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Cloning and characterization of the heart muscle isoform of sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) from crayfish

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Summary

This paper describes the cloning and functional characterization of the heart muscle isoform of Sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) from crayfish *Procambarus clarkii*. The complete crayfish heart SERCA, identified by reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE), consists of 4495 bp with a 3060 bp open reading frame, coding for 1020 amino acids. This isoform differs from the previously identified axial abdominal (tail) muscle SERCA solely in its C-terminal amino acids. The last nine amino acids of the tail muscle isoform are replaced by 27 hydrophobic amino acids in the heart isoform that have the potential to form an additional transmembrane domain. Consistent with other invertebrate studies, Southern blot analysis suggested that the heart and tail muscle isoforms are encoded from the same gene that is equally related to *SERCA-1*, *-2* and *-3* of vertebrates. The tissue distributions of these two isoforms have been assessed using isoform-specific probes and northern analysis. A cardiac-specific probe bound only to a 5.8 kb species in heart and had minimal cross-hybridization with 7.6 and 5.8 kb species in eggs and no hybridization with tail muscle. A tail-isoform-specific probe hybridized with a 4.5 kb species in tail muscle and cross-hybridized with a 4.5 kb species in eggs and 8.8 kb in heart muscle. Both isoforms are expressed in eggs suggesting that transcripts are formed early in development and are subsequently broadly expressed in all tissue types. Expression of the cardiac muscle SERCA isoform varied with the stage of moulting. Expression was high in intermoult and decreased in premoult. However, expression was restored rapidly in postmoult (within 2 days) unlike expression of tail muscle SERCA, which remained downregulated for weeks. Differences in contractility between the two muscle types in the postmoult period may explain these expression patterns.

Key words: heart muscle, cDNA sequence, mRNA expression, tissue specific distribution, moulting cycle, crayfish, *Procambarus clarkii*.

Introduction

The Sarco/endoplasmic reticulum Ca^{2+} adenosine triphosphatase (SERCA) is a Ca^{2+} pump located on the internal membranes of the cell that temporarily removes cytosolic Ca^{2+} by sequestering it in the SR/ER. In muscle it functions to resequester Ca^{2+} in the SR lumen following muscle contraction; in non-muscle tissue it regulates intracellular (IC) Ca^{2+} during routine signaling events or mass transit of Ca^{2+} during periods of transepithelial flux. SERCA is an integral membrane protein with 1000 amino acid residues. The transmembrane domain consists of 10 α-helices that include the Ca^{2+} binding and translocating domain. A small and a large hydrophilic loop are exposed to the cytoplasm and connected to the membrane via a stalk-like cluster of α-helices (Brandl et al., 1986).

Mammalian SERCA is encoded by a family of three homologous and alternatively spliced genes that encode multiple isoforms with a distinct pattern of tissue expression (MacLennan et al., 1985; Brandl et al., 1986, 1987; Gunteski-Hamblin et al., 1988; Burk et al., 1989; Wu and Lytton, 1993; Wuytack et al., 1994; Wu et al., 1995). SERCA1 is expressed in fast twitch skeletal muscle. SERCA2 generates two isoforms that differ in their C-terminal tail. SERCA2a is a muscle-specific isoform with a short 4-amino-acid (aa) tail; the ubiquitous SERCA2b has an extended tail of 50 residues that provides an additional membrane-spanning stretch placing the terminus in the ER lumen (Campbell et al., 1992). The gene encoding *SERCA3* is expressed in endothelial and epithelial cells of a variety of muscle and non-muscle tissue. SERCA genes have also been cloned in other vertebrates such as birds, frogs and fish (Karin et al., 1989; Campbell et al., 1992; Vilsen and Andersen, 1992; Tullis and Block, 1996).

SERCA has also been characterized in several invertebrate arthropods including the fruit fly, *Drosophila melanogaster* (whole body and presumed primarily muscle; Varadi et al., 1989; Magyar and Varadi, 1990; Magyar et al., 1995), the brine shrimp, *Artemia franciscana* (whole body and presumed

muscle; Palmero and Sastre, 1989; Escalante and Sastre, 1993, 1994) and the crayfish, *Procambarus clarkii* (axial abdominal muscle; Zhang et al., 2000). A single SERCA gene was identified in *Drosophila* that had low abundance in all tissues. In *Artemia* two SERCA mRNAs (4.5 and 5.2 kb), which are developmentally regulated, are originated by alternative splicing of a single gene (homologous to *SERCA2a* and *-b* in vertebrates). In *Artemia*, the last six amino acids of one isoform are replaced by 30 aa in the other isoform. The 30 aa extension of the *Artemia* isoform does not show significant homology with the 49 aa extension of the mammalian SERCA2b; however, both exhibit hydrophobicity.

In our laboratory we have employed the moulting cycle of a freshwater crustacean, the crayfish *Procambarus clarkii*, as a non-mammalian model to study regulation of expression of Ca^{2+} pumps (Wheatly, 1996, 1999). As arthropods, crustaceans exhibit incremental growth at ecdysis that results in discontinuous patterns of muscle growth and transepithelial Ca^{2+} flux. Skeletal muscles can exhibit different growth patterns during moulting depending upon location (Mellon et al., 1992). Abdominal and leg muscles undergo longitudinal growth during immediate postmoult with cross-sectional growth occurring in late postmoult/intermoult due to longitudinal myofibrillar splitting in response to stretching of the new cuticle (El Haj et al., 1992). Meanwhile claw muscles undergo a preprogrammed premoult atrophy to allow removal through the narrow basi-ischial joint (Mykles and Skinner, 1990; Mykles, 1997).

In an earlier study we characterized the crayfish SERCA from axial abdominal muscle (Zhang et al., 2000). Expression was greatest in intermoult and decreased around ecdysis in response to either hormonal or mechanical stimuli. In order to determine whether this pattern of expression is replicated in all crustacean muscle types we elected to characterize a novel SERCA isoform in cardiac muscle and its expression throughout the moulting cycle. Unlike skeletal muscle, heart is continuously contractile. Further, since it is not encased in cuticle, heart muscle may grow continuously rather than incrementally during the moulting cycle.

Materials and methods

Animal material

Crayfish (*Procambarus clarkii* Girard) were obtained from Carolina Biological Supply and maintained in 40 l aquaria in filtered aerated water at room temperature (21 °C). Tissues were removed from animals at various stages in the natural moulting cycle. Premoult status was determined from the gastrolith index (McWhinnie, 1962). Postmoult status was classified in reference to the day of ecdysis (shedding). Following decerebration, the following tissues were dissected out: heart muscle, axial abdominal muscle and unfertilized eggs.

Isolation of total RNA and mRNA

After dissection, tissues were frozen immediately in liquid nitrogen and stored at –80 °C. Total RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). Messenger RNA was separated from total RNA using an oligo-dT cellulose column (Stratagene; Sambrook et al., 1989). RNA or mRNA was quantified spectrophotometrically at wavelengths of 260 and 280 nm. Only RNAs with an absorbance ratio A_{260}:A_{280} of greater than 1.8 were used for further experiments. The integrity of RNA was confirmed on a 0.72 mol l^{-1} formaldehyde, 1 % agarose denaturing gel run in Mops buffer (5 mmol l^{-1} sodium acetate, 1 mmol l^{-1} EDTA, 20 mmol l^{-1} Mops, pH 6.6).

Amplification of central 460 bp fragment by RT-PCR

First strand cDNA was reverse transcribed from 400 ng of heart muscle mRNA using the SuperScript II RNase H-reverse transcriptase (Gibco BRL) with oligo$(dT)_{12\text{-}18}$ as primer. Based on two highly conserved regions of the published *Artemia* muscle SERCA sequence (corresponding to nucleotides 2176–2195 and 2617–2636, Palmero and Sastre, 1989), two non-degenerate primers (5′-GAAATTTCC-GCTATGACTGG-3′ sense and 5′-ACAGTGGCAGCACC-AACATA-3′ antisense) were designed using Oligo 4.0 software (American Biotechnology Laboratory). These primers targeted a fragment of approximately 460 base pairs (bp) located between the 5′-(*p*-fluorosulphonyl)benzoyladenosine (FSBA) binding site and transmembrane region 7 of a typical SERCA. These primers had been successful in amplification of a 460 bp fragment of axial abdominal muscle SERCA (Zhang et al., 2000) and were subsequently used to amplify SERCA from heart. Polymerase chain reactions (PCR; total volume 50 µl) included 2 µl of first strand cDNA reaction, 20 mmol l^{-1} Tris-HCl, pH 8.4, 50 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} dNTP mix, 0.1–0.2 µmol l^{-1} of each primer and 2.5 units of *Taq* DNA polymerase (Gibco BRL). PCR was performed by the hot-start method in an MJ Research thermal cycler. PCR cycles were: 94 °C, 3 min followed by 25 cycles of 94 °C for 30 s, 53 °C for 1 min, 72 °C for 1 min, and a final cycle of 72 °C for 5 min. Negative controls in which reactions contained only one primer or no template cDNA were included. PCR products were analyzed by electrophoresis on a 0.8–1.0 % agarose gel with 0.5 µg ml^{-1} of ethidium bromide in 1× TAE buffer (40 mmol l^{-1} Tris, pH 7.2, 40 mmol l^{-1} sodium acetate and 1 mmol l^{-1} EDTA). The DNA bands were visualized with ultraviolet light.

