FOUR DIFFERENTIALLY EXPRESSED cDNAs CONTAINING THE REBERS-RIDDIFORD CONSENSUS SEQUENCE IN *CALLINECTES SAPIDUS*

Anna Wynn

A Thesis Submitted to the
University of North Carolina at Wilmington in Partial Fulfillment
Of the Requirements for the Degree of
Master of Science

Department of Biological Sciences

UNCW

2004

Approved by

Advisory Committee

Dr. Robert Roer_____   Dr. Michael McCartney_____
Dr. Robert Roer   Dr. Michael McCartney

Dr. Thomas Shafer_ _____
Dr. Thomas Shafer, Chair

Accepted by

Dr. Robert Roer_____
Dean, Graduate School

This thesis has been prepared in the style and format

consistent with the journal

Journal of Experimental Biology

TABLE OF CONTENTS

ABSTRACT

Decapod crustaceans such as *Callinectes sapidus,* the blue crab, provide a unique

opportunity to study proteins involved in biomineralization. Subsequent to each molt, the

majority of the exoskeleton (e.g. dorsal carapace) calcifies while morphologically similar cuticle

at the joints (arthrodial membrane) remains uncalcified. Several proteins from both types of

cuticle contain the chitin-binding Rebers-Riddiford (RR) consensus sequence,

$Gx_8Gx_7YxAxExGYx_7Px_2P$. This study obtained sequence and expression data for four

transcripts containing the RR consensus sequence from hypodermal tissue of *C. sapidus*.

Expression analyses using Northern blots and quantitative PCR revealed that two of the

transcripts, CsAMP8.1 and CsAMP6.0, are found only in arthrodial membrane and expressed

uniformly both before and after the molt. Analyses of the remaining two transcripts, CsCP8.5

and CsCP8.2, revealed that both are expressed solely in pre-molt carapace, indicating possible

involvement in the postmolt mineralization of the pre-exuvial cuticle. NCBI BLAST results for

CsAMP8.1 and CsAMP6.0 identified sequence homology with proteins containing the RR

consensus sequence found in the uncalcified membranes of *Cancer pagarus*, *Penaeus japonicus*,

and *Homarus americanus*. NCBI BLAST results for CSCP8.5 and CSCP8.2 identified sequence

homology with calcification-associated peptides containing the RR consensus sequence obtained

from the calcified cuticle of *Procambarus clarkii*. These results add *C. sapidus* to the list of

arthropods containing cuticular proteins with the characteristic RR consensus sequence. The

differential expression of these four genes is consistent with the hypothesis that RR-containing

proteins play an important role in regulating calcification.

ACKNOWLEDGEMENTS

I would like to thank all the people who helped me with this research. To my advisor Dr. Thomas Shafer, thank you for your support and continued understanding. I had a number of false starts but you kept me going. To my committee members, Drs Roer and McCartney thank you for your interest and suggestions.

A huge thank you to my partners in crime in the lab: Liz Buda, Francie Coblentz and Mark Gay. Thank you Liz and Francie for your unending help troubleshooting techniques that refused to work consistently, and to Mark for having answers to obscure questions. My research is only as good as the help I had, and because of all of you it is something I can be proud of.

Murry Bridges at Endurance Seafood in Kill Devil Hills, NC deserves a resounding thank you for his continued support of this research as well as for putting up with us on our annual crab trip.

To Wade, my family and friends thank you for your love and support.

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Biomineralization is the process by which living organisms form inorganic solids. It is responsible for important biological functions in many organisms including mollusks, echinoderms, sponges, crustaceans and vertebrates (Lowenstam and Weiner, 1989). Biomineralization always occurs in conjunction with an organic matrix and crystal development is believed to be regulated both temporally and spatially by an array of acidic macromolecules that control crystal initiation and growth (Addadi and Weiner, 1985). In some mineralizing systems a single extracellular acidic macromolecule can be responsible for both nucleation and modification of crystal growth depending on its orientation (Addadi and Weiner, 1985). Clearly, a more complete knowledge of the organic matrix responsible for the timing and structure of crystal growth is a key to understanding biomineralization.

Calcium carbonate crystals are an integral part of crustacean exoskeletons such as that of *Callinectes sapidus*, the blue crab. These organisms must periodically shed their mineralized exoskeleton to allow for growth (Roer and Dillaman, 1984). At each molt cycle, they produce and calcify the organic matrix of the cuticle layers in a defined sequence. However, while certain regions of the exoskeleton calcify, morphologically similar regions at the joints (arthrodial membrane) remain uncalcified (Neville, 1975; Williams et al., 2003). These unique features of crustaceans provide an opportunity to study organic matrix molecules that regulate biomineralization both temporally and spatially.

The decapod cuticle is composed of four layers which are, from the outside in, the epicuticle, exocuticle, endocuticle, and membranous layer. Underlying and in direct contact with the cuticle is the hypodermis, an epithelial layer responsible for the formation of the new cuticle layers during both pre- and postmolt stages (Roer and Dillaman, 1984). The molt cycle is

divided into five stages (A – E) with subdivisions within each stage. Postmolt is denoted by A_1, A_2, B_1, B_2, C_1, C_2, and C_3. Intermolt is C_4 and premolt is D_0, D_1', D_1'', D_1''', D_2, D_3, and D_4. The actual act of molting or ecdysis is stage E (Drach and Tchernigovtzeff, 1967). Adult decapods spend the majority of each cycle in the intermolt stage (C_4), molting only once or twice yearly (Passano, 1960).

Preparation for ecdysis involves many modifications of the crustacean integument. Premolt is initiated when the hypodermis separates from the existing cuticle at apolysis. The outer two layers (epi- and exocuticle) of the new cuticle are synthesized beneath the old exoskeleton prior to ecdysis (Roer and Dillaman, 1984). Epicuticular deposition occurs during molt stage D_1, while exocuticular deposition occurs during molt stages D_1 and D_2. These layers are referred to as pre-exuvial layers. Mineralization of cuticle destined to calcify does not begin in the pre-exuvial layers until postmolt stages. At this time calcification initiates at the epicuticle-exocuticle boundary and within exocuticular regions termed the interprismatic septa (Giraud-Guille, 1984; Dillaman et al., in press) After the molt, the hypodermis begins to deposit the new endocuticle, which is mineralized as it is formed.

The timing of cuticle deposition for the arthrodial membrane in *C. sapidus* appears to be identical to the timing of cuticle deposition in the calcified cuticle despite differences in biomineralization (Williams et al., 2003). Again, the outer two layers (epi- and exocuticle) of the new cuticle are manufactured beneath the old exoskeleton. Epicuticular deposition is complete and exocuticlar deposition begins in late stage D_1. Post-exuvial deposition of the endocuticle accounts for three times as much cuticle as pre-exuvial deposition and is observed in arthrodial membranes until stage C_4 (Williams et al., 2003).

Although the timing is similar, the composition of arthrodial membranes is thought to be significantly different from calcified cuticle (Hepburn and Chandler, 1976). There are noticeable hematoxylin and eosin staining differences between arthrodial and calcified cuticle as well as within the layers of both cuticles (Williams et al., 2003). The exocuticle of the calcified cuticle is basophilic indicating a high concentration of acidic proteins. The endocuticle of calcified cuticle is eosinophilic indicating a high concentration of basic proteins. The arthrodial membrane is weakly basophilic, although still easily distinguishable from the exocuticle of calcified cuticle (Williams et al., 2003). These results illustrate a marked difference in protein compositions between cuticle types. Because the calcified cuticle composition is dominated by endocuticle, proteins extracted from it might be expected to be more alkaline with a small proportion of acidic proteins. In contrast, the proteins extracted from arthrodial membrane might be expected to be more acidic (Williams et al., 2003). A model has been proposed incorporating the differences in organic matrix composition between calcified and arthrodial cuticle (Andersen, 1999). Hydrophilic cuticular proteins found in arthrodial membranes are intimately bound to a chitin scaffold and interact with other proteins present to form a non-covalent network providing a rigid but pliant membrane. In comparison, the hydrophobic cuticular proteins of calcified cuticle tend to aggregate with each other and with the N- and C- terminal regions of the chitin-linked proteins, decreasing the available space for water and providing a much more rigid membrane (Andersen, 1999).

Several studies have investigated matrix proteins that may be responsible for the temporal and spatial control of biomineralization. An increase in calcium carbonate nucleation sites in *C. sapidus* cuticle occurs between one and three hours after ecdysis in conjunction with major changes in aqueous-soluble cuticular glycoproteins (Shafer et al., 1995). Specifically, two large

3

dense bands disappear from lectin blots and gels stained for carbohydrates during this early

postecdysial time (Shafer et al., 1995). These large macromolecules may be shielding nucleation

sites from calcium and/or carbonate ions or in some other way effectively inhibiting crystal

formation prior to ecdysis. Whatever the nature of the change after ecdysis, it causes the

nucleation sites to be exposed and the inhibition to be lifted (Coblentz et al., 1998). The

glycoprotein involved has been purified from 0-hour cuticle (Tweedie et al., 2004).

Immunohistochemistry using a polyclonal antibody raised against a peptide portion of the

glycoprotein detected a uniform distribution of antigen in the exocuticle at ecdysis and at one

hour postmolt but decreased binding in the interprismatic septae (IPS) in two and three hour

postmolt samples (Tweedie et al., 2004). The location and timing of the loss of antigen correlate

with the beginning of calcification in the exocuticle IPS (Dillaman et al., in press).

Investigations of less soluble cuticular proteins from crustacean calcified cuticles have

recovered proteins from *Cancer pagurus* (Andersen, 1999) and *Homarus americanus*

(Nousiainen et al., 1998) which contain an 18-residue motif ($xLxGPSG\varphi_2x_2DGx_3Q\varphi$; x=any

residue; φ=hydrophobic residue) that is not found in uncalcified crustacean cuticle nor in insects

that do not mineralize. Proteins containing this motif are suspected to play a role in the

regulation of crystal growth (Andersen, 1999). Eleven cDNA transcripts with multiple copies of

this 18-residue motif were also discovered in *C. sapidus* calcified cuticle (Kennedy, 2004).

Less soluble cuticular proteins have also been extracted from insect cuticles. Many of

these have a highly conserved motif known as the Rebers-Riddiford consensus sequence (RR).

The RR consensus sequence ($Gx_8Gx_7YxAxExGYx_7Px_2P$; x=any residue) was first reported in

seven insect cuticular protein sequences (Rebers and Riddiford, 1988). Although insect cuticle

does not undergo biomineralization, two distinct cuticle types exist; soft and sclerotized (hard).

