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A Highly Conserved Red Pigment-Concentrating Hormone Precursor in the Blue Crab *Callinectes sapidus*+

Jörg M. Klein, Carl J. Mohrherr, Frank Sleutels, Nicola Jaenecke, John P. Riehm and K. Ranga Rao*

Department of Cellular and Molecular Biology, The University of West Florida, 11000 University Parkway, Pensacola, Florida 32514-5751

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SUMMARY. A cDNA library was established from the eyestalk ganglia of the blue crab *Callinectes sapidus*. One clone was isolated (644 bp excluding the poly(A) tail) which encodes the red pigment-concentrating hormone (RPCH)-precursor, consisting of the 25 amino acid residue signal peptide, the RPCH, and a 73 amino acid residue RPCH-precursor related peptide. This clone displays high sequence similarity with a clone isolated from an eyestalk cDNA library of the shore crab *Carcinus maenas*, in accordance with the close phylogenetic relationship between these species. Northern blot experiments indicated the presence of two different mRNA transcripts which hybridized with a specific RPCH-cDNA probe pointing to the possibility of multiple RPCH isoforms in the blue crab. Although crustacean RPCH and the insect adipokinetic hormones (AKH) are structurally related, their precursors show little similarity.

The crustacean red pigment-concentrating hormone (RPCH) and the insect adipokinetic hormones (AKHs) are a family of structurally related peptides with diverse functions. RPCH (pELNFSPGWamide) has only been found in crustaceans where it triggers chromatophoral pigment concentration and dark-adaptational eye pigment migration (1). Additional roles as neurotransmitter and/or neuromodulator have been identified by combined immunocytochemical and electrophysiological studies (2-7). Identical RPCHs have been isolated and sequenced from the crustacean species *Pandalus borealis* (8), *Cancer magister*, *Carcinus maenas*, and *Orconectes limosus* (9). Based on biological assays, amino acid compositions and similar chromatographic characteristics there is strong evidence that the RPCHs from *Palaemon squilla* (10),

+The nucleotide sequence data reported in this paper are available through the GSDB, DDBJ, EBI and NCBI databases with accession number L 36824.

*To whom correspondence should be addressed. Fax: 904-474-3130.
Email: RKROTHAP@UWF.CC.UWF.EDU.

Abbreviations: AKH: adipokinetic hormone; APRP: AKH-precursor related peptide; ORF: open reading frame; PRP: precursor related peptide; RPCH: red pigment-concentrating hormone; RPRP: RPCH-precursor related peptide.

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Paleaemonetes pugio (11), *Homarus americanus* (12), *Cardisoma carnifex* (13), *Liocarcinus puber, Nephrops norvegicus, Pacifastacus leniusculus* (9), and *Procambarus clarkii* (14) share an identical octapeptide structure. In contrast, the insect adipokinetic hormones show considerable sequence heterogeneity, ranging from 8 to 10 amino acid chain lengths, with more than 20 different forms isolated from the species investigated. RPCH has not been found in insects, although RPCH-related octapeptides do occur.

The study of the evolutionary and phylogenetic relationships of the AKH/RPCH peptides requires the knowledge of the corresponding precursor structures. Prohormone structures of peptides of the RPCH/AKH family were first reported for AKHs. Single AKH-precursors are known from *Manduca sexta* (15) and *Drosophila melanogaster* (16), respectively. From the grasshoppers *Schistocerca gregaria* (17, 18) and *Schistocerca nitans* (19) two different cDNAs encoding two different AKH-precursors (AKH I and AKH II) have been found in each species. In contrast, only one RPCH-precursor structure has been determined in crustaceans (20).

In the present study a recently established cDNA library from the eyestalk ganglia of the blue crab *Callinectes sapidus* was screened for RPCH clones. We wished to identify the presence of isoforms as has been observed in orthopteran insects (18, 19) and to compare AKH- and RPCH-precursors relative to structure and phylogenetic relationships.

MATERIALS AND METHODS

cDNA library construction. Poly(A)$^+$RNA from 120 eyestalks of the blue crab *Callinectes sapidus* was isolated by the use of guanidine thiocyanate and oligo(dT)cellulose (Stratagene). About 5 μg of poly(A)$^+$RNA were used for creating the cDNA-library with the Uni-ZAP XR vector (Stratagene) according to the manufacturer's instructions.