Marathon^{TM} rapid amplification of cDNA ends (RACE)

Rapid amplification of cDNA ends (RACE) was employed to complete the 3′ and 5′ regions of the cardiac SERCA. Briefly first strand cDNA was reverse transcribed by MMOL/LLV reverse transcriptase using a modified lock-docking oligo(dT) primer. Following creation of blunt ends with T_4 DNA polymerase, the double stranded cDNA was ligated to the Marathon cDNA adaptor that has the complementary sequence to the Clontech adaptor primer. PCR amplification of the 5′ region was performed on the resulting template using the Clontech adaptor primer and the gene-specific primer (5′-TCA

GCT TTC TTC AGG GCA GGT GCA TC-3′, antisense), which is designed based on the amplified 460 bp fragment of crayfish heart muscle SERCA fragment. PCR amplification of the 3′ region was carried out using the gene-specific primer (5′-GAT GCA CCT GCC CTG AAG AAA GCT GA-3′, sense) and the Clontech adaptor primer. PCR conditions were as follows: one cycle at 94 °C for 3 min; five cycles at 94 °C for 30 s, 72 °C for 4 min; 25 cycles at 94 °C for 30 s, 68 °C, for 4 min; followed by one cycle at 72 °C for 5 min. PCR products were ligated to PCR 2.1 vector (Invitrogen) for transformation into INVαF host cells (Invitrogen). Each clone was digested with the appropriate restriction enzymes and subcloned for sequencing. Two or three independent clones containing the appropriate insert were sequenced from both ends.

DNA sequencing and sequence analysis

The cDNA clones were sequenced by automated sequencing (Applied Biosystems Division Model 377, University of Cincinnati, OH). The complete sequence was analyzed with MacDNASIS software (Hitachi). Sequence homology was revealed through a GenBank database search using the BLAST algorithm (Altschul et al., 1990). Hydropathy analysis was performed with MacDNASIS software (Kyte and Doolittle, 1982).

Northern blot analysis

Northern blot analysis was performed to delineate tissue-specific distribution of the two SERCA isoforms in intermoult crayfish tissues. Total RNAs (0.2–15 μg) from each tissue (heart muscle, axial abdominal muscle, eggs) examined was fractionated by electrophoresis through 0.72 mol l^{-1} formaldehyde, 1 % agarose denaturing gel run in Mops buffer and transferred overnight to a Nytran Plus membrane (Schleicher & Schuell) by capillary elution in 10× SSC (1× SSC is 150 mmol l^{-1} NaCl, 15 mmol l^{-1} sodium citrate). RNA was fixed by ultraviolet crosslinking using a UVC-515 ultraviolet multilinker from Ultra-Lum (120,000 μJ cm^{-2}). RNA molecular mass markers (a 0.24–9.5 kb ladder) were run along with the samples, then visualized with UV light after staining with ethidium bromide, and used for the standard curve. The membrane was prehybridized for 4 h at 68 °C in 6× SSC, 2× Denhardt's reagent (0.4 g Ficoll type 400, 0.4 g polyvinylpyrrolidone, 0.4 g bovine serum albumin in 1 l water), 0.1 % SDS and 100 ng ml^{-1} of denatured salmon sperm DNA. Hybridization was performed overnight at 68 °C in the prehybridization solution with 20 ng of SERCA cDNA probe (outlined below) that was randomly labeled with [α-^{32}P]dATP. The membrane was washed four times for 15 min at 60 °C in 0.1× SSC and 0.1 % SDS. Membrane was exposed to X-ray film with intensifying screens at –80 °C. The following fragments from different regions of crayfish SERCA isoforms were used as probes: (A) a 460 bp fragment corresponding to crayfish nucleotides 2086–2546 that is common to both heart and axial abdominal muscle isoforms; (B) a 723 bp *Xba*I to poly(A) tail fragment (nucleotides 3772 to poly(A) tail) from the 3′ untranslated region of crayfish heart muscle SERCA; and (C) a 515 bp *Xho*I to poly(A) tail fragment nucleotide 3205 to poly(A) tail from the 3′ untranslated region of crayfish axial abdominal muscle SERCA (Zhang et al., 2000).

These fragments were purified from the positive clones using a QIAquick gel extraction kit (Qiagen) and labeled for 3 h with [α-^{32}P]dATP to a specific activity of 1×10^9 cts min^{-1} μg^{-1} using a random labelling kit (Gibco BRL). The labelled probe was then separated from unincorporated nucleotides by chromatography on a Sephadex G-50 Nick column (Pharmacia Biotech). Following high stringency washes (four times for 15 min at 60 °C in 0.1× SSC and 0.1 % SDS), membranes were exposed to X-ray film with intensifying screens at –80 °C.

Expression of the heart-specific SERCA was quantified in heart muscle as a function of stage in the moulting cycle using northern blotting. Heart muscle total RNA was isolated from six crayfish in each moulting stage (intermoult, late premoult, 1–2 days postmoult) and hybridized to the heart-specific SERCA probe (B, 723 bp) with exposure to X-ray film for 24 h. To confirm equal loading between samples, 18S RNA was quantified on a corresponding formaldehyde-agarose gel. Total RNA content was determined by OD_{260} and shown by ethidium bromide staining.

Southern blot analysis

Southern blotting was used to determine whether the heart- and tail-specific isoforms originated from one or multiple genes. Total genomic DNA was purified from crayfish muscle following a previously described protocol (Sambrook et al., 1989). After electrophoresis, the DNA gel was denatured in denaturing solution (1.5 mol l^{-1} NaCl, 0.5 mol l^{-1} NaOH) for 45 min and neutralized in 1 mol l^{-1} Tris-Cl, pH 7.4, 1.5 mol l^{-1} NaCl for 30 min. The DNA was then transferred to a Nytran Plus membrane (Schleicher & Schuell) by capillary elution in 10× SSC and hybridized under the same conditions as described above for the northern blot except that the probe used was from the 3′ terminal of crayfish axial muscle SERCA (Zhang et al., 2000), corresponding to nucleotides 2785–3019. The membrane was washed twice for 20 min in 2× SSC, 0.1 % SDS at 65 °C and once for 20 min in 0.2× SSC, 0.1 % SDS at 65 °C, then was exposed to X-ray film with intensifying screen at –80 °C.

Results

Amplification of central 460 bp fragment by RT-PCR

A pair of non-degenerate primers were as successful in amplification of a discrete 460 bp product from crayfish heart muscle cDNA as they had been in axial muscle (Zhang et al., 2000) and as they proved to be in other tissues such as gill and antennal gland (kidney). The successful amplification of this 460 bp fragment from a variety of crayfish tissues demonstrates that this fragment is highly conserved among the different SERCA isoforms. In this heart sequence only two bases were different from that of axial abdominal muscle, and these

substitutions did not result in any change in the amino acid sequence. A search of GenBank confirmed that the nucleotide sequence matched exclusively with SERCA from *Drosophila* (80 %), *Artemia* (73 %) and many other vertebrates. Its amino acid sequence showed 85 % homology with that of *Artemia* and *Drosophila*, and 82 % with rabbit fast twitch muscle SERCA (1a or 1b). This partial sequence provided crucial DNA sequence information required for the 5′ and 3′ RACE cloning of the complete crayfish cardiac SERCA cDNA. Based on the 460 bp partial sequence, two gene-specific primers were used along with Clontech's adaptor primers to perform Marathon RACE of crayfish cardiac muscle cDNA. The cloning strategy is described previously (Zhang et al., 2000).