The mechanical properties of these cuticle types are dictated by the proportion of chitin and the type of proteins present. A variation of the original RR consensus sequence characterized by several conserved residues upstream of the RR is denoted RR-1 and is found in the soft cuticles of insects (Andersen, 1998a) and crustaceans (Andersen, 1988b), while a second more degenerate variant called RR-2 is only found in insect sclerotized (hard) cuticle (Anderson, 1998a). A third variation, RR-3, which differs from RR-1 and RR-2 in the N-terminal region has been identified in both insect and crustacean hard cuticles (Andersen, 2000). The RR-2 consensus has been shown to bind chitin *in vitro* (Rebers and Willis, 2001).

Numerous crustacean cuticle proteins containing RR consensus sequence variations have been identified. Six *Homarus americanus* arthrodial cuticle proteins containing perfect or near perfect RR-1 consensus sequences have been discovered (Andersen, 1998b). Sequence alignments with a variety of insect proteins from soft tissue verified homology of the complete RR-1 consensus sequence among taxa. This alignment also recognized the presence of a conserved sequence in the region preceding the RR-1 sequence. These data provide further support that the RR-1 subgroup occurs in soft, pliant cuticle and may be necessary for flexible cuticle function (Andersen, 1998b). In addition, two proteins from calcified cuticle of *H. americanus* were identified that contained partial RR consensus sequences which can not be categorized as either RR-1 or RR-2, although they are most like the RR-1 subgroup. Similarity between the two calcified and six arthrodial proteins is restricted to the RR consensus region. Five arthroidal cuticle proteins, very similar to the arthrodial proteins found in *H. americanus* (Anderson, 1998b) and containing the RR-1 consensus sequences, have been extracted and sequenced from *Cancer pagurus* (Anderson, 1999). One of these arthrodial proteins was also extracted from calcified cuticle.

Two uncalcified cuticle protein transcripts, DD9A and DD9B, with deduced amino acid sequences containing partial RR-1 consensus sequences have been identified in *Marsupanaeus japonicus* (Watanabe et al., 2000). These transcripts are found in the lateral uncalcified exoskeleton region of the tail blade of shrimp, and not in the calcified medial region. DD9A and DD9B may function to prevent calcification of the lateral endocuticle by negatively regulating calcium carbonate crystallization (Watanabe et al., 2000). Sequence alignments of the deduced amino acid sequences demonstrate homology with crustacean and insect uncalcified cuticle proteins containing the RR-1 consensus sequence including *H. americanus* and *C. pagurus*.

Two calcification-associated peptides, CAP-1 and CAP-2, containing an RR consensus sequence similar to RR-1 have been extracted from the calcified cuticle of *Procambarus clarkii* (Inoue et al., 2001, 2004). Sequences have also been determined for CAP-1 and CAP-2 cDNA transcripts (Inoue et al., 2003, 2004). These transcripts are only present in tail fan blade RNA (Inoue et al., 2003, 2004) during postmolt, the time the shrimp exoskeleton calcifies. Protein sequence alignments show homology among the CAP proteins and RR-containing *H. americanus* and *C. pagurus* proteins within the RR regions. There is very little similarity between the N- and C- terminal regions of the CAP proteins and other RR proteins because the CAPs contain a high proportion of acidic amino acid residues in these areas. The distinct sequence differences combined with the *in vitro* anti-calcification and chitin binding activity of the CAP proteins and the cDNA expression patterns indicate that these unique N- and C-terminal regions may be associated with calcification of the exoskeleton *in vivo* (Inoue et al., 2001, 2003, 2004). An additional transcript, crustocalcin, has been identified in *Marsupanaeus japonicus* calcified cuticle (Endo et al., 2004). The encoded protein contains a Rebers-Riddiford-like motif in the N-terminus region that exhibits homology with the CAP proteins of

P. clarkii. Like the N- and C- terminals of the CAP proteins, the crustocalcin region downstream from the RR motif is highly acidic. *In vitro* assays indicate that crustocalcin promotes the formation of calcium carbonate crystals (Endo et al., 2004). The sequence homology combined with the *in vitro* activity has led to a description of crustocalcin as a "putative promoter of calcification in the crustacean exoskeleton" (Endo et al., 2004).

The wide distribution of the Rebers-Riddiford consensus sequence among arthropod cuticle proteins suggests its presence in *Callinectes sapidus* cuticle proteins. This investigation was designed to identify genes responsible for the production of cuticle proteins that are differentially expressed in either calcified or uncalcified cuticle and that contain the RR consensus sequence in *C. sapidus*.

<center>MATERIALS AND METHODS</center>

Organisms and Tissue

Premolt (D_2 and D_3) and postmolt (0, 2, 4, 6, 12, 24, 48 hr and 4, 8, 16, and 32 days after ecdysis) adult *Callinectes sapidus* were obtained at a shedding operation in Kill Devil Hills, NC. Early premolt (D_1') and intermolt (C_4) crabs were purchased locally in Wilmington, NC. Hypodermis was dissected from above the cardiac chamber (mid-dorsal hypodermis). This location was chosen because the epithelium can be obtained with little non-epithelial contamination and is synthesizing cuticle destined to calcify. Hypodermis was dissected from the carpus joints of both chilepeds (arthrodial hypodermis). This location was chosen because the carpus epithelium produces an uncalcified cuticle morphologically similar to mid-dorsal cuticle. Hepatopancreas was obtained dorsal to the branchial chamber. Blood was collected in a

<center>7</center>

syringe containing anti-coagulant solution (Leonard et al., 1985) and hemocytes were obtained

by centrifugation at 800 x g for 10 min. Hypodermis tissue was preserved in RNA Later

(Ambion, Austin, TX, USA) and stored at -20°C. RNA from hepatopancreas and blood was

immediately extracted after collection.

Total RNA Extraction

Total RNA was extracted using the spin-column RNeasy Protect Mini Kit (Qiagen,

Valencia, CA, USA), with the following modifications to increase yield and quality of RNA.

Tissue was homogenized in 1ml TRIzol (Invitrogen, Carlsbad, CA, USA). RNA was eluted

from the column in 30 µl nuclease-free water (Ambion, Austin, TX, USA), and the eluate was

passed through the column a second time to increase the yield. RNA was quantified by

absorbance at 260 nm. RNA quality was evaluated on a 2% formaldehyde denaturing gel. Only

RNA with sharp ribosomal bands and little visible degradation was used in further applications.

Rapid Amplification of cDNA Ends (RACE)

Nested 3' RACE and 5' RACE were performed via First Choice RLM-RACE Kit

(Ambion, Austin, TX, USA) using BD Advantage 2 Polymerase Mix (BD Biosciences, San Jose,

CA, USA) for PCR amplifications.   The 3' RACE protocol used 1 µg total RNA reverse

transcribed after priming with oligo (dT) ligated to a universal adaptor. The 5' RACE protocol

used a series of phosphatase reactions to target full length mRNA in 2 µg total RNA for ligation

to a 5' RACE adapter. The RNA was then reverse transcribed using random decamers as

primers. Gene-specific primers for RACE were designed using Primer 3 software (Table 1).

PCR products were stained with ethidium-bromide and visualized on 1.6% agarose gels.

Cloning

PCR products of appropriate sizes were purified using QIAquick spin-columns (Qiagen, Valencia, CA, USA). The pGEM-T Easy Vector System (Promega, Madison, WI, USA) was used to ligate these products into plasmids and to transform *E. coli* cells. Transformed cells were plated on carbenicillin-containing LB agar plates using sterile beads to spread 100 µl of the undiluted culture. The plates were incubated at 37°C for 24 hours. PCR was performed with individual colonies as templates to verify and amplify the inserts. The conditions were 1 min at 94°C followed by 30 sec at 94°C, 1 min at 60°C, and 1 min at 72°C for 30 cycles with BD Advantage 2 Polymerase Mix (BD Biosciences, San Jose, CA, USA).

Sequencing

Cloned PCR targets were purified using QIAquick spin-columns (Qiagen, Valencia, CA, USA). Cloned inserts from a recently developed expressed sequence tag (EST) library (Coblentz et al., 2005) were obtained by purification of plasmids using the Wizard Plus SV Miniprep DNA System (Promega, Madison, WI, USA). Sequence reactions contained 1 µl of the purified product, 2 µl Big Dye Terminator Ready Reaction Mix version 3 (Applied Biosystems (ABI), Foster City, CA, USA), 2 µl dilution buffer, 3.4 µl nuclease free water, and 1.6 µl of either SP6, T7, M13F or M13R vector primer (1 µM). Reaction conditions were 45 sec at 94°C, 45 sec at 50°C, and 4 min at 60°C for 25 cycles. Sequence reaction products were purified by centrifugation through G-50 sephadex at 2,000 x G for 2 min and analyzed on a capillary DNA sequencer (ABI 3100).

Table. 1. Nucleotide sequences for primers used in 3' RACE, 5' RACE, DIG probes and
Q-PCR

Primer	Direction	Primer Sequence 5' – 3'	Location[1]
3'Outer-1	F	AGCTCCTTGCCATCGTAGAA	354- 373
3'Inner-1	F	GCTCACCCGCAGAAAGTAAT	417-437
5'Outer-1	R	TGCCATCGTAGAAGAACTCAGG	360-382
5'Inner-1	R	CAACTCTGCATTCATCCCCA	304-322
5'gsp-1	F	AACATGCAGGGAGACTTTGG	217-237
DIGF-1	F	AACTCCACACACCGACAACA	13-31
DIGR-1	R	GCAGAAAGTAATTGCCGAC	425-444
QPCRF-1	F	TGCAGAGACCATAGTGGACGAA	96-118
QPCRR-1	R	AATTTCCAATACCGCTTCGAGAC	140-162
3'RR-2[2]	F	GCYCAYGAGAACGGYTAC	130-150
5'Outer-2	R	TGAGTACGTCCAGAGGCAGA	309-328
5'Inner-2	R	CAGCACAGCAACATTCACATT	233-254
5'gsp-2	F	GCTCTGAGGGACAGAGCAAC	179-197
DIGF-2	F	GCCCCGATAAGGATGCCACCAT	62-82
DIGR-2	R	GACACTACGCCCCAGGACACAA	457-477
QPCRF-2	F	TCCAGAGGCAGATCGACTTG	316-337
QPCRR-2	R	CGTTAAACCTTCGACCAATGAACT	358-380
DIGF-3	F	TCTTGACATGGACATCGATGACG	146-169
DIGR-3	R	CCATCTCACTCGTTCACCTCTCC	648-670
QPCRF-3	F	CCCTGAAGGCGAGAAGTTCTT	203-224
QPCRR-3	R	GGATACCGCATCGTGGAGTC	250-269
DIGF-4	F	ATCACCACCGCCGACACCAT	8-28
DIGR-4	R	AGTACCTCACCGCAGCACCA	366-386
QPCRF-4	F	GGCACCTACCGCTGGATGT	152-170
QPCRR-4	R	TTTCGTGCGCTACATTGCTG	209-190
QPCRF-5	F	GTGGCCAGGGTGCAGATC	409-425
QPCRR-5	R	TCAATGACATGGGCCTTGTG	481-463

---

[1] Locations refer to the nucleotide sequences of CsAMP8.1 (Fig. 1), CsAMP6.0 (Fig. 3), CsCP8.5 (Fig. 5), CsCP8.2
(Fig. 6) and ribosomal protein L10 for primers 1, 2, 3, 4, and 5 respectively.

