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Molecular cloning of a cDNA encoding a crustacean hyperglycemic hormone from eyestalk ganglia of the blue crab, *Callinectes sapidus*

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Abstract

Crustacean hyperglycemic hormone (CHH), a polypeptide with multiple physiological effects, was first identified in the X-organ/sinus gland neurosecretory system of the eyestalks. In studies reported here, we used a PCR-based cloning strategy (RT-PCR followed by 5′- and 3′-RACE) to clone from blue crab (*Callinectes sapidus*) eyestalk ganglia a cDNA (*CsCHH-1*) encoding a putative CHH preprohormone. Sequence analysis revealed the preprohormone included all structural features previously reported for CHH preprohormones: a signal peptide, a CHH precursor-related peptide (CPRP), the CHH polypeptide, and a C-terminal basic processing site. Further, the deduced amino acid sequence of the mature polypeptide included all signature domains previously reported for CHH. The primary structure of blue crab CHH is most closely related to CHH from other brachyurans. RT-PCR revealed the CsCHH-1 transcript was present in eyestalk ganglia, but was undetectable in other tissues tested. A transcript encoding a similar CHH-like preprohormone was detected in thoracic ganglion, ventral nerve cord, and brain, but was not detected in eyestalk ganglia.

Keywords: Crustacean hyperglycemic hormone; Neurohormone; *Callinectes sapidus*

1. Introduction

Crustacean hyperglycemic hormone (CHH) is a polypeptide neurohormone with multiple physiological effects. CHH was first identified as a "diabetogenic factor" in extracts of fiddler crab (*Uca pugilator*) eyestalks (Abramowitz et al., 1944). Since then, significant progress has been made in understanding the role of CHH in regulating carbohydrate metabolism (review: Santos and Keller, 1993). Other studies have implicated CHH in regulation of additional physiological processes including reproduction (De Kleijn and Van Herp, 1998; Khayat et al., 1998), osmoregulation (Serrano et al., 2003; Spanings-Pierrot et al., 2000), lipid metabolism (Santos et al., 1997), and molting (Chung et al., 1999). Of particular interest to our laboratory is the finding that CHH may be involved in the regulation of ecdysteroid production by crustacean molting glands (Y-organs) (Webster, 1993).

CHH was first purified and sequenced from eyestalks of the green crab, *Carcinus maenas* (Kegel et al., 1989). Subsequently, the primary structure of CHH has been directly determined by protein sequencing or deduced from cloned cDNAs for a number of crustaceans, including crayfish, lobsters, shrimp, an isopod, and additional crabs (reviews: De Kleijn et al., 1995; Chang, 2001; Lacombe et al., 1999; Soyez, 1997). CHH, along with molt-inhibiting hormone (MIH), vitellogenesis-inhibiting hormone (VIH), and mandibular organ-inhibiting hormone (MOIH), constitute a family of structurally related neurohormones (Keller, 1992) that appear to fall into two major subgroups (Lacombe et al., 1999). An intriguing aspect of CHH structure is the existence of multiple molecular forms of the neurohormone within a species

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(Bulau et al., 2003; Chen et al., 2004; Dircksen et al., 2001; Gu and Chan, 1998; Soyez et al., 1994; Yasuda et al., 1994). Although it has been reasonably hypothesized that the structural variants may have distinct physiological effects, the significance of the structural polymorphism has not been fully resolved.

The eyestalk source of CHH is a cluster of neurosecretory cells, the medulla terminalis X-organ, from which axons project distally and terminate in the neurohemal sinus gland (Dircksen, 1992; Dircksen et al., 1988; Gorgels-Kallen et al., 1982; Jaros and Keller, 1979). Several recent reports have revealed the existence of possible extra-eyestalk sources of CHH, including the gut, pericardial organ, subesophageal and thoracic ganglia, heart, antennal glands, and gills (Chang et al., 1999; Chen et al., 2004; Chung et al., 1999; Webster et al., 2000; Dircksen et al., 2001).

Blue crabs (*Callinectes sapidus*) are widely distributed along the Atlantic coast and in the Gulf of Mexico (Churchill, 1921; Steele and Perry, 1990). They occupy a variety of habitats, primarily in brackish and shallow oceanic water (Churchill, 1921; Steele and Perry, 1990), and play critical roles in the estuarine food web (Hines et al., 1990; Laughlin, 1982; Van Engel, 1958). In addition, blue crabs support valuable commercial and recreational fisheries along the Atlantic and Gulf coasts (Guillory and Perret, 1998; Rugolo et al., 1998; Steele and Perry, 1990).

However, despite the importance of CHH in regulating a variety of physiological processes in Crustacea, and the ecologic and economic significance of blue crabs, the structure of the CHH polypeptide and gene are not known in this species. In the present study, we report the molecular cloning of a cDNA encoding CHH from eyestalks of the blue crab. The results provide comparative data on the structure of CHH, and given the possibility that CHH may play a role in the control of ecdysteroidogenesis, complement our ongoing studies of the endocrine regulation of growth and molting in crustaceans.