Cloning of the complete cDNA sequence by RACE

Following RACE, a 2.3 kb RACE product was obtained from both the 5′ RACE and 3′ RACE amplifications. The bands were cloned into pCRII vector, respectively. Two independent clones containing an insert of appropriate size were sequenced from both directions following subcloning. The complete nucleotide sequence and the deduced amino acid sequence is shown in Fig. 1. The complete crayfish heart SERCA consists of 4495 bp with a 3060 bp open reading frame, coding for 1020 amino acids. The 5′-terminal 195 bp noncoding region is GC rich. An in-frame stop codon is situated 25 bases upstream from the start codon. The initiator Met codon was part of the longer sequence, -CCACCATGG-, which contains a purine at position –3 and a G at position +4, both of which are necessary for efficient initiation of translation (Kozak, 1984). There is an extremely long 1435-nucleotide 3′ terminal noncoding region with a poly(A) tail. The poly(A) addition signal AATAAA begins 13 bp from the poly(A) tail.

Surprisingly, the nucleotide sequence of this clone is almost identical to that of the crayfish axial abdominal muscle SERCA clone up to nucleotide 2980, except for a couple of conservative nucleotide changes and three non-conservative changes (the D in amino acid 57 of axial muscle is changed to Y in cardiac muscle; the T in amino acid 191 of axial muscle is changed to A in cardiac muscle; the H in amino acid 683 of axial muscle is changed to R in cardiac muscle; Fig. 1B). Importantly, from nucleotide 2981 to the end of the clone the cardiac muscle sequence differs completely from that of the axial muscle SERCA. In axial muscle the divergent sequence includes the region coding for the terminal nine amino acids; the heart SERCA sequence codes for 27 additional amino acids in the C-terminal region of the protein that are highly hydrophobic. The relationship between these two crayfish SERCA isoforms is similar to that observed for the two SERCA isoforms in *Artemia* and