[2] Rebers-Riddiford degenerate 3' RACE primer is indicated by "RR".

Sequence Analysis

Conceptual translation of the cDNA clones with the RR consensus sequence in frame was performed using VectorNTI software v 9.0. Signal sequences were predicted using web-based SignalP 3.0. Similarities among the virtual translations and the sequences contained in NCBI's non-redundant protein database were revealed using BLASTp. Alignments were constructed using VectorNTI software v.9.0.

Nomenclature

The cDNA transcripts are numbered according to the predicted molecular weights (ExPASy – ProtParam Tool) of the virtual translations of the mature proteins. These numbers are preceded by the letters CsAMP (*C. sapidus* arthrodial membrane protein) for those transcripts found only in arthrodial membrane hypodermis and by the letters CsCP (*C. sapidus* cuticular protein) for those transcripts found associated with calcifying cuticle.

Quantitative PCR

Quantitative PCR was performed using an ABI 7500 Real Time PCR System with three independent crab RNA samples from each time-tissue combination. These combinations consisted of either calcified or arthrodial hypodermis from either premolt stages (D_2 and D_3), or postmolt stages (0, 2, 4, 6, 12, 24 and 48 hours). Two µg of each RNA were reverse transcribed into cDNA. Reactions contained 2 µl oligo(dT) (0.5 µg/µl), 2 µl dNTP mix (10mM), 4 µl Q solution (Qiagen, Valencia, CA, USA), 2 µl RNA (1 µg/µl) and 14 µl water. These were heated to 70°C for 5 min and held at 48°C. A mix of 8 µl 5X First-Strand Buffer (Invitrogen, Carlsbad, CA, USA), 4 µl DTT (0.1M) (Invitrogen, Carlsbad, CA, USA), and 2 µl RNaseOUT

Recombinant Ribonuclease Inhibitor (40units/µl) (Invitrogen, Carlsbad, CA, USA) was pre-warmed to 48°C and added to each reaction. After a 2 min incubation at 48°C, 1 µl Superscript II (Invitrogen, Carlsbad, CA, USA) was added. The conditions were 50 min at 48°C, 15 min at 70°C, and 25 min at 35°C. Five minutes into the 35°C incubation 1 µl *E. coli* RNaseH (2 units) (Invitrogen, Carlsbad, CA, USA) was added. Quantitative PCR primer sets with amplicons between 50 and 80 base pairs were designed using ABI Primer Express software for each of the transcripts and for an unpublished *C. sapidus* ribosomal protein, L10 (GenBank #AY82260), as an endogenous control (Table 1). L10 was experimentally determined to be constituently expressed throughout the molt cycle and between arthrodial and mid-dorsal hypodermis. PCR reactions contained 12.5 µl SYBR Green PCR Master Mix (ABI, Foster City, CA, USA), 1 µl (0.5 ng) cDNA, 7.5 µl water, 4 µl primer mix (200 nM each). Data were analyzed using the ABI 7500 System Sequence Detection Software v. 2.1.

DIG-Labeled Riboprobes

Primers sets were designed to yield PCR products between 400 and 500 base pairs for each of the transcripts (Table 1). PCR templates were either 2 hr arthrodial cDNA (see Q-PCR) or cDNA purified from clones in an EST library (see Sequencing). Products were cloned into the pGEM-T Easy Vector System (Promega, Madison, WI, USA). Colonies containing sense and anti-sense inserts were identified by sequencing and grown in TB broth overnight at 37°C. Plasmids were purified using the Wizard Plus SV Miniprep kit (Promega, Madison, WI, USA). One µg purified plasmid was linearized with SPE 1 restriction enzyme (Roche, Indianapolis, IN, USA) following the manufacturer's protocol. Riboprobes were transcribed from the T7

promoter and labeled with dioxygenin (DIG) using the DIG RNA labeling kit (Roche, Indianapolis, IN, USA).

Northern Blots

One μg of total RNA from each time period and tissue type was complexed with RNA loading dye (GenHunter, Nashville, TN, USA) containing ethidium bromide and fractionated on a 1.0% agarose gel with 2% formaldehyde as a denaturant. The RNA was allowed to migrate at 35 volts for 4-5 hours before the gel was evaluated under UV light. The RNA was transferred with 20X SSC (3M sodium chloride, 0.3M sodium citrate, pH 7.0) via capillary action to a Millipore Immobilon-NY$^+$ membrane (Billerica, MA, USA) according to the manufacturer's protocol. The RNA was UV cross-linked to the membrane and hybridized with DIG-labeled probes overnight at 68°C. Probe binding was detected using the Roche DIG Nucleic Acid Detection Kit (Indianapolis, IN, USA), which utilizes CSPD, an alkaline phospatase-activated chemiluminescent substrate. Luminescence was detected by a 10 min exposure to x-ray film.

RESULTS

Sequences of Rebers-Riddiford cDNAs

Previous unpublished work in this laboratory identified a short *C. sapidus* cDNA fragment that appeared to be differentially expressed in postmolt arthrodial hypodermis. The virtual translation of this fragment revealed a RR-1 consensus sequence. 3' RACE with primers 3'Outer-1 and 3'Inner-1 (Table 1) verified that this sequence exists in 3 hr postmolt arthrodial hypodermis and that the fragment is the 3' end of the transcript. 5' RACE with primers 5'Outer-1, 5'Inner-1 and 5'gsp-1 (Table 1), based on the 3' RACE sequence, identified an overlapping

sequence. The continuity of the 3' and 5' sequences was verified using primers DIGF-1 and DIGR-1 (Table 1) with 2 hr cDNA as a template. The cDNA is 580 base pairs and contains a 402 base-pair open reading frame beginning at nucleotide 31 and a polyadenylation signal beginning at nucleotide 560 (Fig. 1). The first 18 amino acid residues of the putative polypeptide are a signal sequence. The RR motif is between amino acid residues 49 and 95 (Fig. 1). The deduced molecular mass is 8.1 kDa and the pI is 4.74. After the transcript was proven to be differentially expressed in arthrodial membrane (see below) it was named CsAMP8.1 and submitted to GenBank as accession number AY752733.

A BLASTp search of CsAMP8.1 in NCBI's non-redundant protein data base revealed homology with CpAMP12.39 from *C. pagurus* (70% amino acid identity), DD9A and DD9B from *M. japonicus* (56% and 55% amino acid identity, respectively) and HaAMP1A from *H. americanus* (59% amino acid identity). Similarity with these RR-1 containing proteins is conserved both within and outside of the RR consensus region (Fig.2).

Transcripts for additional *C. sapidus* RR-containing polypeptides were sought using a degenerate 3' RACE primer, 3'RR-2 (Table 1). A fragment was identified with a deduced amino acid sequence containing a partial RR. Nested 5' RACE using primers 5'Outer-2, 5'Inner-2, and 5'gsp-2 (Table 1) designed to this 3' RACE product produced an overlapping fragment. The 3' and 5' sequences were verified as contiguous using primers DIGF-2 and DIGR-2 (Table 1) and 2 hr cDNA as a template. The cDNA is 519 base pairs and contains a 333 base-pair open reading frame beginning with nucleotide 27, and a polyadenylation signal beginning with nucleotide 500 (Fig. 3). The first 11 amino acid residues of the putative polypeptide are a signal sequence. The RR motif is between amino acid residues 49 and 83 (Fig. 3). The deduced molecular mass is 6.0 kDa and the

```
  1  attctgacacgaactccacacaccgacaacatgaagcttgtggttctctcctgcctgctg  60
-18                                       M  K  L  V  V  L  S  C  L  L  -9

 61 gctttcgccgtcgctgccccacgccctgatggagatgcagagaccatagtggacgaacgc 120
-10  A  F  A  V  A  A  P  R  P  D  G  D  A  E  T  I  V  D  E  R   12

121 agcgataacggcgatggaaatttccaataccgcttcgagacttccaacggcattgttgag 180
 13  S  D  N  G  D  G  N  F  Q  Y  R  F  E  T  S  N  G  I  V  E   32

181 cagaggctgggcgctccaggatcagagggacagagcaacatgcagggagactttggcttc 240
 33  Q  R  L  G  A  P  G  S  E  G  Q  S  N  M  Q  G  D  F  G  F   53

241 accctccctgaaggtgatcgttttgacttgacctatgtcgctgacgagaacggttaccag 300
 54  T  L  P  E  G  D  R  F  D  L  T  Y  V  A  D  E  N  G  Y  Q   74

301 cccaactctgcattcatccccaccgaccaccctctgcccgcccacgtagttgagctcctt 360
 75  P  N  S  A  F  I  P  T  D  H  P  L  P  A  H  V  V  E  L  L   94

361 gccatcgtagaagaactcaggcgtcagggcgccacctggaacgaccaaggcgaaaggctc 420
 95  A  I  V  E  E  L  R  R  Q  G  A  T  W  N  D  Q  G  E  R  L  114

421 acccgcagaaagtaattgccgacctatgtattcctatgtatctcatctcgaccatctctc 480
115  T  R  R  K  *                                                118

481 cagacactggcgctgcctctcagctttcatgtacatagcaatcctgatggcggcacaatc 540


541 catgcctttcatatctatataataaactgcgttaaacatt                      580
```