PCR-based library screening. Based on the amino acid sequence of *Pandalus borealis* RPCH (8) a degenerated oligonucleotide (R1-CSA, 5'-GGGAATTC(A/C/G/T)CCCCA(A/C/G/T)CC(A/C/G/T)GG(A/C/G/T)(C/G)(A/T)(A/G)AA(A/G)TT-3', antisense) corresponding to amino acids 3-8 of RPCH was designed and used in combination with primer BS2 (5'-AGCGGATAACAA TTTCACACAGGA-3', sense) corresponding to nucleotides 824-847 of the pBluescript II SK- vector for a PCR-based screening of the *Callinectes* eyestalk cDNA library. Ten different fractions of the library, 5 μl each containing 5000 independent clones, were used as template in a PCR with a total volume of 50 μl (100 pmol primer R1-CSA, 20 pmol primer BS2, 2 mM $MgCl_2$, 200 μM each dNTP, 50 mM KCl, 10 mM Tris-HCl, pH 8.3). The PCR was performed 5 cycles with an annealing step at 68°C, 5 cycles at 64°C and 40 cycles at 60°C. The denaturation step in each cycle was 40 s at 94°C, the amplification step 3 min at 72°C. Fractions which gave single amplification products with a size of about 350 bp were plated in appropriate dilutions on 150 mm plates. Phage DNA was transferred to nylon membranes. The replicas were suspended in 5 ml lambda dilution buffer (0.1 M NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5, 0.1% gelatin; (21)). Five μl samples of the supernatant were used for a second round of amplification. Plates with a positive PCR result were then divided in 4 parts and phages of these parts were again tested by PCR. Thus smaller amounts of phages were screened in subsequent steps until individual clones were subjected to PCR analysis.

DNA sequencing. The conversion of recombinant Uni-ZAP XR clones to a pBluescript phagemid was performed according to the manufacturer's instructions. The sequences were determined by the use of Sequenase (USB) and RPCH-cDNA-specific oligonucleotides as primers.

Synthesis of cRNA probes and northern blot analysis. Poly(A)$^+$RNA was isolated from 10 eyestalks using deoxythymidylate covalently attached to superparamagnetic polystyrene beads (Dynabeads Oligo(dT)$_{25}$). The RNA was separated by electrophoresis on a denaturing 6% formaldehyde/1% agarose gel. After transfer to a nylon membrane (Magnagraph) the RNA was linked to the membrane by baking for 2 h at 80°C. The RPCH clone was digested with Bam HI and the 300 bp fragment (corresponding to nucleotides 1-274 of the RPCH-cDNA (Fig. 1)) was subcloned into the pBluescript II SK(+) vector (Stratagene). The generation of digoxigenin-labelled antisense probes was performed according to the manufacturer's instructions (Boehringer Mannheim). Hybridization was performed at 68°C for 16 h and the membrane was washed with 0.1x SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and 0.1% sodium dodecyl sulfate at 68°C. Detection was performed according to the instructions of the manufacturer (Boehringer Mannheim).

RESULTS AND DISCUSSION

One clone encoding a RPCH-prepropeptide was isolated from the *Callinectes sapidus* eyestalk ganglia cDNA library. The cDNA and deduced amino acid sequences are presented in Fig. 1. The open reading frame (ORF) encodes 109 amino acids. It consists of a signal peptide (25 amino acid residues), the RPCH (8 amino acid residues), a glycine residue, a dibasic cleavage site, and a peptide with 73 amino acid residues, the RPCH-precursor related peptide (RPRP). The amino acid sequence of *Callinectes* RPCH is identical with the RPCHs from *Pandalus borealis* (8), *Cancer magister*, *Carcinus maenas*, and *Orconectes limosus* (9), previously characterized by Edman degradation. This interpretation assumes that the N-terminal glutamine residue is processed posttranslationally into pyroglutamate and the C-terminal glycine residue serves as an amide donor (15).

There are striking similarities between the sequences of the *Callinectes* RPCH clone and a RPCH clone previously isolated from a MTXO (Medulla terminalis X-organ) cDNA library from the shore crab *Carcinus maenas* (20). A comparison of the complete cDNA sequences reveals a similarity of about 86%, whereas a comparison of the ORFs of both RPCH-clones shows a similarity of 89% (Fig. 2). The signal peptides and the untranslated regions of the RPCH-cDNAs of both crustacean species are nearly identical. In contrast, the pigment-dispersing hormone (PDH)-cDNAs from *Callinectes* and *Carcinus* showed greater differences (22, 23). In decapod crustaceans there appears to be a tendency to conserve RPCH at both the peptide and mRNA levels.