A

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                                                        ATCCTAATACGACTC  -181
ACTATAGGGCTTGAGCGGCCGCCCGGGCAGGTGCTGGACAGGGCAAGGCGGGCGTTCCTT  -121
TTCCTTTCTGTGTCTCTTTCCTGGGAGCCAGTCATTTTGGCCCAGGCTCGTGTCTTTAGA   -61
GTAGCAGAAGTGGACGGAGCAAGAGAGAGAGAATAGATCGCCCGCCATCACCGAGCCACC    -1
ATGGATGATGCACATTGCTTTCCCGTCGAGGACGTCGTCGCGAAATTTGGCGTGAACATT    60
 M  D  D  A  H  C  F  P  V  E  D  V  V  A  K  F  G  V  N  I     20
GAGAATGGCCTCTCCGCGTCTCAAGTGAAGGATTATCAGGCCAAATATGGCCCCAACGAG   120
 E  N  G  L  S  A  S  Q  V  K  D  Y  Q  A  K  Y  G  P  N  E     40
CTACCCGCCGAGGAAGGCAAGTCTCTCCTCCAGCTCATCCTGGAGCAGTTCTACGACTTG   180
 L  P  A  E  E  G  K  S  L  L  Q  L  I  L  E  Q  F  Y  D  L     60
CTTGTTAAAATCCTTCTTCTCGCAGCCATTATTTCATTCGTCCTGGCGTGTTTCGAAGAA   240
 L  V  K  I  L  L  L  A  A  I  I  S  F  V  L  A  C  F  E  E     80
GGTGAAGAAACCGTCACCGCCTTCGTGGAACCCTTCGTCATCCTGCTTATCCTGATCGCT   300
 G  E  E  T  V  T  A  F  V  E  P  F  V  I  L  L  I  L  I  A    100
AACGCCATCGTGGGCGTGTGGCAGGAACGCAATGCCGAATCGGCCATCGAGGCGCTGAAG   360
 N  A  I  V  G  V  W  Q  E  R  N  A  E  S  A  I  E  A  L  K    120
GAGTACGAGCCCGAGATGGGCAAGGTANTGCGCTCCAACAAGCATGGTGTGCAGAAGGTC   420
 E  Y  E  P  E  M  G  K  V  X  R  S  N  K  H  G  V  Q  K  V    140
CGTGCCAGGGAGATAGTCCCGGGGGACATCGTTGAGGTCTCTGTTGGNGACAAGATTCCT   480
 R  A  R  E  I  V  P  G  D  I  V  E  V  S  V  G  D  K  I  P    160
GCTGACATTCGCCTTGTCAAGATTTTTTCCACGACCCTACGTATTGACCAGTCTATCCTG   540
 A  D  I  R  L  V  K  I  F  S  T  T  L  R  I  D  Q  S  I  L    180
ACTGGAGAGTCTGTTTCGGTCATCAAGCACGCTGATGCCATTCCCGACCCCAAAGGCTGTC  600
 T  G  E  S  V  S  V  I  K  H  A  D  A  I  P  D  P  K  A  V    200
AACCAGGACAAGAAGAACATCCTCTTCTCAGGAACCAATGTTTCTGCCGGCAAGGCACGT   660
 N  Q  D  K  K  N  I  L  F  S  G  T  N  V  S  A  G  K  A  R    220
GGTGTNGTCATTGGTACAGGTCTCGCAACTGCCATTGGTAAGATCCGCACCCAAATGGCT   720
 G  V  V  I  G  T  G  L  A  T  A  I  G  K  I  R  T  Q  M  A    240
GAGACTGAAGAAATCAAGACTCCACTACAACAGAAACTTGATGAATTTGGCGAACAATTA   780
 E  T  E  E  I  K  T  P  L  Q  Q  K  L  D  E  F  G  E  Q  L    260
TCCAAGGTTATCTCCATTATTTGTGTTGCTGTCTGGGCTATCAATATTGGACATTTCAAT   840
 S  K  V  I  S  I  I  C  V  A  V  W  A  I  N  I  G  H  F  N    280
GACCCTGCTCATGGTGGTTCCTGGATCAAGGGTGCTATTTATTATTTCAAGATTGCTGTT   900
 D  P  A  H  G  G  S  W  I  K  G  A  I  Y  Y  F  K  I  A  V    300
GCCTTGGCTGTGGCTGCTATTCCCGAAGGCCTTCCCGCTGTTATTACTACTTGTTTGGCT   960
 A  L  A  V  A  A  I  P  E  G  L  P  A  V  I  T  T  C  L  A    320
CTGGGTACCCGTCGTATGGCTAAGAAGAATGCCATTGTTAGGTCTCTTCCCTCTGTTGAA  1020
 L  G  T  R  R  M  A  K  K  N  A  I  V  R  S  L  P  S  V  E    340
ACTCTGGGCTGCACTTCTGTCATCTGCTCTGATAAAACCGGCACGCTCACCACCAACCAG  1080
 T  L  G  C  T  S  V  I  C  S  D  K  T  G  T  L  T  T  N  Q    360
                          phosphorylation
ATGTCTGTGTCTCGTATGTTCATCATGGACAAGGTTGAGGGTAACGATTCCTCTCTTCTT  1140
 M  S  V  S  R  M  F  I  M  D  K  V  E  G  N  D  S  S  L  L    380
GAATTTGAAGTTACTGGCTCCACCTATGAACCTATTGGTGATGTATACCTGAAAAATACT  1200
 E  F  E  V  T  G  S  T  Y  E  P  I  G  D  V  Y  L  K  N  T    400
AAAGTTAAGGGATCTGACTTTGAGGGATTACAAGAACTCTCTACCATTTCTTTTATGTGT  1260
 K  V  K  G  S  D  F  E  G  L  Q  E  L  S  T  I  S  F  M  C    420
AATGACTCTTCCATTGACTTTAATGAATTCAAGAATGTGTTTGAGAAGGTTGGTGAGGCA  1320
 N  D  S  S  I  D  F  N  E  F  K  N  V  F  E  K  V  G  E  A    440
ACTGAGACAGCTCTTATTGTCCTTGGTGAGAAGATCAACCCATACAACATGTCTAAATCT  1380
 T  E  T  A  L  I  V  L  G  E  K  I  N  P  Y  N  M  S  K  S    460
GGCTTGGATCGTCGCTCTGCTGCCATTATTGCTAGGCACGACATGGAGACAAAATGGAAG  1440
 G  L  D  R  R  S  A  A  I  I  A  R  H  D  M  E  T  K  W  K    480
AAAGAATTCACCCTCGAGTTCTCACGTGATCGCAAATCCATGTCTTCATACTGTGTTCCA  1500
 K  E  F  T  L  E  F  S  R  D  R  K  S  M  S  S  Y  C  V  P    500
CTCAAACCTACCCGCTTGGGAACTGGACCAAAGATGTTCTGCAAAGGAGCCCCTGAGGGT  1560
 L  K  P  T  R  L  G  T  G  P  K  M  F  C  K  G  A  P  E  G    520
                                    FITC
GTACTTGATCGCTGCACTCACGTGCGTGTTGGCACTCAAAAGGTCCCTCTTACTGCTGGT  1620
 V  L  D  R  C  T  H  V  R  V  G  T  Q  K  V  P  L  T  A  G    540
GTGAAAGAGAAGATTCTGTCCGTCACCCGTGATTATGGCTGTGGTCGTGACACTCTTCGC  1680
 V  K  E  K  I  L  S  V  T  R  D  Y  G  C  G  R  D  T  L  R    560
TGCTTGGGTCTTGCTACCATCGATAATCCAATGAAACCTGAAGATATGGATCTGGGAGAA  1740
 C  L  G  L  A  T  I  D  N  P  M  K  P  E  D  M  D  L  G  E    580
GCTTCTAAGTTCTATACATATGAAGTTAATATGACATTTGTTGGCGTAGTTGGTATGCTT  1800
 A  S  K  F  Y  T  Y  E  V  N  M  T  F  V  G  V  V  G  M  L    600
GACCCACCACGTAAGGAAGTTAAAGATTCAATCCAGAGATGTCGTGATGCTGGTATCCGT  1860
 D  P  P  R  K  E  V  K  D  S  I  Q  R  C  R  D  A  G  I  R    620
GTTATTGTCATTACTGGAGACAATAAGGCAACTGCTGAGGCTATCTGCCGTCGTATTGGA  1920
 V  I  V  I  T  G  D  N  K  A  T  A  E  A  I  C  R  R  I  G    640
GTTTTTAAAGAAGATGAAGATACAACTGGTATGTCATATTCTGGCCGTGAGTTTGACGAG  1980
 V  F  K  E  D  E  D  T  T  G  M  S  Y  S  G  R  E  F  D  E    660
CTAAGTCCTGAAGAACAGAGGCAGGCATGTATTCGTTCCCGCCTCTTCTCTCGTGTAGAG  2040
 L  S  P  E  E  Q  R  Q  A  C  I  R  S  R  L  F  S  R  V  E    680
CCCTTCCGTAAGTCAAAGATTGTTGAATATCTTCAAGGAGAGAACGAGATCTCAGCCATG  2100
 P  F  R  K  S  K  I  V  E  Y  L  Q  G  E  N  E  I  S  A  M    700
ACAGGTGATGGTGTGAATGATGCACCTGCCCTGAAGAAAGCTGAAATTGGCATTGCTATG  2160
 T  G  D  G  V  N  D  A  P  A  L  K  K  A  E  I  G  I  A  M    720
      FSBA
GGATCTGGTACAGCTGTGGCCAAGTCTGCCTCTGAAATGGTGCTGGCTGATGACAACTTC  2220
 G  S  G  T  A  V  A  K  S  A  S  E  M  V  L  A  D  D  N  F    740
TCCTCTATTGTGGCTGCTGTTGAAGAAGGTCGTGCTATTTACAACAACATGAAGCAGTTC  2280
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 S  S  I  V  A  A  V  E  E  G  R  A  I  Y  N  N  M  K  Q  F    760
ATCCGTTACCTCATTTCTTCCAATGTTGGTGAGGTTGTTTCCATCTTTTTGACTGCTGCT  2340
 I  R  Y  L  I  S  S  N  V  G  E  V  V  S  I  F  L  T  A  A    780
CTAGGTCTTCCAGAAGCTCTTATCCCAGTCCANCTCCTGTGGGTCAACCTTGTAACTGAT  2400
 L  G  L  P  E  A  L  I  P  V  X  L  L  W  V  N  L  V  T  D    800
GGCTTGCCTGCTACTGCCTTGGGCTTCAACCCTCCAGATCTTGATATTATGGACAAACCT  2460
 G  L  P  A  T  A  L  G  F  N  P  P  D  L  D  I  M  D  K  P    820
CCCCGCAGAGCTGACGAGTCCCTCATCTCTGGCTGGCTATTCTTCCGTTACATGGCCATT  2520
 P  R  R  A  D  E  S  L  I  S  G  W  L  F  F  R  Y  M  A  I    840
GGTGGCTATGTTGGTGCANCCACCGTTTTTGCTGCATCATGGTGGTTCATGTATGATCCT  2580
 G  G  Y  V  G  A  X  T  V  F  A  A  S  W  W  F  M  Y  D  P    860
ACTGGCCCTCACCTAAACTACTATCAACTCTCTCACCATCTGCAATGTCTTGGAGATCCT  2640
 T  G  P  H  L  N  Y  Y  Q  L  S  H  H  L  Q  C  L  G  D  P    880
GAAAACTTTGAAGGACTGGACTGCAACATTTTCAGTCACCCTGCTCCAATGACAATGGCT  2700
 E  N  F  E  G  L  D  C  N  I  F  S  H  P  A  P  M  T  M  A    900
CTGTCTGTGCTGGTCACCATTGAAATGCTCAATGCTCTAAACAGCTTGTCTGAGAACCAG  2760
 L  S  V  L  V  T  I  E  M  L  N  A  L  N  S  L  S  E  N  Q    920
TCGCTGCTGATAATGCCTCCCTGGGTCAACTTCTGGCTGCTGGCGGCTATGGCCCTGTCC  2820
 S  L  L  I  M  P  P  W  V  N  F  W  L  L  A  A  M  A  L  S    940
ATGACCCTCCACTTCATCATCCTCTACATTGACATCCTCAGTACTGTGTTCCAGGTGATG  2880
 M  T  L  H  F  I  I  L  Y  I  D  I  L  S  T  V  F  Q  V  M    960
CCACTGTCTGTTGCCCAGTGGGTTGCTGTCTTGAAGATTTCCTTCCCTGTATTGCTGCTG  2940
 P  L  S  V  A  Q  W  V  A  V  L  K  I  S  F  P  V  L  L  L    980
GACGAGACTCTTAAGTTCATCGCACGTAATTACACCGACG/GTGAGAACAATCTGTATAAA 3000
 D  E  T  L  K  F  I  A  R  N  Y  T  D   G  E  N  N  L  Y  K   1000
TGCCACTGGATTGTGCTAGCCTGGGCTACCTATTTTGCCTACATCAAGATTTACTTCTTT  3060
 C  H  W  I  V  L  A  W  A  T  Y  F  A  Y  I  K  I  Y  F  F   1020
TAAACCTCAGTCACCACCCTAACAAAATGTACTGTCGGGAATCAGACCCGTTTTATTTTGA  3120
 *
AAACTTAAAAAAAGACTTAGTTGGGAGCAAGGAAAAACATTGTGATTTGACTTTCACAGA  3180
ATTTTCTCATTTGAAGAGGTTTGTGCAGAACAGCCTCTCATTCATGGAGAGATTAAGCTT  3240
TTTACAATGTTTATAGGTGCTACTTGAGCACTGTTGTGCTCCCTCTTACTGAAAGGTACA  3300
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CTGTTTGCTGCTGTTGCCACTGCTGTTTGCTTAAGTTGGGGGCAGTGTGAGTGTCGAGTA  3480
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AACGACTATTCTTATTAATGATACGACCAGAAAGCAGTAATAATTAAAAAAATTTTTTTT  4260
TTTTTTAAATCCTGTACGTGTATGATCACGAGACCGTTAAAGAGTAATTGCTTAAGTACT  4320
GTAGTTTAAACAAATTTGCCATCATTACCCATTGCATCCTTAAGCCCCCCCTTATTCACA  4380
AGAATCCTTTTAAAGACCTTTATTTATGGACTGTGAGATTGAGTTGTTTGCATGTCATAA  4440
TTAGTTATTTGACAGATTTTGGAATAAATTCATCATAGTTCAAAAAAAAAAAAAA       4500
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B