Fig. 1 Nucleotide sequence of cDNA CsAMP8.1 (GenBANK #AY752733). Conceptual translation of the open reading frame of 118 amino acids is shown in the one-letter representation below the respective codons. The signal sequence is underlined, the RR-1 consensus sequence is bold and underlined, the asterisk indicates a stop codon, and the consensus polyadenylation signal (AATAAA) is in a box. The poly (A) has been omitted from the figure.

```
              1                20               40               60
CsAMP8.1   (1) MKLVVLSCLLAFAVAAPRPDGDAETIVDERSDNGDGNFQYRFETSNGIVEQRLGAPGSEG
CpAMP12.39 (1) -------------------H---------------Q---------R--------AE-------
DD9A       (1) ----VVLLVCLAAVAFARPDGDAELLLDEREDQGDGNFRYVFETSNGIYQETVGTPGASG
DD9B       (1) ----VVLLVCLAAVAFARPDGDARLLLDEREDQGDGNFRYVFETSNGIFKETVGTPGAEG
HaAMP1A    (1) ------------------DRDAQTLTDERSDQGDGNFRYEFETSNGIYTQKTGTPGSEG

              61               80               100              120
CsAMP8.1  (61) QSNMQGDFGFTLPEGDRFDLTYVADENGYQPNSAFIPTDHPLPAHVVELLAIVEELRRQG
CpAMP12.39(42) QSNMKGGYSFNLPDGSRFQLSFAADENGYNADSPFIPTDHPLPAHVIELLALVEELKRQG
DD9A      (57) QSNMVGSYRFTEPDGNVIEVRFTADENGFVPESDAIPQPPPLPAHVYELLEIAEQQRRDG
DD9B      (57) QSNMVGSYRFTDPGGNVVEVRFTADENGFVPESDAIPQPPPLPAHVYELLEIADQQRREG
HaAMP1A   (42) QSNYQGSFRFTLEDGTIAEVTYIADENGFQPSSDLLPVGPPA P  Q     A  A
RR consensus         G         G        Y A E GY      P  P

              121       134
CsAMP8.1 (121) ATWNDQGERLTRRK
CpAMP12.39(102)A       VR T---
DD9A     (117) RTFDGQGFEI----
DD9B     (117) IEFDDQGFPI----
HaAMP1A  (102) ITFD----------
```

Fig. 2 Deduced CsAMP 8.1 amino acid sequence alignment with most similar sequences in DCBI database as determined by BLAST: *Cancer pagurus* CpAMP12.39, *Marsupanaeus japonicus* DD9A and DD9B, and *Homarus americanus* HaAMP1A. Black highlight indicates identical residues, dark gray indicates conserved residues, and light gray indicates a block of similar residues. The Rebers-Riddiford consensus sequence is beneath the alignment in bold.

```
  1 cctcacacgatcctcacaccgacaacatgaagctcgtcgtgctggctttggctgccgcc  59
-11                                M  K  L  V  V  L  A  L  A  A  A   -1

 60 cgccccgataaggatgccaccatcctgacggacgaacgtgaagaccgcggggacggaaac 119
  1  R  P  D  K  D  A  T  I  L  T  D  E  R  E  D  R  G  D  G  N   20

120 ttcttctaccgcttcgagacgagcaacggcattcagaaggagaagactggcacccctggc 179
 21  F  F  Y  R  F  E  T  S  N  G  I  Q  K  E  K  T  G  T  P  G   40

180 tctgagggacagagcaacatggtcggctcattccaattccctctggacgacggcagcaca 239
 41  S  E  G  Q  S  N  M  V  G  S  F  Q  F  P  L  D  D  G  S  T   60

240 gcaacattcacattcgtggccgacgagaatggctaccgcgttgagtccccgctgctccct 299
 61  A  T  F  T  F  V  A  D  E  N  G  Y  R  V  E  S  P  L  L  P   80

300 cccatccctgagtacgtccagaggcagatcgactttgctaattcccagggcaagcgccgt 359
 81  P  I  P  E  Y  V  Q  R  Q  I  D  F  A  N  S  Q  G  K  R  R  100

360 taaaccttcgaccaatgaacttacaccttcacactattcgaacctcttgccactcctgta 419
     *

420 cataacctgtgccagccatcagcttacctaaagttggacactacgccccaggacacaatc 479

480 atgttaacactttttttttccaaaataaacagcaatatagc                     519
```

Fig. 3   Nucleotide sequence of cDNA CsAMP6.0 (GenBANK #AY752734). Conceptual translation of the open reading frame of 100 amino acids is shown in the one-letter representation below the respective codons. The signal sequence is underlined, the RR-1 consensus sequence is bold and underlined, the asterisk indicates a stop codon, and the consensus polyadenylation signal (AATAAA) is in a box. The poly (A) has been omitted from the figure.

17

pI is 4.66. After the transcript was proven to be differentially expressed in arthrodial membrane (see below) it was named CsAMP6.0 and submitted to GenBank as accession number AY752734.

The deduced amino acid sequence of CsAMP6.0 is similar to CsAMP8.1 (54% amino acid identity). However, a BLASTp search of CsAMP6.0 in NCBI's non-redundant protein database revealed sequence homology with a slightly different selection of proteins than the search using CsAMP8.1. Cs.AMP6.0 is homologous with CpAMP11.14 from *C. pagurus* (74% amino acid identity), HaAMP4 and HaAMP3 from *H. americanus* (60% and 59% amino acid identity, respectively) and DD9A from *M. japonicus* (54% amino acid identity). Similarity with these RR-1 containing proteins is conserved both within and outside of the RR consensus region (Fig.4).

Additional transcripts were discovered when partial sequences of randomly chosen clones from a normalized *C. sapidus* hypodermis cDNA library were compared to the NCBI non-redundant protein database using BLASTx (Coblentz et al., 2005). A search of these EST data revealed two sequences containing the RR consensus that share homology with *P. clarkii* proteins, CAP-1 and CAP-2 (Inoue et al., 2001, 2004). The clones were grown and the inserts sequenced completely. The first cDNA is 911 base pairs and contains a 315 base-pair open reading frame; however, a polyadenylation site could not be identified (Fig. 5). The first 18 amino acid residues of the putative polypeptide are a signal sequence. The partial RR consensus sequence is between amino acid residues 42 and 58 (Fig. 5). The deduced molecular mass is 8.5 kDa and the pI is 4.25. After the transcript was proven to be differentially expressed (see below) in mid- dorsal hypodermis it was named CsCP8.5 and submitted to GenBank as accession

```
                        1                20                40                60
CsAMP6.0      (1)  --MKLVVLALAAARPDKDATILTDEREDRGDGNFFYRFETSNGIQKEKTGTPGSEGQSNM
CpAMP11.14    (1)  ---------------     T     T                S      QDT       A
HaAMP4        (1)  --------------DRDAQTLTDERNDQGDGNFRYEFETSNGIYTQKTGTPGSEGQSNY
HaAMP3        (1)  --------------DRDAQTLTDERNDQGDGNFRYEFETSNGIYTQKTGTPGSEGQSNY
DD9A          (1)  VVLLVCLAAVAFARPDGDAELLLDEREDQGDGNFRYVFETSNGIYQETVGTPGASGQSNM

                        61               80               100              120
CsAMP6.0     (59)  VGSFQFPLDDGSTATFTFVADENGYRVESPLLPP---IPEYVQRQIDFANSQGKRR----
CpAMP11.14   (46)  NGDFSFPLDDGSTASFTYVADENGYHVESPLLPS---IPEYVQKQIDFAAEQRARGVIFD
HaAMP4       (46)  QGSFRFPLEDGTIAEVTYIADENGFQPSSDLLPVGPPAPPHVQRLLEIAEDQRRQGITFD
HaAMP3       (46)  QGSFRFPLEDGTIAEVTYIADEYGFQPSSDLLPVGPPAPPHVQRLLEIAEDQRRQGITFD
DD9A         (61)  VGSYRFTEPDGNVIEVRFTADENGFVPESDAIPQPPPLPAHVYELLEIAEQQRRDGRTFD
RR consensus          G        G      Y A E GY      P  P
```

```
                        121
CsAMP6.0    (112)  ------
CpAM11P.14  (103)  ------
HaAMP4      (106)  ------
HaAMP3      (106)  ------
DD9A        (121)  GQGFEI
```

Fig. 4 Deduced CsCAMP 6.0 amino acid sequence alignment with most similar sequences in DCBI database as determined by BLAST: *Cancer pagurus* CpAMP11.14, *Homarus americanus* HaAMP4 and HaAMP3, and *Marsupanaeus japonicus* DD9A. Black highlight indicates identical residues, dark gray indicates conserved residues, and light gray indicates a block of similar residues. The Rebers-Riddiford consensus sequence is beneath the alignment in bold.

```
  1 cttctaccagctcctctcctgcctcaacccacaccaccaccaccaccaccaccatgaagtgc 62
-18                                                           M   K   C  -16

 63 accgctattcttctcctggccctcgctgctgtcgccttcgcccgccctgactccatcttc 122
-15  T   A   I   L   L   L   A   L   A   A   V   A   F   A   R   P   D   S   I   F    5

123 gacttctctgatgaggacatgcatcttgacatggacatcgatgacgacaacacctacact 182
  6  D   F   S   D   E   D   M   H   L   D   M   D   I   D   D   D   N   T   Y   T   25

183 ggttcctacagctggacatcccctgaaggcgagaagttcttcgtcaagtacattgctgac 242
 26  G   S   Y   S   W   T   S   P   E   G   E   K   F   F   V   K   Y   I   A   D   45

243 aagcgtggataccgcatcgtggagtccaatgccatccccatcactgccaacggagtgccc 302
 46  K   R   G   Y   R   I   V   E   S   N   A   I   P   I   T   A   N   G   V   P   65

303 gccgacggtacccagggcgccttctcctccgaggaaaacgacagcttcgactcccgcgac 362
 66  A   D   G   T   Q   G   A   F   S   S   E   E   N   D   S   F   D   S   R   D   85

363 agggattaagcacaccatcttgatcttgactgtctgagcggcgcgcggttaagagagtga 422
 86   R   D   *                                                               88

423 gagttcgattctcgctatgaagtcaaattgatgttgtcttcttgattccttttgtattct 482

483 tcgttttttcatgtttttatttcttgatttctcttcagttagagtctaatctttcctgttttt 542

543 tttttctctctctattcactcacacaaaccaatacactgtttatttattacacaaaaaga 602

603 tacacactatttataaacacacacaacaacaacacaattttctatccatctcactcgttc 662

663 acctctccctctattccgccgccgccgcaacgccctcctcccccacccaccatgatctaa 722

723 acctgccgccttcctcgcctccttcgggaagcctcgagccagtgttttgtctgtccctca 782

783 gccctctttctggtatttgcttgcccgctcccccggtgtgtgcgtgtgtgtgcgagcgtg 842

843 cgagtgtgtgtgtcagtgtgtctgtgtgtgtgtatgtatgtatgagatctcagtaaaggt 902

903 attggttgg                                                      911
```

Fig. 5 Nucleotide sequence of cDNA CsCP8.5 (GenBANK #AY752735). Conceptual
translation of the open reading frame of 88 amino acids is shown in the one-letter
representation below the respective codons. The signal sequence is underlined, the
partial RR consensus sequence is bold and underlined, the asterisk indicates a stop codon.
The poly (A) has been omitted from the figure.

number AY752735.

The second cDNA identified from the EST database is 778 base pairs and contains a 321 base-pair open reading frame beginning at nucleotide 26 and a polyadenylation signal beginning at nucleotide 762 (Fig. 6). The first 18 amino acid residues of the putative polypeptide are a signal sequence. The partial RR consensus sequence is located between amino acid residues 25 and 57 (Fig. 6). The deduced molecular mass is 8.2 kDa and the pI is 3.85. After the transcript was proven to be differentially expressed in mid-dorsal hypodermis (see below) it was named CsCP8.2 and submitted to GenBank as accession number AY752736.