Northern blot analysis (Fig. 3), however, indicated the presence of two mRNA transcripts of differing lengths which hybridized with the RPCH-specific probe. Whereas the size of the

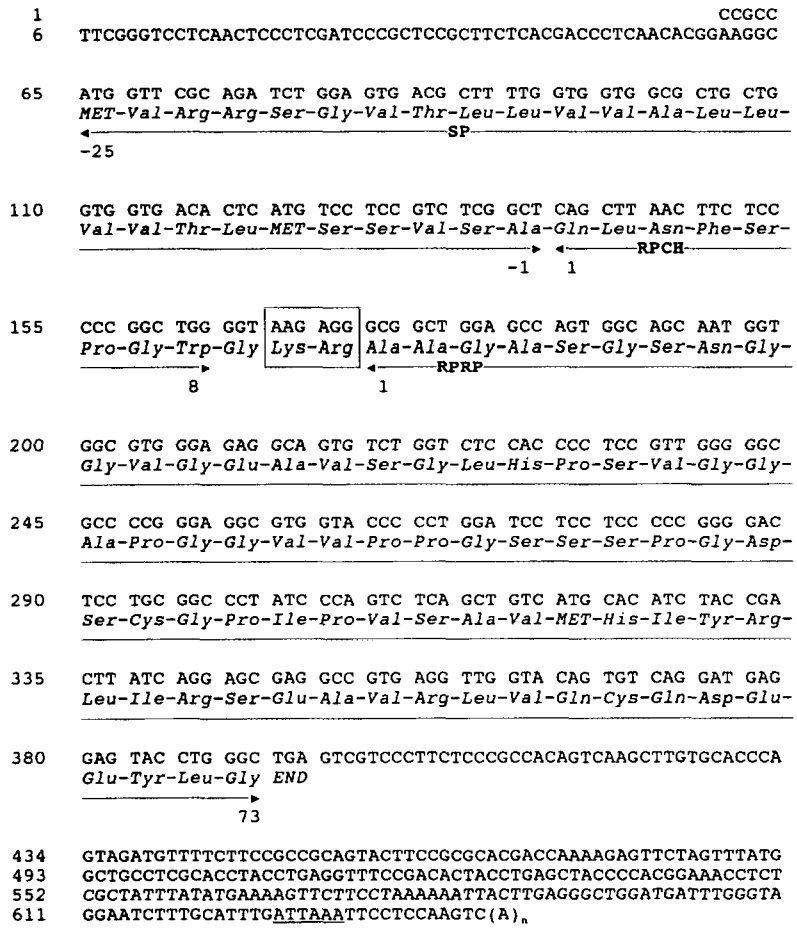

Fig. 1. Nucleotide and deduced amino acid sequences of the RPCH-precursor-cDNA from *Callinectes sapidus*. The prepropeptide which consists of signal peptide (SP), RPCH and RPCH-precursor related peptide (RPRP) is underlined. The dibasic cleavage site between RPCH and RPRP is boxed. The putative polyadenylation site is underlined.

smaller hybridization product (660 bp) is in accordance with the isolated clone (644 bp without the polyA tail) the origin of the second hybridization product (850 bp) remains unclear. This is in contrast to our findings of only one RPCH clone. Intensive library screening did not reveal any other cDNAs. Further experiments are in progress to examine the genetic basis of this second band.

The classes of Crustacea and Insecta represent two distinct groups of mandibulate arthropods which share many common aspects of anatomy and biochemistry. On this basis it is generally assumed that the origins and phylogeny of RPCH/AKH neuropeptides share a common ancestry (24). The features common to these peptides are: pyroglutamic acid at the N-terminus; an amide at the C-terminus; a tryptophane residue at position 8; and a second aromatic amino acid residue at position 4 (Phe or Tyr). Furthermore, RPCH and many AKHs