2. Methods

2.1. Experimental animals

Blue crabs, *C. sapidus*, were purchased from local markets or from Gulf Specimen Marine Laboratories, Inc. (Panacea, FL). Crabs were maintained in compartmented tanks containing artificial seawater as previously described (Han and Watson, 2005). Intermolt crabs were used for all experiments.

2.2. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Degenerate primers were designed from highly conserved regions of CHH from the marbled crab (*Pachygrapsus marmoratus*, Accession No. AY094983), mud crab (*Scylla serrata*, Accession No. AY372181), and green crab (*C. maenas*, Accession No. X17596). The sequence of the forward primer (5′-CA[AG]AT [CT]T[AT]CG A[CT][AT][CG][GT] TC[CGT]TG CA-3′) was based on nucleotides 222–243; the sequence of the reverse primer (5′-AGGTC [AT]TCCA T[AG]CA[CT] TG[CG]CG-3′) was based on nucleotides 369–388. Eyestalk ganglia were dissected from ice-anesthetized intermolt crabs. Total RNA was extracted from eyestalk ganglia using an RNAgents Total RNA Isolation System (Promega Corp., Madison, WI). Reverse transcription and PCR amplification were performed using a SuperScript One-Step RT-PCR with Platinum Taq Kit (Invitrogen, Carlsbad, CA). Reverse transcription was performed at 50 °C for 30 min, followed by heating at 94 °C for 2 min to terminate the reaction. After an initial denaturation step of 2 min at 94 °C, 35 amplification cycles were performed. Each cycle involved denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 45 s. A final extension step of 7 min at 72 °C was performed to complete the reaction. A single PCR product of the expected size (167 bp) was obtained. The PCR product was isolated, cloned into a pGEM-T Easy Vector (Promega), and sequenced (UAB Core Sequencing Facility).

2.3. Rapid amplification of 3′- and 5′-cDNA ends (RACE)

For RACE reactions, total RNA was isolated from eyestalk ganglia of intermolt crabs as described above. With 1 μg of total RNA as template, 5′-RACE-Ready cDNA and 3′-RACE-Ready cDNA were generated using protocols and reagents provided in a SMART RACE cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA). Gene-specific primers were selected from the 167 bp PCR product obtained by RT-PCR. For 5′-RACE, the 50-μl PCR mixture was as follows: 2.5 μl of 5′-RACE-Ready cDNA, 5 μl of 10× Universal Primer Mix (UPM), 1 μl of 10 μM 5′-Gene-Specific Primer 1 (5′-GSP 1: 5′-TTGAT CTGCA TCCAC TGGCC A-3′), and 41.5 μl of Master Mix. Positive and negative controls were prepared according to the instructions of the supplier. PCR was carried out for 38 cycles as follows: 94 °C for 45 s for denaturation, 58 °C for 45 s for primer annealing, and 72 °C for 1 min for extension, followed by 1 cycle of 7 min at 72 °C for extension. The primary PCR product was diluted 1:100 in TE buffer (10 mM Tris–HCl, pH 8.0), and used as template for a second (nested) round of amplification with 5′-GSP 2 (5′-AGCTC GTTGA AGATG GCTCT-3′) as primer. PCR conditions were as described above. The final PCR product was amplified, cloned into pGEM-T Easy Vector, and sequenced.

For 3′-RACE, the 50-μl PCR mixture contained 3′-RACE-Ready cDNA, 3′-Gene-Specific Primer (3′-GSP: 5′-TGGCC AGTGG ATGCA GATCA A-3′), UPM, and Master Mix as described above. Positive and negative controls were prepared according to the instructions of the supplier. PCR conditions were as previously described. The PCR product was amplified, cloned into pGEM-T Easy Vector, and sequenced.

2.4. Sequence analysis

DNA and deduced amino acid sequences data were analyzed using MacDNASIS Software (Hitachi, Japan).

2.5. Analysis of the tissue distribution of CsCHH-1 mRNA by RT-PCR

Total RNA was extracted from eyestalk ganglia and control tissues (thoracic ganglion, ovary, ventral nerve cord, muscle, gill, hepatopancreas, and brain) as described above. For each tissue, 1 μg total RNA was treated with RQ1 RNase-Free DNase prior to RT-PCR. Primers (FullCHH-F: 5′-C ATGC AATCC ATCAA AACCG TG-3′; FullCHH-R: 5′-TACTT CTTGC CGACA ACCTG TA-3′) were designed to amplify the full CHH transcript. Equal amounts of RNA (as determined spectrophotometrically) from the various tissues were used for reverse transcription. RT-PCR was performed using a SuperScript One-Step RT-PCR with Platinum Taq Kit (Invitrogen). 18S rRNA primers (18SrRNA-F: 5′-TCAAG TGTCT GCCTT ATCAG CT-3′; 18SrRNA-R: 5′-TAGTC ATTAC CTCGG GTTCA GA-3′) were used in the control reactions (Kim and Abele, 1990).

3. Results

3.1. Cloning and sequence analysis of a cDNA encoding a putative crustacean hyperglycemic hormone from eyestalks of C. sapidus

A PCR-based cloning strategy (RT-PCR followed by 3′- and 5′-RACE) was used to clone a cDNA (*CsCHH-1*)