The deduced amino acid sequence of CsCP8.5 is similar to CsCP8.2 (54% amino acid identity). A BLASTp search of CsCP8.5 in NCBI's non-redundant protein data base revealed homology with CAP-1 and CAP-2 from *P. clarkii* (66% and 35% amino acid identity, respectively). A search with CsCP8.2 also revealed homology with CAP-1 and CAP-2 from *P. clarkii* (80% and 60% amino acid identity, respectively). A comparison between the *C. sapidus* and *P. clarkii* sequences illustrates amino acid conservation between species both within and outside of the RR consensus region (Fig.7).

Quantitative PCR

Relative quantification was determined for each of the four transcripts (CsAMP8.1, CsAMP6.0, CsCP8.5, and CsCP8.2) in both calcified and arthrodial hypodermis throughout the molt cycle after normalizing to ribosomal protein L10 transcription as an endogenous control. CsAMP8.1 and CsAMP6.0 exhibited no measurable expression in mid-dorsal hypodermis at any time throughout the molt cycle

```
  1 cagagccatcaccaccgccgacaccatgaagacccttgtgctctcccttctcgccctggct 61
-18                              M  K  T  L  V  L  S  L  L  A  L  A  -7

 62 gtcctcgtcgctgcacggcccgagaacgtcctgaatctggatctggacgacatccaccag 121
 -6  V  L  V  A  A  R  P  E  N  V  L  N  L  D  L  D  D  I  H  Q   14

121 gacatggacatcgacgacaccaccatcaccggcacctaccgctggatgtctcccgagggc 181
 15  D  M  D  I  D  D  T  T  I  T  G  T  Y  R  W  M  S  P  E  G   34

181 actgagtatttcgtgcgctacattgctgacgagaagggttaccgcgtgctggagtccaac 241
 35  T  E  Y  F  V  R  Y  I  A  D  E  K  G  Y  R  V  L  E  S  N   54

242 gctgtgcccgtcaccgctgatggtacctttgctaacggcgctcaggggtctttgtcttca 301
 55  A  V  P  V  T  A  D  G  T  F  A  N  G  A  Q  G  S  L  S  S   74

302 gaggaggatgattcgaatgactctgatgatcgatttgattttgactgagtgtggggtttt 361
 75  E  E  D  D  S  N  D  S  D  D  R  F  D  F  D  *               89

362 atacagtacctcaccgcagcaccaccgcagccacaacaggaccagagcatcactgtaccg 421

422 cgccacaccaaacaccaccgtcacctcaaatacactacgccacaataccgtgacacaata 481

482 tacacaccaccactacaacacaaagtatgaagtcatctcaatacagcaacaccacaatca 541

542 ctgcaccactccacacaaaacaccataaaaaatacactacaccacaatacacatcacacc 601

602 accaccacaccattacattacaaagcatatccccccccccggggcctgcctcttcctccc 661

662 tccctttccccatcacccctccctcccccttttagtcatcacaaggcgggggtcgccgcg 721

722 ccttttttcaaactcaaccatgtatttcaactaataaatt aataaa agattgattcc    778
```

Fig. 6 Nucleotide sequence of cDNA CsCP8.2 (GenBANK #AY752736). Conceptual translation of the open reading frame of 89 amino acids is shown in the one-letter representation below the respective codons. The signal sequence is underlined, the partial RR consensus sequence is bold and underlined, the asterisk indicates a stop codon, and the consensus polyadenylation signal (AATAAA) is in a box. The poly (A) has been omitted from the figure.
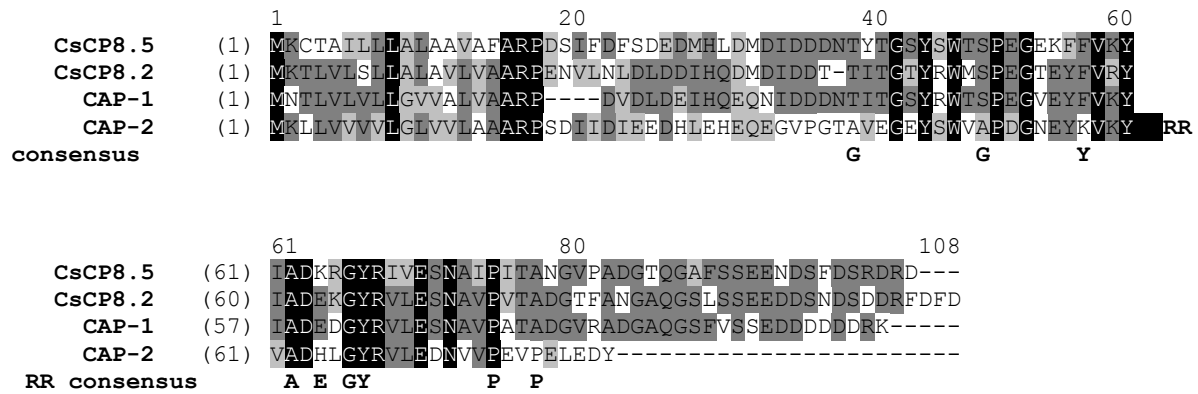
```
               1                    20                    40                    60
CsCP8.5   (1) MKCTAILLLALAAVAFARPDSIFDFSDEDMHLDMDIDDDNTYTGSYSWTSPEGEKFFVKY
CsCP8.2   (1) MKTLVLSLLALAVLVAARPENVLNLDLDDIHQDMDIDDT-TITGTYRWMSPEGTEYFVRY
CAP-1     (1) MNTLVLVLLGVVALVAARP----DVDLDEIHQEQNIDDDNTITGSYRWTSPEGVEYFVKY
CAP-2     (1) MKLLVVVVLGLVVLAAARPSDIIDIEEDHLEHEQEGVPGTAVEGEYSWVAPDGNEYKVKY RR
consensus                                               G         G        Y

               61                   80                    108
CsCP8.5  (61) IADKRGYRIVESNAIPITANGVPADGTQGAFSSEENDSFDSRDRD---
CsCP8.2  (60) IADEKGYRVLESNAVPVTADGTFANGAQGSLSSEEDDSNDSDDRFDFD
CAP-1    (57) IADEDGYRVLESNAVPATADGVRADGAQGSFVSSEDDDDDRK-----
CAP-2    (61) VADHLGYRVLEDNVVPEVPELEDY-----------------------
RR consensus     A E GY      P  P
```

Fig. 7 Deduced CsCP8.5 and CsCP8.2 amino acid sequences alignment with most similar
     sequences in NCBI database as determined by BLAST: *Procambarus clarkii* CAP-1 and
     CAP-2. Black highlight indicates identical residues, dark gray indicates conserved
     residues, and light gray indicates a block of similar residues. The Rebers-Riddiford
     consensus is beneath the alignment in bold.

(Figs 8b, 9b). Both transcripts showed continuous premolt and postmolt expression in arthrodial

hypodermis with an increase after 24 hr (Figs 8a, 9a). CsCP8.5 was not expressed in arthrodial

hypodermis either pre- or postmolt or in postmolt mid-dorsal hypodermis (Fig. 10a,b). However,

it exhibited relatively high expression levels in mid-dorsal premolt stage D_2 and measurable

expression in stage D_3 (Fig. 10a). CsCP8.2 also exhibited high expression levels in mid-dorsal

hypodermis during stage D_2 and measurable expression in stage D_3 (Fig. 11b). In this case,

expression was also recorded in at least one of the three crabs in mid-dorsal 0 hr and 48 hr tissue

as well as arthrodial D_2 and 2 hr tissue (Fig.11a,b).

Northern Blots

        Before transcript expression was assessed with Northern blots the specificity of the four

DIG-probes was verified with a virtual Northern, which showed that each probe only bound with

its appropriate cloned cDNA (data not shown). Northern blot analysis was performed to

corroborate quantitative PCR results and expand the expression pattern analysis. Single RNA

bands of approximately 575 nucleotides for both CsAMP8.1 and CsAMP6.0 were detected in

arthrodial RNA samples D_2 through 32 days postmolt, but were absent in all mid-dorsal RNA

samples (Fig. 12a,b). Signal intensity in the arthrodial RNA was high from premolt to 48 hours

postmolt with no discernable changes in intensity until a decrease at 4 days and a continuing

lower expression level through 32 days postmolt. Single bands for both CsCP8.5 and CsCP8.2

transcripts at approximately 1050 nucleotides were detected in mid-dorsal premolt (D_2 and D_3)

RNA samples, but neither CsCP8.5 nor CsC8.2 was detected in arthrodial hypodermis either pre-