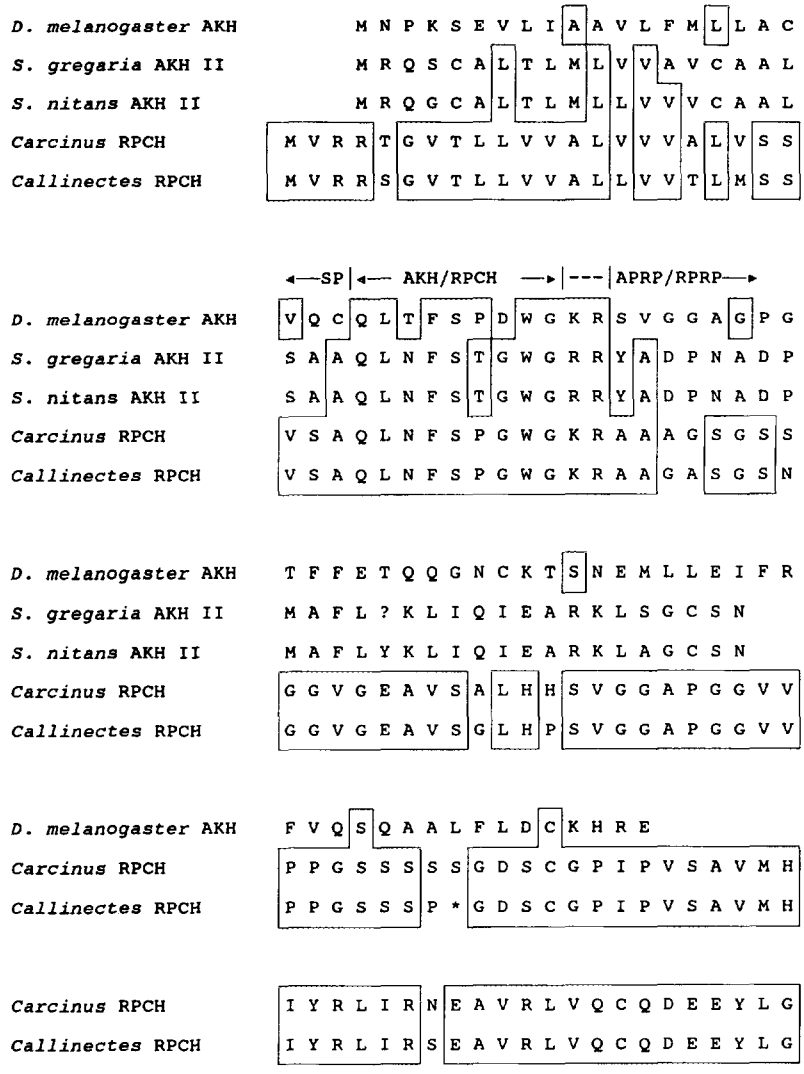

Fig. 2. Comparison of the amino acid sequences of the AKH-precursor from *Drosophila melanogaster* (16), the AKH II-precursors from *Schistocerca gregaria* (18), *Schistocerca nitans* (19) and the RPCH-precursors from *Carcinus maenas* (20) and *Callinectes sapidus*. Identical positions (relative to the RPCH-precursors) are boxed. The blank in the *Callinectes*-RPRP was introduced in order to get maximum sequence matches. SP, signal peptide; AKH, adipokinetic hormone; RPCH, red pigment-concentrating hormone; APRP, AKH-precursor related peptide; RPRP, RPCH-precursor related peptide. For this comparison only known precursors of AKH/RPCH octapeptides are listed. The question mark in the APRP of *S. gregaria*-AKH II stands for Y (18, determined by cDNA cloning) or T (29, determined by peptide sequencing).

strongly crossreact in crustacean and insect bioassays (25). The amino acid sequence of RPCH exhibits a high degree of similarity to known AKH octapeptides. RPCH differs by only one amino acid exchange in the AKH II peptides of *Schistocerca gregaria* (Thr^6), *Schistocerca nitans* (Thr^6), and *Locusta migratoria* (Ala^6), and there are two amino acid exchanges for several other

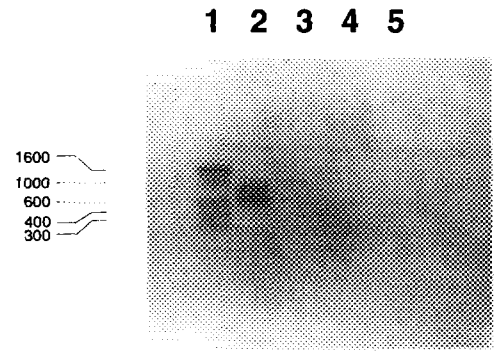

Fig. 3. Northern blot analysis of different tissues from the blue crab *Callinectes sapidus* by use of a specific digoxigenin-labelled RPCH-cRNA probe. Lane 1: RNA molecular weight marker. The fragment sizes are indicated. Lane 2: blotted RNA from eyestalk ganglia; two hybridization products are observed. No hybridization products were observed in Lane 3 (heart), 4 (muscle), and 5 (hepatopancreas).