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                                                G/TACCCGAACAGATTAAGCAA  3000
                                                  V  P  E  Q  I  K  Q
CAGTGGTAAAGATTGCAAGTTAACATCTGCTCCAGCTTTTACAATTTCCTCACAGCCAGT  3060
 Q  W  *
AGCAATAGCTGTACTAACCTGCTAACTGTCAGTGCCACGCCTGTGATGAGCTGAACACCA  3120
CCCCACCATGGCCTCCACAGCAAGAGAACGTCTGTCAATACATCAGGGGGTTTCCTATCC  3180
CTAGAGATGATGATGCCCATGGCTCTCGAGAAGCAGTGGCCATGGGTCAATGGAGAGGGG  3240
GCAGCAGCATATGGCAGTGGTGTGCGGGGTCCTTCACCTCCACCAGAGATCTCAGCCTCC  3300
CCTTGCAATGCCTCGGGCCCTCTTCATGCCTGATGCAACACTTGGATTTGGCACACTTGC  3360
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GTAGAGAATGAATATGTACATTTACTTGTGTTGTAATTTGATTTAGAGTAACTAGACTAT  3480
TTTGATTCCCTTGTTAAAATAAACTGTTAGCCAAGCTACTCTTGGGCAAACATTACCAAA  3540
AGTCGCACAAAGTCTGTTGTGTCCTGTGTTCGTGCTCGTGTCAATTCGCCCTTCAGAGAG  3600
CAATGAACGACCATACAAACATCCACTTGTTCAAGTACAAAGATGTGGTGCAATTTTGTA  3660
CCTCATTTTGTACATGCCACTCATTTATTGTTGATAGAATAAATAATCAAAAGTA(n)     3720
```

Fig. 1. (A) The complete nucleotide and deduced amino acid sequence of crayfish cardiac muscle SERCA cDNA (accession number AF025848). Nucleotides and amino acids are numbered to the right of the sequence. The phosphorylation site, fluorescein isothiocyanate (FITC) site and the 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA)/γ-[4-(*N*-2-chloroethyl-*N*-methylamino)]benzylamide ATP (CIRATP) binding site are underlined and labeled. The start codon and the stop codon are indicated in bold letters. The slash in the sequence at nucleotide 2980 is the site at which the heart muscle sequence diverges from the axial abdominal muscle sequence (Zhang et al., 2000; accession no. AF025849). (B) The nucleotide sequence of crayfish axial abdominal muscle SERCA (Zhang et al., 2000) beginning from nucleotide 2980.

mammalian *SERCA2* isoforms (Fig. 2). The additional sequences do not show homology to each other among these different animals. However, they share a marked hydrophobic character, which may result in an additional transmembrane domain (Fig. 3).

A GenBank search using the BLAST algorithm (Altschul et al., 1990) revealed that the deduced amino acid sequence of crayfish heart SERCA matched with more than 30 SERCAs from various invertebrates and vertebrates, of which *Drosophila* SERCA showed the highest homologous score of 80 %, *Artemia* SERCA had 79 % identity, frog fast-twitch skeletal SERCA had 73 % identity. Mammalian SERCA1 and SERCA2 genes showed approx. 71–72 % identity, and SERCA3 had 68 % identity with crayfish heart SERCA.

Northern blot analysis of the tissue distribution of SERCA isoforms

To distinguish the tissue distribution of cardiac muscle SERCA isoform from axial muscle SERCA isoform, a northern blot of mRNA from axial muscle, cardiac muscle and egg was hybridized in individual experiments with cDNA probes specific to either crayfish heart SERCA (probe B, Fig. 4B) or crayfish axial abdominal muscle SERCA (probe C, Fig. 4C) and compared with a probe that was common to both isoforms (probe A, Fig. 4A), respectively.

Probe A was the 426 bp fragment corresponding to crayfish nucleotides 2086–2546 common to both isoforms. When hybridized with probe A, four bands

```
A   Crayfish axial abdominal muscle
    IARNYTD/VPEQIKQ
B   Crayfish heart muscle
    IARNYTD/GENNLYKCHWIVLAWATYFAYIKIYFF

C   Artemia 4.5 kb isoform
    VARKYTD/EFSFTK
D   Artemia 5.2 kb isoform
    VARKYTD/GMPLSSYFVDAWGLVLAWALFFGVIFYSPL

