Summer 2007

Three Variants of Sarcoplasmic Calcium-Binding Protein DNA Found in Axial and Cardiac Tissues of Procambarus clarkii

Michael Northcutt

Three Variants of Sarcoplasmic Calcium-Binding Protein DNA Found in Axial and Cardiac Tissues of *Procambarus clarkii*

Michael J. Northcutt '08, with Dr. Christopher M. Gillen

Kenyon College Summer Science 2007, Gambier, OH

Introduction

•The crayfish *Procambarus clarkii* is an established model organism for the study of calcium transport because of the mass transport of calcium during the molting process (Gao et *al*., 2006).

•*P. clarkii* has been used to better understand the regulation of genes encoding proteins involved in Ca^{2+} homeostasis such as SCP (Sarcoplasmic Calcium-binding Protein) and PMCA3 (Plasma Membrane Calcium ATPase) (Gao *et al*., 2006).

•SCP binds and buffers cytosolic Ca^{2+} but does not transport it.

•SCP1 expression in *P. clarkii*, as measured by real-time PCR, is greatest in the intermolt stage. A considerable decrease in expression is observed during premolt, followed by another decline in postmolt (Gao *et al.,* 2006).

•The functions of crayfish SCP1 and PMCA3 have been inferred from sequence homology to different vertebrate genes. Currently, no experimental evidence relating to protein function or information regarding specific biological and chemical properties exists.

•The introduction of a functional expression system will open the door to a comparative approach through which we can investigate, for example, calcium binding affinity and function/coordination in the presence of an ATPase inhibitor.

Methods

Constructing an Expression Vector

•SCP1 was amplified from cDNA reverse-transcribed from axialabdominal tissue and heart mRNA (Gao *et al*., 2006).

•Crayfish total RNA from axial and cardiac muscle (collected by M. Niehaus-Sauter) was converted to random hexamer primed cDNA (Gao *et al*., 2006).

• Real-time PCR primers were designed for SCP1 based on the published sequence (Gao *et al*., 2006) using DNASTAR computer software.

SCPF1	5'	TGCGGCTTCGGCTTCTGAGAAACAAGAGGT 3'
SCPF2	5'	CAAGAGGTCCTTTAAGGTTTAGACGACAAA 3'
SCPF2a	5'	GGCGGTGTTGGTGCCTGTC 3'
SCPF2b	5'	GCGCGGGTAGCGTCTGA 3'
SCPR1a	5'	GCGAAGGCCGGCCCAGGTTGC 3'
SCPR1b	5'	AATGATGCGGTGAGGTACTGGGCACTGGTG 3'

•Primers (above) specific to SCP1 and Platinum PCR SuperMix containing high fidelity DNA polymerase were used for several PCR reactions.

•PCR conditions were optimized. The general reaction mixture contained 1 µl random hexamer primed cDNA (I-tail, J-tail, or R-heart, conc. 1 µg/µl), 1 µl each of forward and reverse primers (200 nm final concentration), and 22 µl Platinum PCR SuperMix High Fidelity, for a 25 µl total reaction volume.

•PCR product were run on a 1% agarose gel and visualized via EtBr under UV illumination.

Methods (cont.)

•PCR products from axial and cardiac muscle were inserted into a TOPO TA cloning vector (PCR2.1, Invitrogen, 2006).

•The resulting vectors were cut using EcoR1 restriction enzyme and analyzed by agarose gel electrophoresis. EcoR1 sites flank the region of PCR product insertion.

•Sequencing (University of Maine) demonstrated the presence of three variants. Variant-specific primers were designed so that only the variable region was amplified. *P. clarkii* cDNAs (A.White) were used for reverse transcriptase PCR, cloning and digestion as previously described.

Results and Discussion

•Preliminary results indicated a product of the expected size (SCP1, ~800 bp) using both axial and cardiac muscle cDNA (Figure 1).

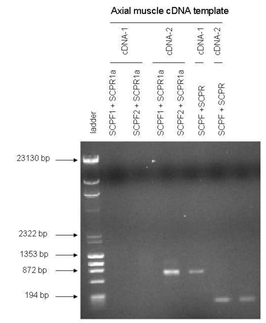

Figure 1. Agarose gel of PCR products using two axial muscle cDNAs (cDNA-1 and cDNA-2) and a variety of primer sets. High fidelity *Taq* polymerase was used for amplification. Expected PCR product sizes were 740 bp for SCPF1/SCPR1a and SCPF2/SCPR1a, and 150 bp for SCPF/SCPR (M. Niehaus Sauter). Negative controls containing no template cDNA were also included for each sample and produced no bands. Bands were visualized via EtBr on a 1% agarose gel.

•It was expected that two bands on the gel would indicate the vector and the dissociated PCR product.

•It was also expected that all bands indicating the vector would be of the same size and all bands indicating the insert would be the same size (~800 bp). Interestingly, our results revealed the presence of three bands for some colonies, which we attributed to an additional EcoR1 site nested within the SCP1 gene itself (Figure 2). Also, the size of the insert band varied among clones (compare SCP1b to SCP1a and SCP1c)

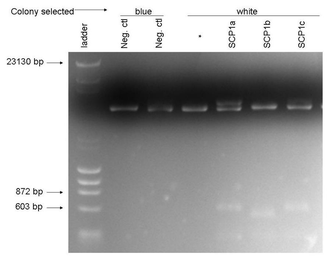

Figure 2. Individual SCP1 clones amplified from axial muscle cDNA by PCR and subcloned into the PCR2.1 vector then digested with Eco R1. Bands shown to the right were a result of subcloning of the SCP PCR product from Lane 3 (SCPF1/SCPR1a with cDNA-2) of Figure 1. * denotes vector without the insert. Bands were visualized via EtBr on a 1% agarose gel.

