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Identification and expression of mRNAs encoding bursicon in the plesiomorphic central nervous system of *Homarus gammarus*

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ABSTRACT

Ecdysis in arthropods is a complex process, regulated by many neurohormones, which must be released in a precisely coordinated manner. In insects, the ultimate hormone involved in this process is the cuticle tanning hormone, bursicon. Recently, this hormone has been identified in crustaceans. To further define the distribution of bursicon in crustacean nervous systems, and to compare hormone structures within the sub-phylum, cDNAs encoding both bursicon subunits were cloned and sequenced from the nervous system of the European lobster, *Homarus gammarus*, and expression patterns including those for CCAP determined using *in-situ* hybridisation, quantitative RT-PCR and immunohistochemistry. Full-length cDNAs encoded bursicon subunits of 121 amino acids (Average M_r: 13365.48) for Burs α, 115 amino acids (Average M_r: 12928.54) for Burs β. Amino acid sequences were most closely related to those of crabs, and for Burs β the sequence was identical to that of the American lobster, *Homarus americanus*. Complete co-localisation with CCAP in the VNC was seen. Copy numbers *burs α*, *burs β* and *CCAP* mRNAs were between 0.5 and 1.5 × 10^5 for both bursicon subunits, 0.5–6 × 10^5 per cdn neurone for CCAP. The terminal abdominal ganglia (AG 6–8) contained about 52 cdn-type neurons, making it the largest bursicon producing region in the CNS. Double labelling IHC using recombinant *Carcinus* Burs α and CCAP antisera demonstrated complete co-localisation in the VNC. On the basis of the results obtained, it is proposed that CCAP and bursicon release occur simultaneously during ecdysis in crustaceans.

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1. Introduction

The hormonal control of somatic changes and behaviour during ecdysis in arthropods is a precisely coordinated process. For insects, this process has been the subject of intense research (reviews, Truman, 2005; Kim et al., 2006; Ewer, 2007; Žitňan et al., 2007), and a complex temporal series of neurohormones including – and this list is probably incomplete – pre-ecdysis triggering hormone (PETH), ecdysis triggering hormone (ETH), kinins, diuretic hormones (DHs), myoinhibitory peptides (MIPs), eclosion hormone (EH), crustacean cardioactive peptide (CCAP)[1] and bursicon act in a tightly controlled cascade before, during and after ecdysis as recently exemplified by an RNAi study on the flour beetle, *Tribolium castaneum* (Arakane et al., 2008). Since many of the recent advances in our understanding of these processes in insects have been made using the genetic resources of model insects, it is unsurprising that our knowledge of analogous events and hormones involved in ecdysis of genetically intractable crustaceans is by comparison, extremely limited. Emergence from the exoskeleton at the start of ecdysis is initiated by a massive release of crustacean hyperglycaemic hormone (CHH) from paraneurones in the fore and hind-gut in *Carcinus maenas*, which leads to dipsogenesis and rapid swelling (Chung et al., 1999; Webster et al., 2000). This is immediately followed by a large, rapid release of CCAP from the pericardial organs (Phlippen et al., 2000), which probably initiates stereotyped motor patterns involved in active ecdysis (escape from the old cuticle) in the same way as has been suggested for insects (Gammie and Truman, 1997). The existence of peptides that initiate pre-ecdysial and other ecdysial behaviours, such as PETH, ETH and EH have not been established in crustaceans, excepting the presence of a transcript encoding an ETH-like molecule in the water flea, *Daphnia pulex* (Gard et al., 2009). Despite these differences, the insect cuticle tanning hormone bursicon, which has long been known to play a pivotal role in tanning and

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[1] *Abbreviations used:* AG, abdominal ganglion; AVLC, anterior ventral–lateral cell cluster; Burs α-IR, Bursicon α-immunoreactivity; CCAP, crustacean cardioactive peptide; CCAP-IR, CCAP immunoreactivity; cdn, CCAP-IR descending neurone; CG, cerebral ganglion; cnc, CCAP-IR neurosecretory cell; CNS, central nervous system; DIG-11-UTP, digoxygenin-11-uridine-5′-triphosphate; EDC, 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide; EST, expressed sequence tag; GSP, gene specific primer; IHC, immunohistochemistry; IPTG, isopropyl β-D-1-thiogalactopyranoside; ISH, *in-situ* hybridisation; ORF, open reading frame; PBS, phosphate-buffered saline; PLC, posterior-lateral cell cluster; PMLC, posterior medial–lateral cell cluster; qRT-PCR, quantitative RT-PCR; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription PCR; SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SOG, sub-oesophageal ganglion; TG, thoracic ganglion; UTR, untranslated region.