E   Rat SERCA2a
    VARNYLEP/AILE
F   Rat SERCA2b
    VARNYLEP/GKECVQPATKSCSFSACTDGISWPFVLL
    IMPLVIWVYSTDTNFSDMFWS
```

Fig. 2. Comparison of the C-terminal amino acid sequences of SERCAs. The amino acid sequence of SERCA C termini shown are for (A) crayfish axial abdominal muscle (Zhang et al., 2000; accession no. AAB82291); (B) crayfish heart muscle (present study; accession no. AAB82290); (C) the 4.5 kb *Artemia* protein (Palmero and Sastre, 1989; accession no. P35316); (D) the 5.2 kb *Artemia* protein (Escalante and Sastre, 1993; accession no. CAA51262); (E) rat SERCA2a (Brandl et al., 1986; accession no. P11508); (F) rat SERCA2b (Lytton et al., 1989; accession no. P11057). The slash mark denotes the point at which the amino acid sequence of each set of proteins diverges.

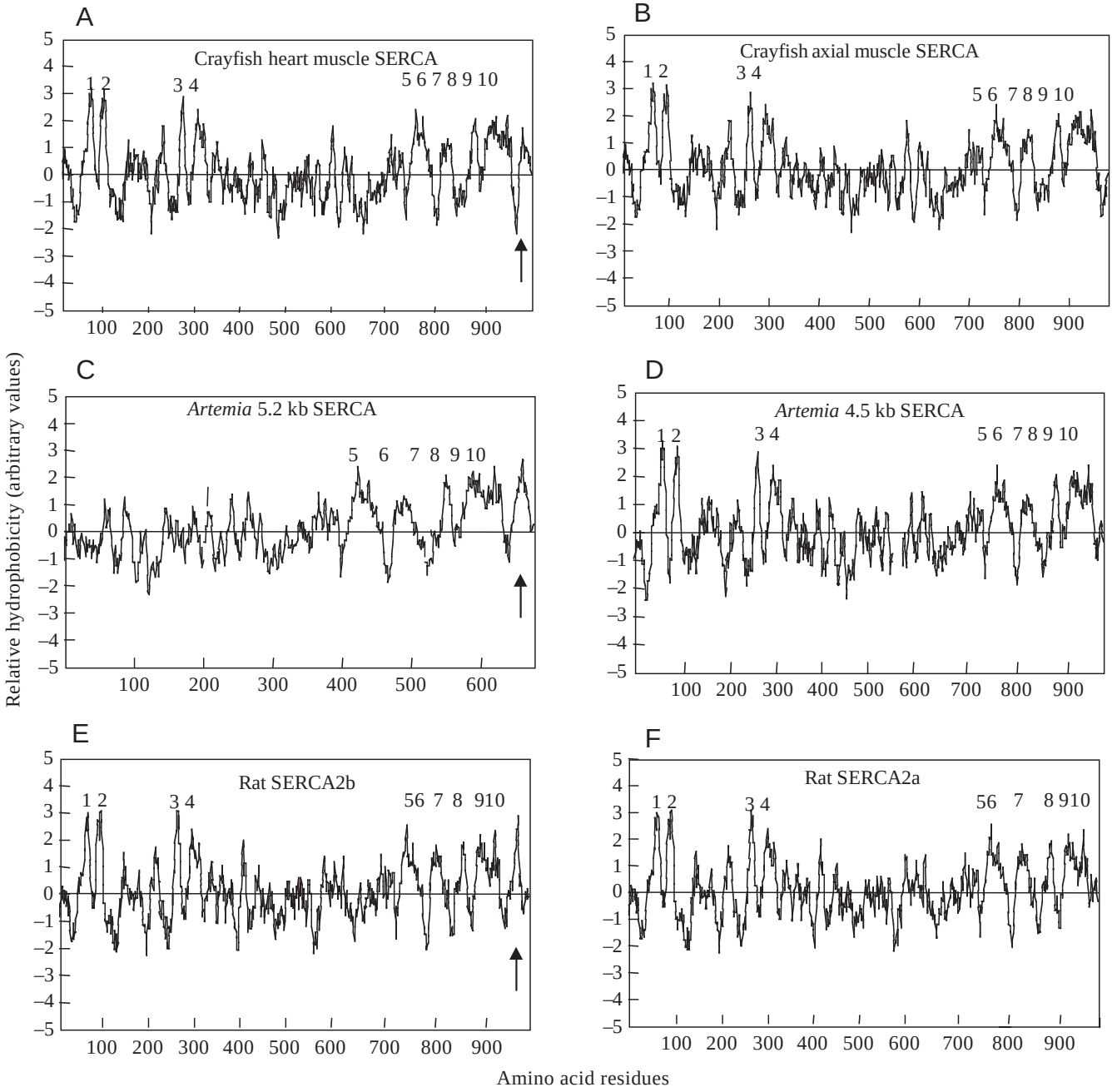

Fig. 3. Hydropathy plot of (A) crayfish cardiac muscle SERCA (present study; accession no. AAB82291) in comparison with (B) crayfish axial muscle (Zhang et al., 2000; accession no. AAB8229), (C) *Artemia* SERCA 5.2 kb isoform (Escalante and Sastre, 1993; accession no. CAA51262), (D) *Artemia* SERCA 4.5 kb isoform (Palmero and Sastre, 1989; accession no. P35316), (E) rat SERCA2b (Lytton et al., 1989; accession no. P11057) and (F) rat SERCA2a (Brandl et al., 1986; accession no. P11508). Hydrophobicity values were determined by the method of Kyte and Doolittle (1982) using a window of 12 residues (MacDNASIS, Rainbow Technologies). Putative transmembrane domains are numbered based on the model of Brandl et al. (1986). The arrows indicate the extra potential transmembrane domain.

were determined with molecular masses of 8.8, 7.6, 5.8 and 4.5 kb. In heart muscle this probe recognized a prominent 5.8 kb band, a secondary band at 7.6 kb, and a weak band at 8.8 kb. By comparison, only one prominent band at 4.5 kb was observed in axial abdominal muscle. This probe recognized a prominent band at 7.6 kb in eggs and two fainter bands at 5.8 and 4.5 kb. In summary, the data suggest that there are as many as four different isoforms of SERCA in the three tissues examined. The difference in molecular mass is due mainly to two factors. First, the 5′ upstream noncoding region may be much longer than the cloned sequence of 145 bp. Second, the 3′ end poly(A) tail often extends to

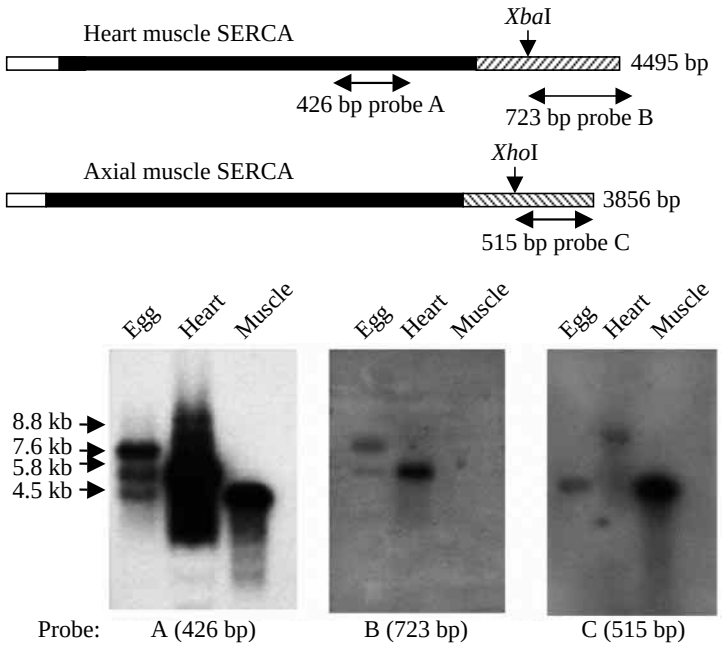

Fig. 4. Northern blot analysis of the distribution of the two crayfish SERCA isoforms (from heart and axial muscle) in intermoult tissues. Messenger RNAs from eggs, heart muscle and axial abdominal muscle were hybridized with the following probes: (A) the 426 bp fragment corresponding to crayfish nucleotides 2086–2546 in both heart and axial muscle SERCA; (B) the 723 bp *Xba*I–poly(A) tail fragment of the putative heart SERCA; (C) the 515 bp *Xho*I–poly(A) tail fragment of the putative muscle SERCA. The positions of marker nucleotides are shown at the left.

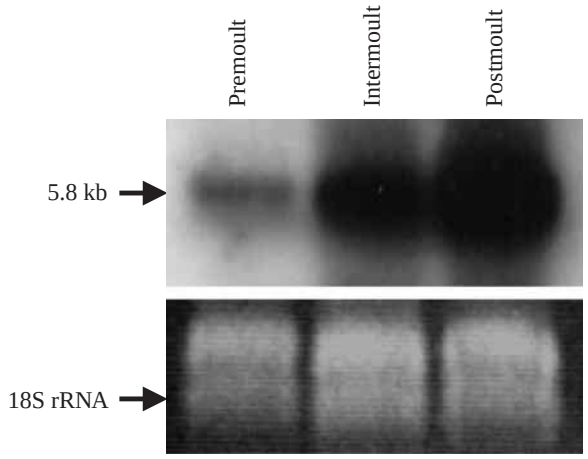

Fig. 5. Northern blot analysis of crayfish heart SERCA mRNA in heart muscle during different stages of the moulting cycle. (Top) Heart muscle total RNA (5 μg) from 18 crayfish at different moult stages was used: intermoult (*N*=6), late premoult (*N*=6) and postmoult (1–2 days, *N*=6). The membrane was hybridized to a heart-specific SERCA probe (723 bp) and exposed to X-ray film for 24 h. (Bottom) 18S RNA concentration on the corresponding formaldehyde/agarose gel before being transferred to the membrane to serve as control (visualized by Ethidium Bromide staining).

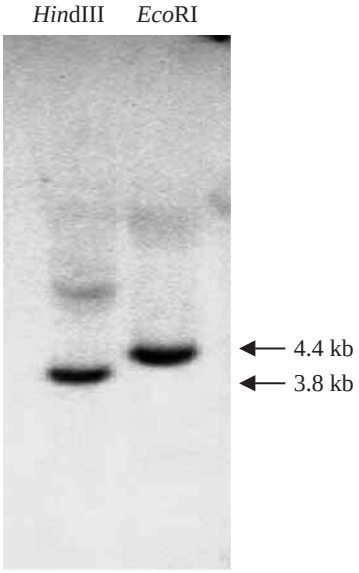

Fig. 6. Southern blot analysis of crayfish *SERCA* gene copy number.15 μg of genomic DNA were digested with *Hin*dIII or *Eco*RI, separated on a 0.8 % agarose gel, transfered to a nylon membrane, and hybridized with a probe that is from nucleotides 2785–3019 of the crayfish tail muscle SERCA.

several hundred bases that are not included as part of the cDNA.

Probe B, the 723 bp fragment specific to the 3′ noncoding region of crayfish heart SERCA, bound only to the 5.8 kb mRNA in heart muscle. Probe B showed minimal cross-hybridization with the 7.6 kb and a 5.8 kb mRNAs in eggs but no hybridization at all with axial abdominal muscle. Probe C, the 515 bp fragment specific to the 3′ noncoding region of the crayfish axial muscle SERCA clone, hybridized strongly with the 4.5 kb mRNA in axial muscle. Probe C also cross-hybridized weakly with a 4.5 kb mRNA in eggs and an 8.8 kb mRNA in heart.

Expression of heart SERCA isoform during moulting stages

Expression of the heart-specific isoform (probe B) was high in intermoult, decreased in premoult and then was restored in postmoult (Fig. 5).

Southern blot analysis of crayfish SERCA gene copy number

The identity of amino acid sequence between crayfish axial and cardiac muscle SERCA isoforms suggested they may be encoded by one gene. To test this hypothesis, the genomic DNAs were digested by two different restriction enzymes, *Eco*RI and *Hin*dIII, and hybridized with a cDNA probe from a region that is conserved. The result (Fig. 6) showed only one hybridization band in each DNA lane, suggesting that these two isoforms (axial abdominal muscle SERCA and heart muscle SERCA) are encoded by one gene (Escalante and Sastre, 1994).

Discussion

This paper has described the cloning of a novel SERCA isoform from the heart of crayfish *Procambarus clarkii*. This is the second SERCA isoform cloned in our laboratory, the first one being from crayfish axial abdominal muscle (Zhang et al., 2000). This cardiac SERCA isoform differs from the axial muscle SERCA solely in its C-terminal amino acids. The last nine amino acids of the axial muscle SERCA are replaced by 27 hydrophobic amino acids in this newly identified heart isoform. The relationship between these two SERCA isoforms in crayfish is surprisingly similar to that of *Artemia* SERCA isoforms (Escalante and Sastre, 1993), where the final six amino acids of one isoform are replaced by 30 amino acids in the other isoform. The crayfish isoforms bear a similar relationship to the gene encoding SERCA2 in mammals (Lytton and MacLennan, 1988) and birds (Campbell et al., 1992) where the last four amino acids in one isoform are replaced by 49–50 amino acids in the other isoform (Fig. 2). Since crayfish and *Artemia* are both crustaceans, it is possible that the crayfish possesses the same splicing mechanism as that described in *Artemia*, which has been found to resemble the splicing mechanism identified in vertebrates. In both the *Artemia* SERCA gene and vertebrate SERCA2 gene, the donor splicing site of the penultimate exon can either be recognized and fused to the last exon, giving rise to the mRNA coding for the shorter protein, or remain unrecognized, in which case a poly(A) site is recognized before the last exon of the gene and the mRNA coding for the longer protein is originated. In the present study the small differences (0.8 %) in sequences 5′ to the putative alternative splice site are not due to sequencing errors. In *Artemia* these minor differences in amino acids between the two SERCA isoforms did not indicate alternative exons encoding the 5′ end of the sequence. Additional studies at the genomic level are needed in order to delineate the splicing mechanism of these two genes in crayfish.