Results and Discussion (cont.)

•The likely explanation was that at least two variants of the SCP1 gene existed.

•After cloning and sequencing of additional samples, analysis of the nucleic acid alignment of the variable region of SCP1 (Figure 3) showed three variants that differed only in a ~123 bp region.

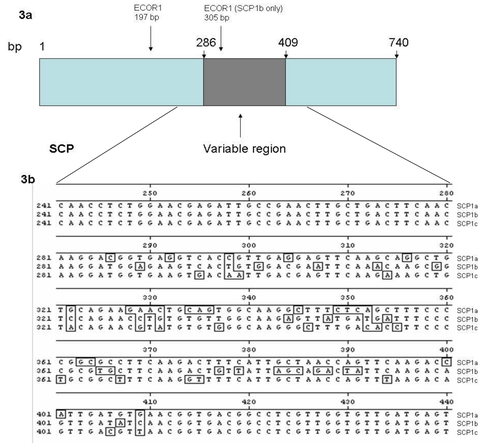

Figure 3a. Schematic of SCP gene and its variable region. Variable region is from 286-409 bp and ECOR1 restriction enzyme sites are located at 197 bp (SCP1a, SCP1b, SCP1c) and 305 bp (SCP1b only).
Figure 3b. Alignment of SCP variants between bp 241 and 440. Boxes indicate regions of disagreement between sequences. DNAStar MegAlign Version 3.1.7 was used for the alignment.

•SCP1a, SCP1b, and SCP1c were amplified from axial muscle tissue using variant-specific primers, and only SCP1c was amplified from cardiac tissue (Figure 4).

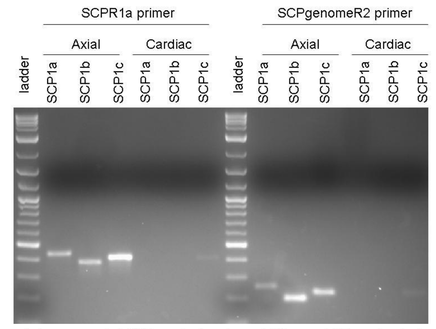

Figure 4. Agarose gel of PCR products using axial tissue cDNA and variant-specific forward primers (SCP1a, SCP1b, SCP1c). Reverse primers were used in the first six reactions (first six lanes), and the reactions were repeated with SCPgenomeR2 reverse primer (second six lanes). The PCR product expected lengths using the SCPR1a primer were 437 bp (SCP1a), 382 bp (SCP1b), and 412 bp (SCP1c). The expected length for products obtained with the genomic primer were 242 bp (SCP1a), 187 bp (SCP1b), and 217 bp (SCP1c). Bands were visualized via EtBr on a 2% agarose gel.

•Relative Quantification Real-Time PCR analysis was used to measure how the SCP1 variants are expressed in cDNA samples from cold-acclimated crayfish (Figure 5).

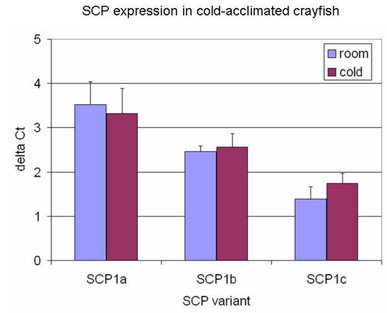

Figure 5. Expression levels determined via real-time PCR. Error bars are the standard error. SCP1a (RQ =1.1), SCP1b (RQ=0.9), and SCP1c (RQ=0.8) showed similar expression trends in room temperature and cold-acclimated samples.

•No significant differences in expression of the three SCP1 variants were measured in cold acclimated crayfish.

Conclusion

•SCP1 was amplified from axial and cardiac tissue of *P. clarkii*.

•Three variants of SCP1 were identified that differed in a 123 bp region.

•RQ Real-Time PCR was used to characterize their expression in the tissues of room temperature and cold-acclimated crayfish, and no differences were found.

•Future work

•Room temperature and cold-acclimated cardiac tissue samples will be used for Real-Time analysis.

•We will amplify SCP1 from the genome of *P. clarkii* using reverse transcriptase PCR with genomic DNA.

•Cell lines will be established by transfecting the expression plasmid into Sf9 cells (derived from fall army worm caterpillar, *Spodoptera frugiperda*). We will use these lines to relate calcium binding affinity to protein function and regulation.

References Cited

Gao, Yongping, Gillen, Christopher M., Wheatly, Michele G. Molecular characterization of the sarcoplasmic calcium-binding protein (SCP) from crayfish *Procambarus clarkii. Comparative Biochemistry and Physiology*, Part B 144 (2006) 478-487.

Invitrogen, Inc. InsectSelect BSD System For Stable Expression of Heterologous Proteins in Lepidopteran Insect Cell Lines using pIB/V5-His. Version G, 30 May 2003.

Invitrogen, Inc. TOPO TA cloning: 5-minute cloning of Taq polymerase-amplified PCR products. Version U, 10 April 2006.

Acknowledgements

I would like to thank Professor Gillen for his advice, insight and assistance in the lab as well as in the presentation of data. Thanks to Professor Powell for suggestions regarding data and loaning equipment. I would also like to thank Ann Downer, Lexie White, and Margaret Niehaus-Sauter.