AKHs: *Drosophila melanogaster* AKH (Thr^3, Asp^7), *Romalea microptera* AKH II (Val^2, Thr^6), and *Locusta migratoria* AKH III (Thr^5, Trp^7; for a recent review see (24)).

Unlike AKH/RPCH peptides, homology is not readily apparent when one examines the AKH-/RPCH-precursor related peptide (APRP/RPRP) sequences. In the RPCH-precursor molecules of both crustacean species, the RPRPs are located at the C-terminal end of the encoding region. The two crustacean RPRPs share a similarity of 90% with a deletion in the *Callinectes sapidus* RPRP and a length of 73 amino acids in contrast to the *Carcinus maenas* RPRP with a length of 74 amino acids (Fig. 2). Database searches did not show any significant similarity to any other known peptides including the AKH-precursor related peptides (APRPs) which are present at corresponding positions of the AKH-precursors from insects. RPRPs have not been isolated or sequenced so far. The analysis of a hydropathy plot (not shown) for these deduced sequences suggests a hydrophobic nature for these molecules.

APRPs have been elucidated by peptide sequencing and cloning AKH-precursors from various insects belonging to different orders. Within a given taxonomic group, a high sequence homology is evident among the APRPs. Thus, the APRPs of the orthopterans *Schistocerca gregaria* AKH II (18) and *Schistocerca nitans* AKH II (19) consist of 28 amino acids and differ only in one position, based on the sequence elucidated by cDNA cloning (Fig. 2). Surprisingly even the corresponding APRPs of the AKH I-precursors of *Schistocerca gregaria* (17) and *Schistocerca nitans* (19), which encode AKH decapeptides, show the same length and some sequence similarity. Four other APRPs with similar sequences have been reported, two from *Locusta migratoria* (26) and two from *Romalea microptera* (24). These putative APRPs have not been found by molecular cloning but by amino acid sequencing. Because of similar sequences they can be considered as APRPs, although it remains to be shown that these peptides

and the corresponding AKHs I and II of *Romalea microptera* and *Locusta migratoria* are encoded by the same mRNAs. However, none of these orthopteran APRPs displays any sequence homology to the crustacean RPRPs.

There is also a lack of sequence similarity between crustacean RPRP and the dipteran APRP from *Drosophila melanogaster* (16). Although *Drosophila melanogaster* AKH differs from RPCH in only one amino acid, the corresponding PRPs differ significantly in length and amino acid sequence (Fig. 2). In this respect it is not surprising that the APRP of the lepidopteran *Manduca sexta* AKH-precursor, which encodees an AKH nonapeptide, also differs in length (34 amino acids) and amino acid sequence (16).

The post-translational events of the APRPs from *Locusta migratoria* and *Schistocerca gregaria* involve the formation of disulfide bonds generating homo- and heterodimers (26, 27; for review see 28). It is not known if this form of processing is retained for the other known APRPs of insects or for the RPRPs of brachyuran crabs which possess appropriate cysteine residues for disulfide bridges. Presently, the physiological function of RPRPs or APRPs remains unknown.

The signal peptides, as was the case with PRPs, show no significant similarity between the insect and crustacean precursors. Only within the corresponding arthropod group are the signal sequences identical (AKH I-SPs of the orthopterans) or highly similar (AKH II-SPs of the orthopterans; RPCH-SPs of the crustaceans).

There seems to be only a limited number of characteristics which are valid for crustacean and insect precursors, and a phylogenetic relationship of the genes is not obvious. All AKH and RPCH-precursors, however, share a basic architectural plan: the encoding region is subdivided into signal peptide, hormone, and precursor related peptide. Additional studies at the molecular level and characterization of precursors from other mandibulates, especially more primitive arthropods, will be required to elucidate the apparent genetic diversity of the SP and PRP portions of the corresponding precursor molecules.

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