Little is known about the biological significance of the existence of two SERCA isoforms. All these genes code for two isoforms that originate by a similar alternative splicing mechanism, but the C-terminal extensions show poor conservation. Comparison of the hydropathy plots of the crayfish SERCA isoforms with that of *Artemia* and mammalian SERCA2 disclosed that all the C-terminal extensions have hydrophobic properties (Fig. 3) that could potentially form an additional transmembrane domain. Campbell et al. (1992) reported that the C-terminal extension of the SERCA2b isoform of birds spans the ER membrane, so that the SERCA2b isoform has 11 transmembrane domains, whereas SERCA2a has 10 transmembrane domains. Immunocytochemical studies demonstrated that SERCA2a and SERCA2b have their C-termini on opposite sides of the ER membrane; the C terminus of SERCA2a is in the cytosol whereas that of SERCA2b is in the ER lumen. However, functional comparisons have not yet revealed any difference between the two isoforms. It is possible that this extra transmembrane domain could change the regulatory properties of the enzyme or its interactions with other cellular components. From the tissue distribution of these isoforms in vertebrates and invertebrates, it is hard to connect this extra domain with any particular tissue. It has been demonstrated that the SERCA2a is expressed mainly in slow-twitch skeletal, cardiac and smooth muscle; SERCA2b is ubiquitously expressed and is referred to as a ‘housekeeping gene’ (Wu et al., 1995). In this study, the isoform with the carboxyl extended terminus was expressed predominantly in heart tissue of crayfish, which seems contrary to the distribution of mammalian SERCA2. In other invertebrates, *Drosophila* and *Artemia*, tissue distribution data for each isoform is lacking owing to the difficulty of isolating individual tissues in such small organisms. Future studies should focus on the tissue distribution of this SERCA family and localization to specific cell types. This may elucidate regulatory factors and selective pressures that have contributed to conservation of SERCA in invertebrates and vertebrates.

The tissue distributions of the crayfish SERCA isoforms seem more complicated than that of *Artemia*. Northern blot analysis revealed a broad expression of this gene in egg, heart and axial muscle. Tissue-specific expression of the isoforms is apparent with a pattern resembling that in vertebrates (Zhang et al., 2000). The isoform-specific probes (B and C) confirmed that the original 3865 bp clone from the axial muscle corresponds to the 4.5 kb transcript and the new 4495 bp clone isolated from cardiac muscle corresponds to the 5.8 kb transcript, respectively. In eggs, the 4.5 kb and 5.8 kb RNAs hybridized to probes B and C, respectively, indicating that the SERCA axial muscle type transcript and cardiac muscle type transcript are formed during the early stage of crayfish development. A strong band shown in eggs (7.6 kb) may be a precursor of these two transcripts suggesting possible developmental regulation of the gene.

Expression of the heart-muscle-specific SERCA isoform varied as a function of the moulting cycle (Fig. 5). The expression pattern was similar to that reported for the axial abdominal muscle isoform in the transition from intermoult to premoult (Zhang et al., 2000), namely that expression was high in intermoult and decreased significantly in premoult. However, in the postmoult period these two isoforms exhibited different expression patterns. While axial abdominal muscle SERCA remained downregulated in the first 1–2 days postmoult and required 2 weeks for recovery to intermoult levels, the cardiac SERCA isoform expression rapidly returned to (and even exceeded) intermoult expression within 2 days.

While the ultrastructure of the intermoult crayfish heart is well described (Komura, 1969; Howse et al., 1971a,b; Anderson and Smith, 1971), and it has been established that the crustacean heart grows indefinitely in proportion to the body mass in species exhibiting indeterminate growth (Wilkens and McMahon, 1994), it is unknown whether the crustacean heart grows incrementally (like somatic muscles) or continuously. Since growth of the heart is not restricted by encasement in cuticle, continuous growth is possible.

Interpretation of the SERCA expression data is probably best explained by considering the relative contractility of the different muscle types during the moulting cycle. A study of

cardiac function during moulting in blue crabs (de Fur et al., 1984) showed that heart rate decreased significantly in premoult; however, within 24 h of ecdysis it had recovered dramatically, associated with the need to deliver oxygen to metabolizing tissues. In immediate postmoult a large increase in hydrostatic pressure was associated with increased stroke volume. The heart muscle is attached *via* alary ligaments to skeletal elements that would impose additional stretching. So, by all indicators, cardiac function may be temporarily reduced in immediate premoult, but recovers rapidly after ecdysis for reasons of physiological necessity. By comparison, contractility of skeletal muscles is visibly reduced for several days surrounding ecdysis as crustaceans appear relatively inactive or quiescent for 2–3 days following ecdysis. Somatic muscles are typically stretched in postmoult following skeletal expansion. Muscle flexion of lobster carpopodite extensor muscle has been shown to upregulate actin mRNA expression and myofibrillar growth within 1–2 weeks (Harrison and El Haj, 1994) coincidentally the time frame for recovery of axial abdominal SERCA expression. Therefore the patterns of SERCA expression in at least these two different muscle types could be directly attributable to muscular contractility. Examination of claw muscle might further our understanding of SERCA expression; claw muscle atrophies in premoult to enable extrication through a narrow opening.

The Southern blot suggested that crayfish axial muscle SERCA and cardiac muscle SERCA are encoded from one gene. This gene shows higher homology with *Drosophila* (80 %) and *Artemia* (79 %) than with the vertebrate genes (72 %); it shows similar homology with the three vertebrate genes. In a separate study of the evolutionary relationships among all SERCA sequences (Wheatly et al., 2001) we have determined that the gene duplications of SERCA into the three homologues (*SERCA1*, *SERCA2* and *SERCA3*) occurred within vertebrates. A single SERCA gene is found in invertebrates that is equally related to vertebrate *SERCA-1*, *-2* and *-3*. These data confirm an earlier hypothesis (Escalante and Sastre, 1993) that there is a unique ancestral SERCA gene that gave rise to the three genes in vertebrates and to a single invertebrate gene. The same alternative splicing was preserved in the invertebrate gene and vertebrate *SERCA2* gene, while it was lost during evolution of vertebrate *SERCA1* and *SERCA3*.

The SERCA sequence from crayfish *Procambarus clarkii* cardiac muscle has been accepted by GenBank (Accession number AF025848).

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References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410.

Anderson, J. G. and Smith, J. E. (1971). The production of conidiophores and conidia by newly germinated conidia of *Aspergillus niger* (microcycle conidiation). *J. Gen. Microbiol.* **69**, 185–197.

Brandl, C. J., Green, N. M., Korczak, B. and MacLennan, D. H. (1986) Two Ca^{2+} ATPase genes: homologies and mechanistic implications of deduced amino acid sequences. *Cell* **44**, 597–607.