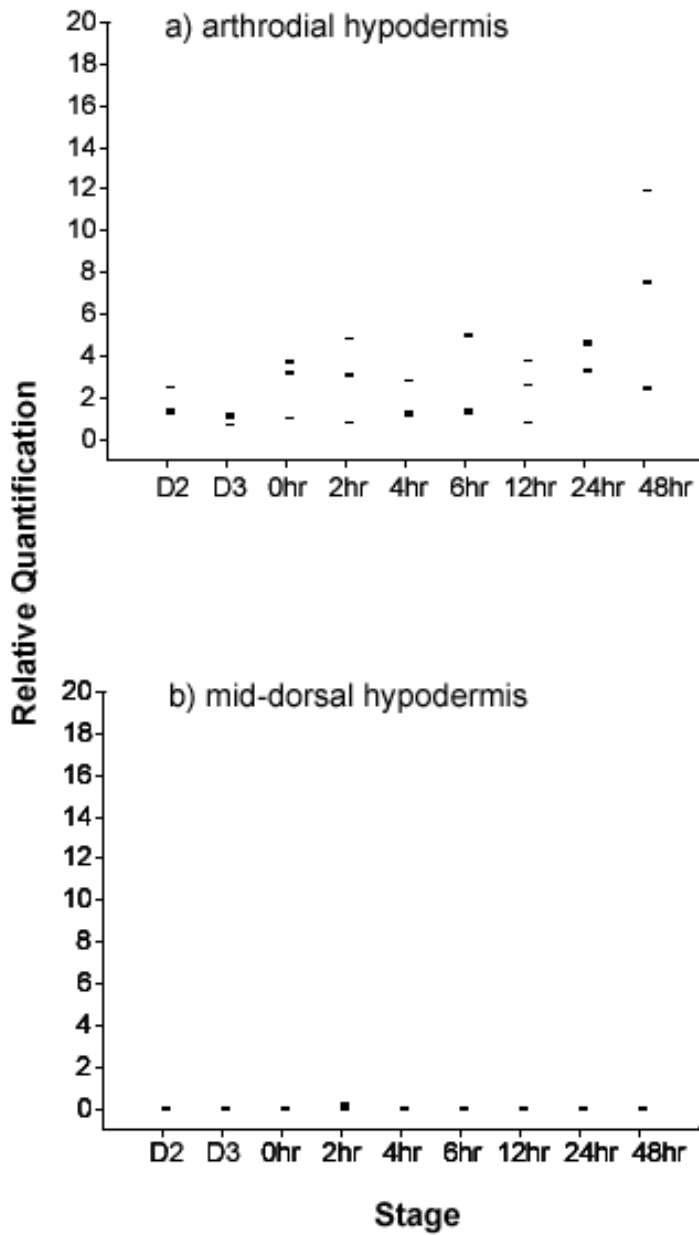or postmolt or in postmolt mid-dorsal hypodermis (Fig. 13a,b).   None of the four transcripts
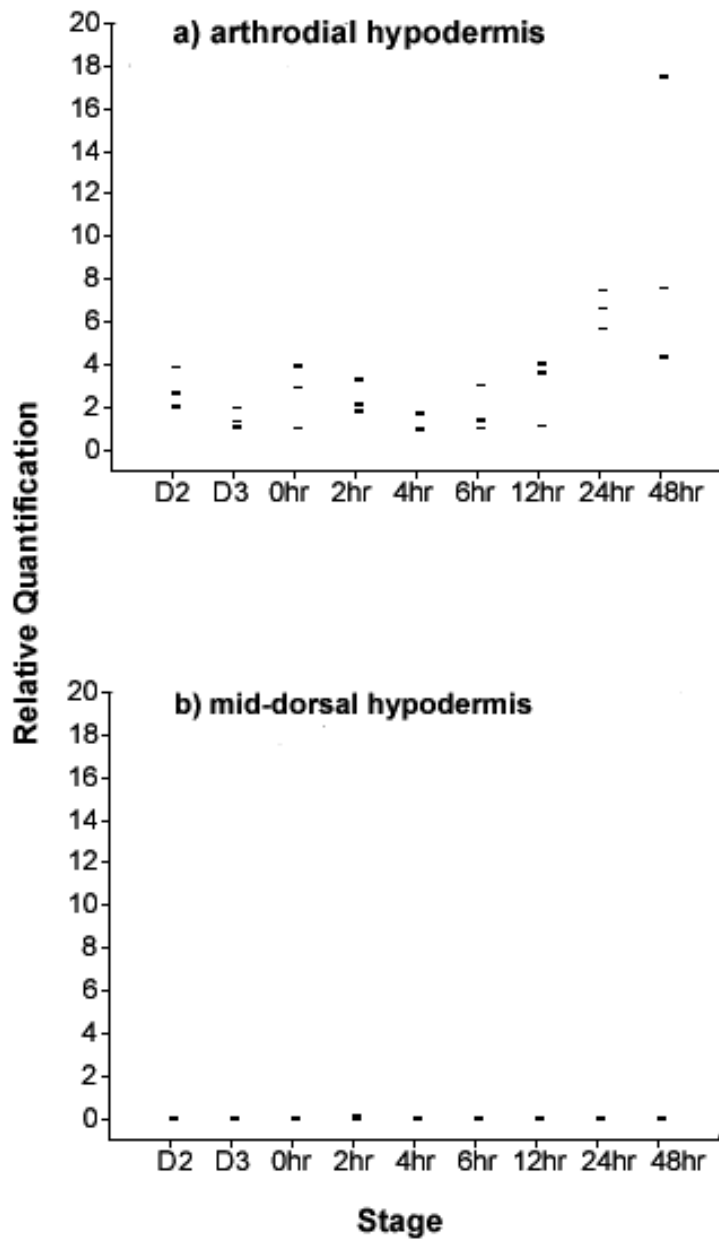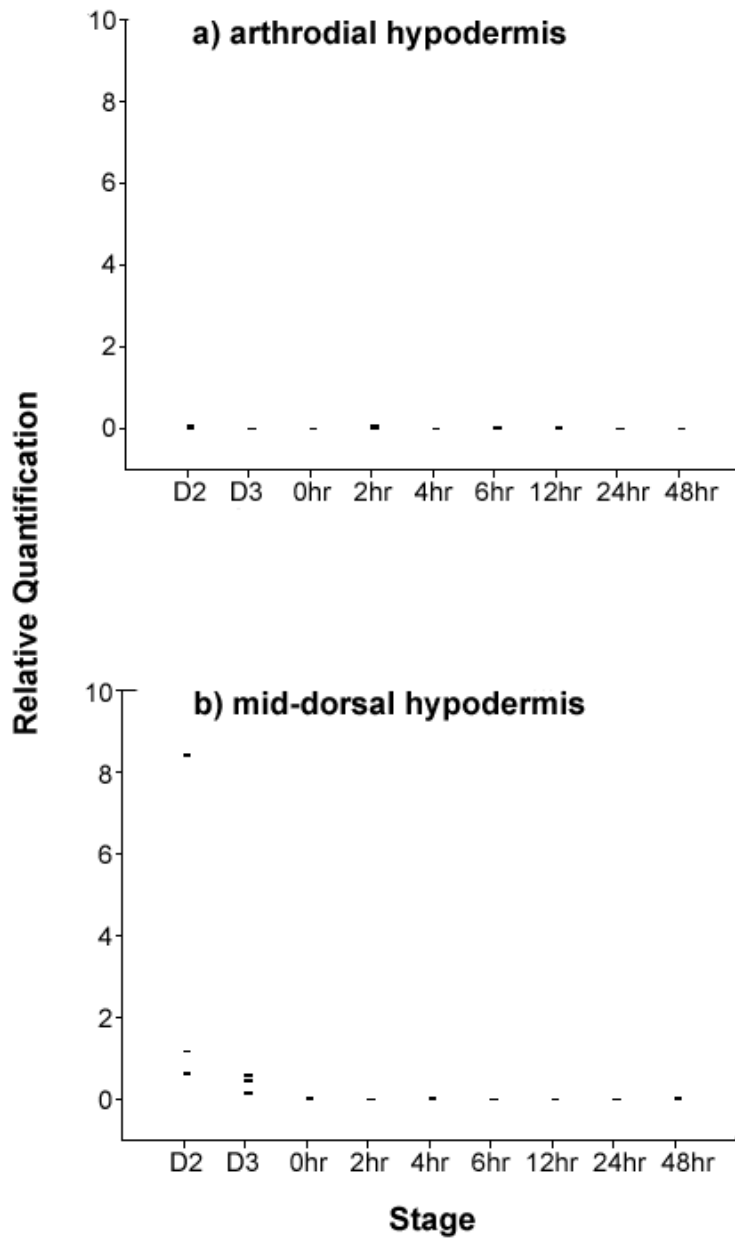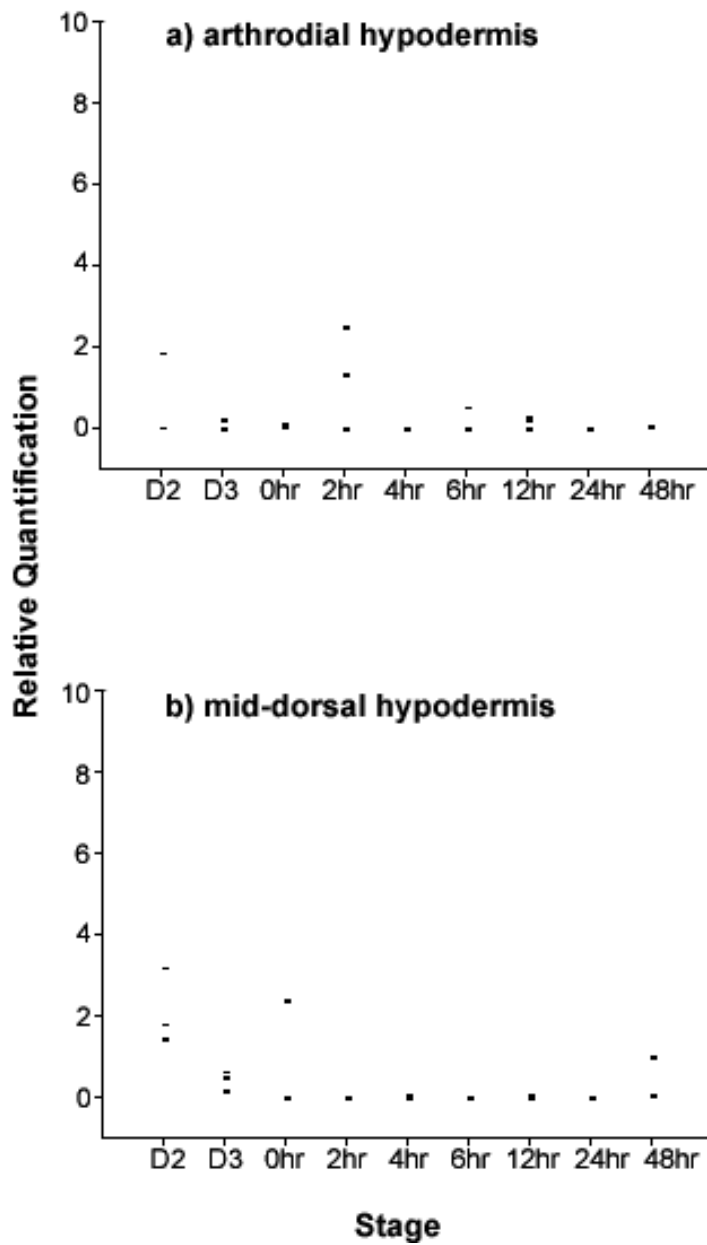
Fig. 8   Relative quantitative PCR for CsAMP8.1. Each data point represents cDNA from an individual crab (3 data points for each time period) analyzed on an ABI 7500 Real Time PCR System. D_2 and D_3 indicate pre-molt stages; times are after ecdysis. a) Arthrodial hypodermis. b) Mid-dorsal hypodermis.

Fig. 9   Relative quantitative PCR for CsCAMP6.0. Each data point represents cDNA from an individual crab (3 data points for each time period) analyzed on an ABI 7500 Real Time PCR System. D_2 and D_3 indicate pre-molt stages; times are after ecdysis. a) Arthrodial hypodermis. b) Mid-dorsal hypodermis.

Fig. 10 Relative quantitative PCR for CsCP8.5. Each data point represents cDNA from an individual crab (3 data points for each time period) analyzed on an ABI 7500 Real Time PCR System. D_2 and D_3 indicate pre-molt stages; times are after ecdysis. a) Arthrodial hypodermis. b) Mid-dorsal hypodermis.

