troponin-T (TnT) (Mykles, 1985a,b). The myofibrillar proteins, including the three TnC isoforms, are differentially expressed in lobster claw and abdominal muscles (Mykles, 1985a,b).

TnC is the calcium (Ca^{2+}) sensor of the striated muscle thin filament Tn/Tm regulatory complex. TnC is a member of a large group of Ca^{2+}-binding proteins (CBPs) that also includes calmodulin, calpain, parvalbumin, calbindin, and myosin light chain (see Gifford et al., 2007 and Gillis et al., 2007 for reviews). CBPs have 1, 2, or 3 pairs of helix-loop-helix structural Ca^{2+}-binding motifs, termed the EF-hand. Each EF-hand motif has a central 12-amino acid (aa) loop; binding of a single Ca^{2+} is coordinated by the side chains of certain

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amino acids at positions #1 (X), #3 (Y), #5 (Z), and #12 (-Z), the backbone carbonyl group at position #7 (-X), and a water molecule hydrogen-bonded to the side chain of the residue at position #9 (-Y) (Gifford et al., 2007; Gillis et al., 2007). TnC contains four EF-hand motifs: two regulatory N-terminal EF-hands (I and II) bind Ca^{2+} with low affinity and regulate muscle contraction; two structural C-terminal EF-hands (III and IV) bind Ca^{2+} and Mg^{2+} with higher affinity and anchor TnC to TnI in the Tn complex. Not all the EF-hands have functional Ca^{2+}-binding loops. For example, sequence substitutions in the vertebrate slow/cardiac TnC render the Ca^{2+}-binding loop in EF-hand I nonfunctional (Gillis et al., 2007). In crustacean muscle TnCs, only EF-hands II and IV have functional Ca^{2+}-binding sites (Wnuk, 1989; Collins et al., 1991). Binding of Ca^{2+} to the TnC regulatory domains results in successive conformational changes in TnC and TnI, causing a shift in the position of Tm away from the myosin-binding sites of actin, removing inhibition of actomyosin interaction and thereby allowing muscle contraction (see Gordon et al., 2000 and Jin et al., 2008 for reviews).

The numbers of TnC isoforms vary among taxa. In crustaceans, three TnC aa sequences have been reported in the American lobster, *H. americanus* (Garone et al., 1991), two TnC aa sequences in barnacle, *Balanus nubilis* (Collins et al., 1991), and five TnC aa sequences in crayfish, *Astacus leptodactylus* (Kobayashi et al., 1989; Wnuk, 1989). cDNAs encoding TnC have been cloned from brine shrimp, *Artemia franciscana* (GenBank accession number EU358105) and the copepods *Caligus clemensi* (#BT080658, #BT080568, and #BT080327) and *Lepeophtheirus salmonis* (#BT078440, #BT078370, #BT078246, #BT078206, and #BT077867). Among other invertebrates, *Caenorhabditis elegans* has two TnC isoforms (Terami et al., 1999) and *Drosophila melanogaster* has four TnC isoforms (Fyrberg et al., 1994; Qiu et al., 2003). The scallop, *Patinopectin yessoensis*, expresses two splice variants of a single TnC gene (Yuasa and Takagi, 2000). Vertebrates express two TnC isoforms, which are encoded by distinct genes (Romero-Herrera et al., 1976; Roher et al., 1986; Parmacek and Leiden, 1989; Parmacek et al., 1990; Gillis et al., 2007).

To better understand the diversity of TnC isoforms in decapod crustaceans, reverse transcription-polymerase chain reaction (RT-PCR) and rapid polymerization of cDNA ends (RACE) were used to clone and characterize cDNAs encoding six isoforms of TnC from lobster skeletal muscles. Primer walking was used to complete the sequencing of expressed sequence tag (EST) clones encoding five additional isoforms. Endpoint PCR was used to determine the expression of the lobster TnC isoforms in four skeletal muscles, heart, and seven other tissues.

2. Materials and methods

2.1. Animals

American lobsters, *H. americanus*, were purchased from a local supermarket or reared at the University of California-Davis Bodega Marine Laboratory, Bodega Bay, California. Intermolt animals were anaesthetized on ice; tissues were dissected, frozen in liquid nitrogen, and stored at -80 °C.

Table 1
Primer sequences used for 5′- and 3′-RACE to clone lobster TnC isoforms.

Primer name	Primer sequence	T_m (°C)
TnC_5′RACE_outer_1	ACTCCACGAACTCCTCGAACTTCA	59.6
TnC_5′RACE_outer_2	CAACTCCACGAACTCCTCGAACTTCAA	60.3
TnC_5′RACE_inner_1	CTCGGAAATCTTGACACCCAT	54.9
TnC_5′RACE_inner_2	TCGGCGATAACCTCCTGAAGGTT	60.1
TnC_3′RACE_outer_1	ATGGGTGTCAAGATTTCCGAGA	56.1
TnC_3′RACE_outer_2	CGAGACTGACGAGGACGGTTC	59.3
TnC_3′RACE_inner_1	CTGATTGAGGAGGACGAGGAGGC	60.9
TnC_3′RACE_inner_2	AGCTGGACAACAGGTTGACT	56.5

Table 2
RT-PCR primers targeted to 5′- and 3′-RACE products that generated contiguous sequences of Ha-TnC_1, $-C_{2a}$, $-C_3$, and $-C_{4''}$.