Brandl, C. J., deLeon, S., Martin, D. R. and MacLennan, D. H. (1987). Adult forms of the Ca^{2+}-ATPase of sarcoplasmic reticulum. *J. Biol. Chem.* **262**, 3768–3774.

Burk, S. E., Lytton, J., MacLennan, D. H. and Shull, G. E. (1989). cDNA cloning, functional expression and mRNA tissue distribution of a third organellar Ca^{2+} pump. *J. Biol. Chem.* **264**, 18561–18568.

Campbell, A. M., Kessler, P. D. and Fambrough, D. M. (1992). The alternative carboxyl termini of avian cardiac and brain sarcoplasmic reticulum/endoplasmic reticulum Ca^{2+}-ATPases are on opposite sides of the membrane. *J. Biol. Chem.* **267**, 9321–9325.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanante-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159.

DeFur, P. L., Mangum, C. P. and McMahon, B. R. (1984). Cardiovascular and ventilatory changes during ecdysis in the blue crab, *Callinectes sapidus*, Rathbun. *J. Crust. Biol.* **5**, 207–215.

El Haj, A. J., Harrison, P. and Whiteley, N. M. (1992). Regulation of muscle gene expression in Crustacea over the moult cycle. In *Molecular Biology of Muscle* (ed. A. J. El Haj), pp. 151–165. Cambridge: Cambridge University Press.

Escalante, R. and Sastre, L. (1993). Similar alternative splicing events generate two sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPase isoforms in the crustacean *Artemia franciscana* and in vertebrates. *J. Biol. Chem.* **268**, 14090–14095.

Escalante, R. and Sastre, L. (1994). Structure of *Artemia franciscana* Sarco/endoplasmic reticulum Ca^{2+}-ATPase gene. *J. Biol. Chem.* **269**, 13005–13012.

Gunteski-Hamblin, A. M., Greeb, J. and Shull, G. E. (1988). A novel Ca^{2+} pump expressed in brain, kidney, and stomach is encoded by an alternative transcript of the slow-twitch muscle sarcoplasmic reticulum Ca^{2+}ATPase gene. *J. Biol. Chem.* **263**, 15032–15040.

Harrison, P. and El Haj, A. J. (1994). Actin mRNA levels and myofibrillar growth in leg muscles of the European lobster (*Homarus gammarus*) in response to passive stretch. *Mol. Mar. Biol. Biotech.* **3**, 35–41.

Howse, H. D., Ferrans, V. J. and Hibbs, R. G. (1971a). A light and electron microscopic study of the heart of a crayfish, *Procambarus clarkii* (Giraud). I. Histology and histochemistry. *J. Morphol.* **131**, 237–252.

Howse, H. D., Ferrans, V. J. and Hibbs, R. G. (1971b). A light and electron microscopic study of the heart of a crayfish, *Procambarus clarkii* (Giraud). II. Fine structure. *J. Morphol.* **133**, 353–373.

Karin, N. J., Kaprielian, Z. and Fambrough, D. M. (1989). Expression of avian Ca^{2+} –ATPase in cultured mouse myogenic cells. *Mol. Cell. Biol.* **9**, 1978–1986.

Komura, T. (1969). The fine structure of the crayfish cardiac muscle. *J. Electron Microsc.* **18**, 291–297.

Kozak, M. (1984). Point mutations close to the AUG initiator codon affect the efficiency of translation of rat preproinsulin in vivo. *Nature* **308**, 241–246.

Kyte, J. and Doolittle, R. F. (1982). A simple method for displaying hydropathic character of a protein. *J. Mol. Biol.* **157**, 105–132.

Lytton, J. and MacLennan, D. H. (1988). Molecular cloning of cDNAs from human kidney coding for two alternatively spliced products of the cardiac Ca^{2+}-ATPase gene. *J. Biol. Chem.* **263**, 15024–15031.

Lytton, J., Zarain-Herzberg, A., Priasamy, M. and MacLennan, D. H. (1989). Molecular cloning of the mammalian smooth muscle sarco(endo)plasmic reticulum Ca^{2+} ATPase. *J. Biol. Chem.* **264**, 7059–7065.

MacLennan, D. H., Brandl, C. J., Korczak, B. and Green, N. M. (1985). Amino-acid sequence of a Ca^{2+}-Mg^{2+}-dependent ATPase from rabbit muscle sarcoplasmic reticulum, deduced from its complementary DNA sequence. *Nature* **316**, 696–700.

Magyar, A. and Varadi, E. (1990). Molecular cloning and chromosomal localization of a sarco/endoplasmic reticulum-type Ca^{2+}-ATPase of *Drosophila melanogaster*. *Biochem. Biophys. Res. Commun.* **173**, 872–877.

Magyar, A., Bakos, E. and Varadi, E. (1995). Structure and tissue-specific expression of the *Drosophila melanogaster* organellar-type Ca^{2+}-ATPase gene. *Biochem. J.* **310**, 757–763.

McWhinnie, M. (1962). Gastrolith growth and calcium shifts in the freshwater crayfish *Orconectes virilis*. *Comp. Biochem. Physiol.* **7**, 1–14.

Mellon, D., Jr, Alones, V. and Lawrence, M. D. (1992). Anatomy and fine structure of neurons in the deutocerebral projection pathway of the crayfish olfactory system. *J. Comp. Neurol.* **321**, 93–111.

Mykles, D. L. (1997). Crustacean muscle plasticity: molecular mechanisms determining mass and contractile properties. *Comp. Biochem. Physiol.* **117B**, 367–378.

Mykles, D. L. and Skinner, D. M. (1990). Atrophy of crustacean somatic muscle and the proteinases that do the job. A review. *J. Crust. Biol*. **10**, 577–594.

Palmero, I. and Sastre, L. (1989). Complementary DNA cloning of a protein highly homologous to mammalian sarcoplasmic reticulum Ca^{2+}-ATPase from the crustacean *Artemia*. *Mol. Biol.* **210**, 737–748.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual* (2nd edn). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Tullis, A. and Block, B. A. (1996). Expression of sarcoplasmic reticulum Ca^{2+}-ATPase isoforms in marlin and swordfish muscle and heater cells. *Am. J. Physiol.* **271**, R262–R275.

Varadi, A., Gilmore-Heber, M. and Benz, E. J. (1989). Amplification of the phosphorylation site-ATP-binding site cDNA fragment of the Na^+, K^+-ATPase and the Ca^{2+}ATPase of *Drosophila melanogaster* by polymerase chain reaction. *FEBS Lett.* **258**, 203–207.

Vilsen, B. and Andersen, J. P. (1992). Deduced amino acid sequence and E1–E2 equilibrium of the sarcoplasmic reticulum Ca^{2+}-ATPase of frog skeletal muscle: comparison with the Ca^{2+}-ATPase of rabbit fast twitch muscle. *FEBS Lett.* **306**, 213–218.

Wheatly, M. G. (1996). An overview of calcium balance in crustaceans. *Physiol. Zool.* **69**, 351–382.

Wheatly, M. G. (1999). Calcium homeostasis in Crustacea: the evolving role of branchial, renal, digestive and hypodermal epithelia. *J. Exp. Zool.* **283**, 277–284.

Wheatly, M. G., Gao, Y., Freire, N. and Zanotto, F. P. (2001). Subcellular calcium homeostasis during transepithelial calcium flux associated with crustacean molting: what role do SERCAs and PMCAs play? In *Proceedings of the 3rd International Satellite Symposium on the Comparative Endocrinology of Calcium Regulation*. http://www.sci.port.ac.uk/cecr.

Wilkens, J. L. and McMahon, B. R. (1994). Cardiac performance in semi-isolated heart of the crab *Carcinus maenas*. *Am. J. Physiol*. **266**, R781–R789.

Wu, K.-D. and Lytton, J. (1993). Molecular cloning and quantification of mRNA and protein encoding Ca^{2+}-ATPase isoform from rat muscles. *Am. J. Physiol.* **264**, 333–341.

Wu, K.-D., Lee, W. S., Wey, J., Bungard, D. and Lytton, J. (1995). Localization and quantification of endoplasmic reticulum Ca^{2+}-ATPase isoform transcripts. *Am. J. Physiol.* **269**, C775–C784.

Wuytack, F., Papp, B., Verboomen, H., Raeymaekers, L., Dode, L., Bobe, R., Enouf, J., Bokkala, S., Authi, K. S. and Casteels, R. (1994). A sarco/endoplasmic reticulum Ca^{2+}-ATPase 3–type Ca^{2+} pump is expressed in platelets, in lymphoid cells and in mast cells. *J. Biol. Chem.* **269**, 1410–1416.

Zhang, Z., Chen, D. and Wheatly, M. G. (2000). Cloning and characterization of sarco/endoplasmic reticulum Ca^{2+} ATPase from crayfish axial muscle. *J. Exp. Biol.* **203**, 3411–3423.