Fig. 11 Relative quantitative PCR for CsCP8.2. Each data point represents cDNA from an individual crab (3 data points for each time period) analyzed on an ABI 7500 Real Time PCR System. D_2 and D_3 indicate pre-molt stages; times are after ecdysis. a) Arthrodial hypodermis. b) Mid-dorsal hypodermis.
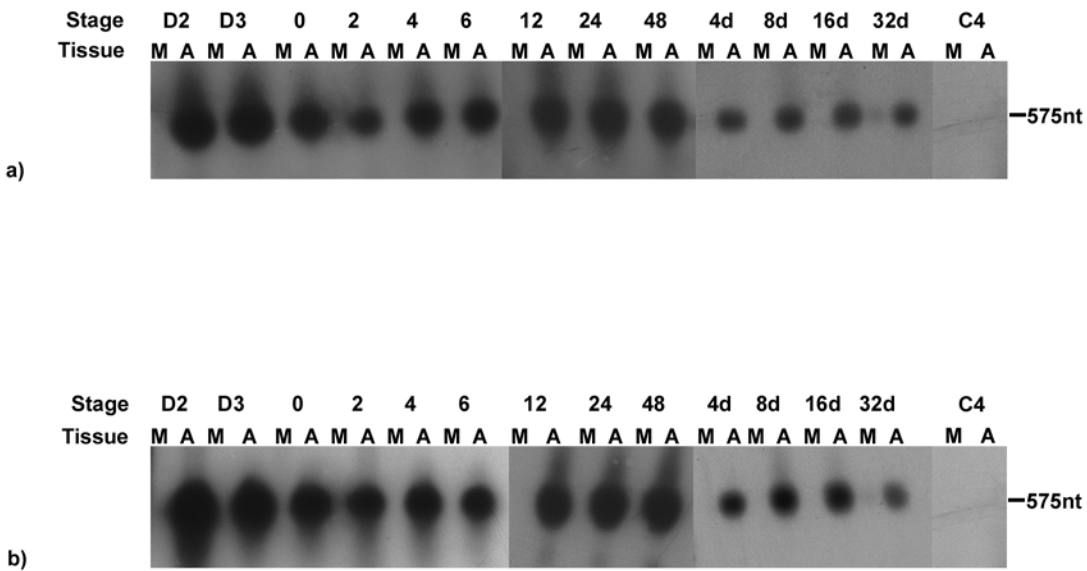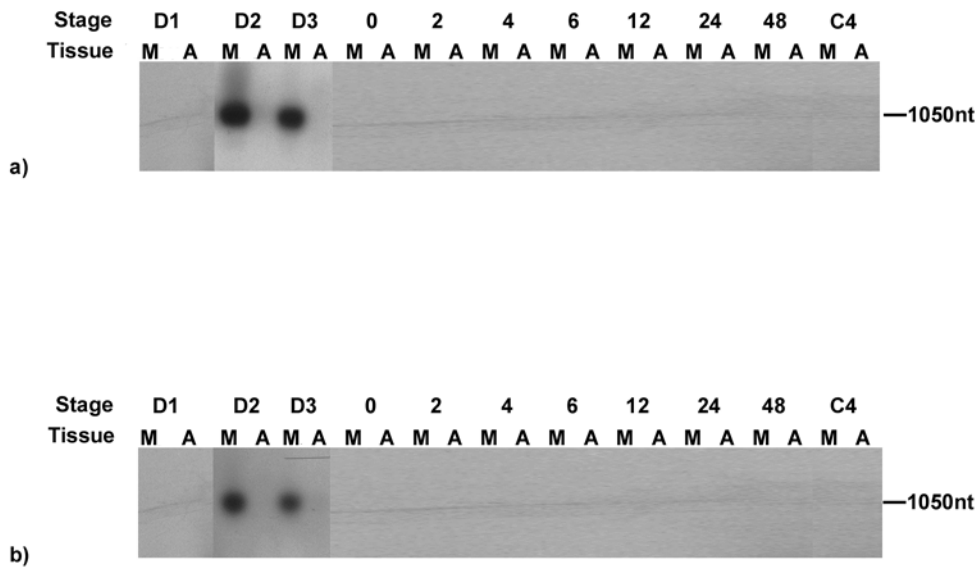
Fig. 12 Northern Blots analysis of (a) CsAMP8.1 and (b) CsAMP6.0 transcripts. RNA (1μg) was separated on a 1% denaturing agarose gel, transferred to Immobilon-Ny+, and hybridized with a DIG-labeled probe. Time course is indicated at the top of each figure. D2 and D3 signify premolt RNA, subsequent numbers represent hours after ecdysis, numbers followed by the letter "d" represent days after ecdysis, and C4 signifies intermolt RNA. M indicates mid-dorsal hypodermis and A indicates arthrodial hypodermis.

Fig. 13 Northern Blots analysis of (a) CsCP8.5 and (b) CsCP8.2 transcripts. RNA (1µg) was separated on a 1% denaturing agarose gel, transferred to Immobilon-Ny+, and hybridized with a DIG-labeled probe. Time course is indicated at the top of each figure. D1, D2 and D3 signify premolt RNA, subsequent numbers represent hours after ecdysis, and C4 signifies intermolt RNA. M indicates mid-dorsal hypodermis and A indicates arthrodial hypodermis.

were detected in intermolt (C_4) mid-dorsal or arthrodial hypodermis, hepatopancreas or hemocyte RNA (data not shown).

DISCUSSION

This study investigated the transcription of genes encoding cuticular proteins containing the Rebers-Riddiford consensus sequence in *C. sapidus*. cDNA sequences have been obtained for four such genes. The deduced polypeptides of all four transcripts contain a signal sequence indicating that they are synthesized in epithelial cells of the hypodermis and secreted to the cuticle to function in the extracellular matrix. Spatial and temporal expression patterns were determined for each of the transcripts by quantitative PCR and Northern blot analysis.

Two of the cDNA transcripts identified in this study, CsAMP8.1 and CsAMP6.0, are similar. Both terminate in three basic amino acid residues. Interestingly, the cDNA transcript of the *P. clarkii* calcified cuticle protein CAP-1 (Inoue et al., 2003) as well as the precursor of ecdysis-triggering hormone associated peptide (ETH-AP) of *Manduca sexta* (Zitnan et al., 1999) end with two basic amino acid residues, RK, that are missing from the mature forms of these proteins. In addition, several cDNAs identified in *C. sapidus* calcified cuticle contain a basic amino acid 4-residue motif, RxKR (Kennedy et al., unpublished) that is absent from the directly sequenced homologous proteins found in *H. americanus* (Kragh et al., 1997; Nousiainen et al., 1998) and *C. pagurus* (Andersen, 1999). In all cases, it has been assumed that these residues are removed by carboxypeptidase B or E, but the significance of this removal is unknown (Inoue et al., 2003). Although there is no

direct evidence at present, it is hypothesized that the C-terminal basic residues in CsAMP8.1 and CsAMP6.0 will not be present in the mature proteins.

The deduced amino acid sequences of both CsAMP8.1 and CsAMP6.0 contain RR-1 consensus sequences. RR-1 is found in the soft cuticles of insects (Andersen, 1998A) and crustaceans (Andersen, 1998B). Expression patterns evaluated with quantitative PCR and Northern blots showed that CsAMP8.1 and CsAMP6.0 are produced only in uncalcified arthrodial hypodermis, as expected for RR-1 containing proteins (Figs 8, 9, 12). These *C. sapidus* transcripts are homologous with a variety of uncalcified crustacean cuticle proteins (Figs 2, 4) within the RR-1 consensus and throughout the length of the sequences. The cDNA transcripts for the homologous *M. japonicus* DD9A and DD9B were expressed in the lateral region of the tail fan where calcification does not occur in this species, but not in the medial calcified regions (Watanabe et al., 2000). These data suggest that a group of related proteins containing the RR-1 consensus sequence are not only essential to crustacean joint arthrodial membrane but, in a broader sense, to all crustacean cuticle that remains uncalcified.

The temporal expression pattern of CsAMP8.1 and CsAMP6.0 indicated by Q-PCR shows the highest levels of expression 24 through 48 hours postmolt (Figs 8, 9). At this time large amounts of endocuticle are being deposited. The transcripts continue to be expressed but with declining intensity in the arthrodial membrane through 32 days postmolt before disappearing from intermolt tissue (Fig. 12). Thus, transcript expression is continuous from the beginning of synthesis of pre-exuvial cuticle through the end of postmolt endocuticle synthesis. This pattern has been recognized in insect cuticle proteins containing the RR-1 consensus sequence. For example, *Manduca sexta* (Riddiford et al., 1986) and *Drosophila melanogaster* (Rebers and Riddiford, 1988) both have transcripts which disappear when larve stop producing

endocuticle. Additionally, DD9A and DD9B from *M. japonicus* are also only expressed during endocuticule synthesis (Watanabe et al., 2000). The high levels of expression of CsAMP8.1 and CsAMP6.0 during endocuticle deposition suggest that these proteins are structurally important to arthrodial membranes. As structural elements of the exoskeleton, these proteins should be continually synthesized until a complete cuticle has been constructed.

The remaining two *C. sapidus* cDNA transcripts identified in this study, CsCP8.5 and CsCP8.2, are also similar to each other. Both deduced polypeptides contain RR consensus sequences. Expression patterns evaluated with quantitative PCR and Northern blots revealed that these transcripts are expressed principally in premolt mid-dorsal hypodermis. CsCP8.5 is expressed in mid-dorsal premolt (D_2 and D_3) only (Fig. 10). Quantitative PCR results for CsCP8.2 showed transcript expression in mid-dorsal 0 hr and 48 hr, arthrodial D_2 and 2 hr as well as in mid-dorsal premolt (D_2 and D_3) tissue (Fig 11).   However, only mid-dorsal D_2 exhibits measurable expression levels in all three crab RNA samples. Expression in mid-dorsal 0 hr (immediately post-ecdysis) tissue is not entirely surprising. Zero hour tissue was removed immediately after the crab was completely free from the old carapace, but there is a great deal of variation among crabs in the time from the first "busting" (suture opening) to this point. It is possible that the individual crab expressing CsCP8.2 at 0 hours postmolt pulled very quickly out of its old exoskeleton and still contained premolt transcripts at the time of sacrifice. The expression shown in D_2 arthrodial hypodermis may be due to contamination with cuticle destined to calcify as it is not always easy to cleanly separate the two regions from the soft pre-exuvial cuticle. The expression levels identified in arthrodial 2-hr hypodermis and mid-dorsal 48-hr hypodermis cannot be explained as easily, except perhaps by individual crab variation. Northern

blot analysis of CsCP8.5 and CsCP8.2, using one single RNA sample per time point, showed

expression for both transcripts only in premolt (D_2 and D_3) mid-dorsal hypodermis (Fig. 13).