Isoform	Primer name	Primer sequence	T_m (°C)
TnC_1	TnC EC6-4*	CAGGTGGCTCCCCTCCATCCC	64.4
	TnC13-1	GGTCTTCTTCAGGTTTCCTTCAGGTAGG	60.1
TnC_{2a}	Tn-C2a readthrough F5*	GGACTCGTTGGATGAAGAACAG	55.5
	Tn-C2a realtime R1	GAGAGACGGTGGTGATGCTGG	59.9
TnC_3	TnC-3a 1 5′*	CTGTCACTTGTCTAGTCTGTCTTCTCGC	59.9
	TnC 5′RACE outer 1	ACTCCACGAACTCCTCGAACTTCA	59.6
$TnC_{4''}$	TnC EC502-1*	CCACATACACACTAACAACCTCCTCC	58.8
	TnC EC302-1	GGAGGAGGTTGTTAGTGTGTATGTGG	58.8

Asterisks (*) denote forward primers.

2.2. Cloning of cDNAs encoding lobster troponin-C

EST cDNA clones encoding a total of five distinct lobster TnCs were sequenced. Two partial Ha-TnC sequences were identified from the lobster EST library generated from whole animals from the Marine Genomics Project website (www.marinegenomics.org) containing the EST sequences HA_mx0_01a01 (GenBank accession number CN852450) and HA_mx0_07c01 (#CN852954) ligated into SPORT vector. Three additional partial sequences were identified from the GenBank database, generated from multiple muscle and non-muscle tissues, corresponding to EST sequences HA_mx1_35g09 (#EX471218), HA_mx2_65f01 (#FD699849) and HA_mx2_86b02 (#FE535729). Plasmids were purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Inserts were sequenced by Davis Sequencing (Davis, CA, USA) in both directions with SP6 and T7 promotor primers initially and sequence-specific primers subsequently.

Table 3
Isoform-specific primers used for endpoint PCR analysis of TnC expression in lobster tissues. EF2 was used as an internal template-loading control.

cDNA	Primer name	Primer sequence	T_m (°C)
TnC_1	Ha-TnC1 Fc2*	CATTGAGGAGGTGGACGAGGAC	59.3
	Ha-TnC1 Rc1	GGTTTCCTTCAGGTAGGCGTAACACTAC	60.3
TnC_{2a}	Ha-TnC 3′RACE outer 2*	CGAGACTGACGAGGACGGTTC	59.3
	Ha-Tn-C2a realtime R1	GAGAGACGGTGGTGATGCTGG	59.9
$TnC_{2b'}$	Ha-Tn-C2b′ F2*	CCTTCAACACACATCAACATGGCC	58.8
	Ha-TnC Hamx135g09 EX R1	GAGGAAGTAGAGGAAGGAGGAAGAGTC	59.0
$TnC_{2b''}$	Ha-Tn-C2b″ F1*	GGATATGCTGGATGAAGAACAGATTGG	57.5
	Ha-TnC Hamx135g09 EX R1	GAGGAAGTAGAGGAAGGAGGAAGAGTC	59.0
TnC_3	Ha-TnC-3a 1 5′*	CTGTCACTTGTCTAGTCTGTCTTCTCGC	59.9
	Ha-TnC 5′RACE outer 1	ACTCCACGAACTCCTCGAACTTCA	59.6
$TnC_{4'}$	Ha-TnC 3′RACE outer 1*	AAGTGAACCAGGTGCGAGTAGTGGG	62.5
	Ha-TnC-4 1 3′	GAGTAGTGGGTGAGTGAGTGTTGTG	54.2
$TnC_{4''}$	Ha-Tn-C4′ F3*	CTGTCTATGTGTCTGTCTGTCTGTC	56.5
	Ha-Tn-C4′ R1	CAAGGTCTGCGTCGTGATGTAG	57.7
$TnC_{4'''}$	Ha-TnC-4‴ EC-302 F1*	GGTTGACTGAGGAGGATCTTGACAGC	60.7
	Ha-TnC-4 1 3′	GAGTAGTGGGTGAGTGAGTGTTGTG	58.9
TnC_5	Ha-TnC5 F67*	CTATATCACTACGCAGACGCTGAAGGAG	59.7
	Ha-TnC5 R46	CAGCAGTAGCAGGTCCTCTCTG	59.0
TnC_6	Ha-Tn-C7 F4*	CCTCAACCCTACACCACACCACC	61.6
	Ha-Tn-C7 R5	CATCGCCTCCTCGTCCTGCTCTTC	63.1
TnC_{6x}	Ha-TnC6x F2*	GTTCGTCCGAGTGGTGGGCCACTC	65.5
	Ha-TnC6x R1	GAGGACAGACAAACAGAAACCCAGACC	60.8
EF2	Ha-EF2 F1*	TTCTATGCCTTCGGACGTGTGTTCTC	60.3
	Ha-EF2 R1	TGATGGTGCCAGTCTTGACCAGGTAC	59.6

Asterisks (*) denote forward primers.