The deduced amino acid sequences of CsCP8.5 and CsCP8.2 are homologous with *P.*

clarkii calcium-associated peptides, CAP-1 and CAP-2 (Fig. 7). They are also similar to the RR-

like region of *M. japonicus* crustocalcin (Endo et al., 2004). Much like the CAP proteins,

CsCP8.5 and CsCP8.2 have highly acidic N- and C- terminal ends. CsCP8.5 has 11 acidic

residues of 41 in the N-terminus and 7 acidic residues of 29 in the C- terminus (Fig. 5), while

CsCP8.2 has 8 acidic residues of 24 in the N-terminus and 10 acidic residues of 32 in the C-

terminus (Fig. 6). These acidic ends have been suggested to facilitate calcium binding and

nucleation (Inoue et al., 2001, 2003, 2004). Crustocalcin also has a highly acidic region located

adjacent to and downstream from the RR-like consensus sequence. It has been hypothesized that

the function of this acidic region is similar to that of the N- and C- terminals of the CAP proteins

(Endo et al., 2004). Crustocalcin, CAP-1 and CAP-2 are chitin-binding, calcium-binding matrix

peptides that may be responsible for crystal nucleation in *M. japonicus* (Endo et al., 2004) and *P.*

clarkii (Inoue et al., 2004) respectively. Although the function of the identified transcripts

CsCP8.5 and CsCP8.2 was not determined in this study, their homology with CAP-1 and CAP-2

suggests that they could be responsible for crystal nucleation in the exocuticle of *C. sapidus*.

Current models assume that calcifying cuticles contain matrix molecules which can facilitate the

initiation and growth of calcium carbonate crystals (Coblentz et al., 1998; Andersen, 1999).

If the *P. clarkii* proteins CAP-1 and CAP-2 and the *C. sapidus* proteins CsCP8.5 and

CsCP8.2 are in fact nucleation sites for calcium, then the peptides in question must be

responsible for nucleation in different cuticle layers because the transcript expression patterns are

different. CAP-1 and CAP-2 transcripts are only expressed in postmolt tissue at which time the

exoskeleton is being calcified (Inoue et al., 2003, 2004). The crustocalcin transcript is also only expressed in postmolt tissue and immunostaining was only able to detect it in endocuticle (Endo et al., 2004). This indicates that these proteins are produced and incorporated into the depositing endocuticle and are immediately active nucleators for calcium carbonate. CsCP8.5 and CsCP8.2 transcripts were not expressed in early premolt (D_1) mid-dorsal tissue during epicuticle deposition. However, both transcripts were expressed in D_2 and D_3 mid-dorsal tissue, at which time the pre-exuvial exocuticle layer is being deposited, but no calcification is occurring. This expression pattern suggests that CsCP8.5 and CsCP8.2 are produced and incorporated into the pre-exuvial exocuticle but remain inactive until postmolt calcification begins. This activation is likely related to the post-ecdysial cuticle alteration (PECA) seen in *C. sapidus* in the early hours post molt, a constellation of events that relates changes in the protein composition to the ability of the cuticle to nucleate calcium carbonate (Shafer et al., 1995). A model has been proposed that small acidic protein nucleation sites are present in the cuticle before ecdysis, but are shielded by larger proteins until after ecdysis (Coblentz et al., 1998). After ecdysis the inhibitory macromolecules are altered during the PECA, leaving the nucleation sites exposed and able to bind ions and initiate crystal growth (Coblentz et al., 1998). The expression patterns of CsCP8.5 and CsCP8.2 combined with their sequence homology with CAP-1 and CAP-2 and small size indicates that these may be the nucleating sites in the Coblentz (1998) *C. sapidus* calcification model.

Expression patterns of the calcified cuticle proteins in both *C. sapidus* and *P. clarkii* show a narrow window of time for message production. If these proteins are acting as nucleators they could be considered a catalytic element, as opposed to structural material necessary for the production of cuticle. As such, they would not need to be present in bulk but rather incorporated

into the cuticle only at specific locations. Once nucleation has been established, crystal growth would continue throughout the exocuticle and additional message for nucleating proteins would be unnecessary.

REFERENCES

Addadi, L. and Weiner, S. (1985). Interactions between acidic proteins and crystals: Stereochemical requirements in biomineralization. *Proc. Natl. Acad. Sci. USA*. 82, 4110-4114.

Andersen, S. O. (1998). Amino acid sequence studies of endocuticular proteins from the desert locust, *Schistocerca gregaria*. *Insect Biochem. Mol. Biol*. 28, 421-434.

Andersen, S. O. (1998)**.** Characterization of proteins from arthrodial membranes of the lobster, *Homarus americanus*. *Comp. Biochem. Physiol.* 121A**,** 375-383.

Andersen, S. O. (1999). Exoskeletal proteins from the crab, *Cancer pagurus. Comp. Biochem. Physiol.* 123A, 203-211.

Andersen, S. O. (2000). Studies on proteins in post-ecdysial nymphal cuticle of locust, *Locusta migratoria*, and cockroach, *Blaberus craniifer. Insect Biochem. Mol. Biol*. 30, 569-577.

Coblentz, F. E., Towle, D. W. and Shafer, T. H. (2005). Expressed sequence tags from normalized cDNA libraries prepared from gill and hypodermal tissue of the blue crab, *Callinectes sapidus*. The Society for Integrative and Comparative Biology. San Diego, CA.

Coblentz, F. E., Shafer, T. H. and Roer, R. (1998). Cuticular proteins from the blue crab alter in vitro calcium carbonate mineralization. *Comp. Biochem. Physiol.* 121B, 349-360.

Dillaman, R. M., Hequembourg, S. and Gay, M. (in press). The Early pattern of calcification in the dorsal carapace of the blue crab, *Callinectes sapidus*. J. Morph.

Drach, P. and Tchernigovtzeff, C. (1967). Sur la methode de determination des stades d'intermue et son application generale aux crustaces. *Vie Milieu Ser. A:Biol. Mar*. 18, 595-610.

Endo, H., Persson, P. and Watanabe, T. (2000). Molecular cloning of the crustacean DD4 cDNA encoding a Ca^{2+}-binding protein. *Biochem. Biophys. Res. Com.* 276, 286-291.

Endo, H., Takagi, Y., Ozaki, N., Kogure, T. and Watanabe, T. (2004). A crustacean Ca^{2+}-binding protein with a glutamate-rich sequence promotes $CaCO_3$ crystallization. *Biochem. J.* 384, 159-167.

Giraud-Guille, M. M. and Quintana, C. (1982). Secondary ion microanalysis of the crab calcified cuticle: Distribution of mineral elements and interactions with the cholestric organic matrix. *Biol. Cell*. 44, 57-68.

Hepburn, H.R. and Chandler, H.D. (1976). Material properties of arthropod cuticles: The arthrodial membrane. *J. Comp. Physiol*. 109, 177-178.

Inoue, H., Ozaki, N. and Nagasawa, H. (2001). Purification and structural determination of a phosphorylated peptide with anti-calcification and chitin-binding activities in the exoskeleton of the crayfish, *Procambarus clakii. Biosci. Biotech. Biochem*. 65, 1840-1848.

Inoue, H., Ohira, N., Ozaki, N. and Nagasawa, H. (2003). Cloning and expression of a cDNA encoding a matrix peptide associated with calcification in the exoskeleton of the crayfish. *Comp. Biochem. Physiol.* 136B, 755-765.

Inoue, H., Ohira, T., Ozaki, N. and Nagasawa, H. (2004). A novel calcium-binding peptide from the cuticle of the crayfish, *Procambuarus clarkii. Biochem. Biophys. Res. Com*. 318, 649-654.

Kennedy, P. J. (2004) Characterization of a gene family associated with calcified structures in the blue crab, *Callinectes sapidus*. M.S. Thesis, University of North Carolina at Wilmington, USA.

Kragh, M., Molbak, L. and Andersen, S.O. (1997). Cuticular proteins from the lobster, *Homarus americanus. Comp. Biochem. Physiol*. 118B, 147-154.

Lowenstam H. A., and Weiner, S. (1989). On Biomineralization. New York: Oxford University Press.

Neville, A. C. (1975). Biology of the Arthropod Cuticle. New York: Springer.

Nousiainen, M., Rafn, K., Shou, L., Røepstorff, P. and Andersen, S.O. (1998) Characterization of exoskeletal proteins from the American lobster, *Homarus americanus. Comp. Biochem. Physiol.* 119B, 189-199.

Passano, L. M. (1960). Molting and its control. In The Physiology of the Crustacea (ed T. H. Waterman), pp. 473-536. New York: Academic Press, Inc.

Rebers, J. E. and Riddiford, L. M. (1988). Structure and expression of a *Manduca sexta* larval cuticle gene homologous to *Drosophila* cuticle genes. *J. Mol. Biol*. 203, 411-423.

Rebers, J. E. and Willis, J. H. (2001). A conserved domain in arthropod cuticular proteins binds chitin. *Insect Biochem. Molec. Biol*. 31, 1083-1093.

Riddiford, L. M., Baeckmann, A., Hice, R. H. and Rebers, J. E. (1986). Developmental expression of three genes for larval cuticular proteins of the tobacco hornworm, *Manduca sexta. Develop. Biol*. 118, 82-94.

Roer, R. D. and Dillaman, R. M. (1984). The structure and calcification of the crustacean cuticle. *Amer. Zool*. 24, 893-909.

Shafer, T. H., Roer, R. D., Miller, C. G. and Dillamen, R. M. (1995). Postecdysial cuticle alteration in the blue crab, *Calliectes sapidus. J. Exp. Zool*. 271, 171-182.

Tweedie, E. P., Coblentz, F. E. and Shafer, T. H. (2004). Purification of a soluble glycoprotein from the uncalcified ecdysial cuticle of the blue crab *Callinectes sapidus* and its possible role in initial mineralization. *J. Exp. Biol*. 207, 2589-2598

Watanabe, T., Persson, P., Endo, H., and Kono, M. (2000). Molecular analysis of two genes, DD9A and B, which are expressed during the postmolt stage in the decapod crustacean *Penaeus japonicus*. *Comp. Biochem. Physiol.* 125B, 127-136.

Williams, C. L., Dillaman, R. M., Elliot, E. A. and Gay, D. M. (2003). Formation of the arthrodial membrane in the blue crab, *Callinectes sapidus. J. Morph*. 256, 260-269.

Zitnan, D., Hollar, L., Spalovska, I., Takac, P., Zitnanova, I., Gill, S.S., and Adams, M.E. (2002). Molecular cloning and function of ecdysis-triggering hormones in the silkworm *Bombyx mori. J. Exp. Biol*. 205, 3459-3473.