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melanisation of the insect cuticle (Cottrell 1962a,b; Fraenkel and Hsiao, 1962, 1965), seems to have a widespread, possibly universal occurrence in arthropods. Following the identification and full characterisation of the bursicon as a heterodimeric cystine knot protein encoded by CG13419 (*burs or burs α*) and CG15284 (*pburs or burs β*) (Luo et al., 2005; Mendive et al., 2005), database searches revealed the presence of a *burs α*-like transcript in the *Daphnia arenata* EST database. Information from this sequence, together with those available from insects allowed us to identify cDNAs (using a strategy involving degenerate PCR 5′ and 3′ RACE) encoding both bursicon subunits in *D. arenata* and the shore crab *C. maenas*, thus firmly establishing bursicon as a hormone common to both subphyla (Wilcockson and Webster, 2008).

For insects, many, but not all bursicon immunoreactive neurons in the CNS co-localise with CCAP (review, Honegger et al., 2008), occasionally some are immunoreactive to only one bursicon monomer (Luo et al., 2005; Dai et al., 2008), and it has been proposed from molecular dissection via enhancer trapping in *Drosophila* that the differential distribution of bursicon and CCAP expressing neurons forms a neural network which controls the sequential activation of bursicon release during the ecdysis programme (Luan et al., 2006a,b). Additionally, for holometabolous insects, adult emergence is associated with a peak in expression and release of bursicon, together with subsequent apoptosis of most of the abdominal neurons that express CCAP (and which co-express bursicon) following eclosion (Ewer et al., 1998; Draizen et al., 1999).

For crustaceans expression patterns of mRNA and the neurons expressing bursicon and CCAP are only known for the shore crab *C. maenas* (Wilcockson and Webster, 2008). Whilst that study suggested that transcripts for both bursicon subunits and CCAP were co-expressed throughout the CNS, the apomorphic nature of the fused abdominal ganglia of the crab (which contain the majority of bursicon expressing neurons) did not easily allow detailed analysis. We therefore reasoned that the more plesiomorphic central nervous system of the lobster, where abdominal ganglia are clearly defined would be ideal to resolve issues concerning co-localisation of bursicon and CCAP. Furthermore, since previous neuronal mapping of CCAP in the ventral nervous system of crayfish (Audehm et al., 1993; Trube et al., 1994) recorded anatomies of each CCAP expressing neuron in every ganglion, and our previous cloning and sequencing of mRNA encoding lobster CCAP (Chung et al., 2006) it would now be possible to determine steady state mRNA copy number per neuron, to accurately determine ratios of bursicon transcript number in relation to CCAP by qRT-PCR in each ganglion of the ventral nerve cord.

Here we report nucleotide and amino acid sequences encoding both bursicon subunits in the European lobster *Homarus gammarus*, and expression patterns of both bursicon and CCAP in individual ganglia of the CNS using *in-situ* hybridisation, immunohistochemistry (IHC) and quantitative RT-PCR.

2. Materials and methods

2.1. Animal collection, tissue preparation

Adult *H. gammarus* (ca. 600 g) were purchased from local fishermen (Anglesey, UK), individually held in a recirculating seawater aquarium under ambient conditions of temperature and photoperiod and fed chopped fish and squid *ad libitum*. Additionally, juvenile lobsters (ca. 60 mm total length) were grown from post-larvae (National lobster Hatchery, Padstow, Cornwall, UK) for immunohistochemical studies of whole mounted VNC. After deep anaesthetisation (>60 min) on ice, nervous systems (eye stalks, cerebral ganglion, thoracic ganglia 1–5, abdominal ganglia 1–5, terminal ganglia) were dissected in chilled saline, snap-frozen in liquid N_2 and stored at −80 °C. For *in-situ* hybridisation nervous systems were fixed immediately in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Following overnight fixation, tissues were dehydrated through a graded methanol/PBS series and stored for 2–3 days at room temperature in methanol before use. Tissues dissected for whole mount immunohistochemistry were fixed in 4% paraformaldehyde in PBS containing 1% 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide (EDC) overnight at 4 °C prior to extensive washing in PBS.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), followed by treatment with DNase1 (37 °C, 1 h, TURBO DNA-free, Ambion, TX, USA) and quantification (ND-1000, NanoDrop Technologies, Wilmington, DE, USA). For rapid amplification of cDNA ends (RACE), mRNA was isolated from extracted total RNA using poly-dT Dynabeads (Dynal, Oslo, Norway) according to the manufacturer's instructions and stored in 10 mmol l^{-1} Tris at −80 °C. Approximately 50 ng mRNA was reverse transcribed in a 20 μl reaction. For 3′ RACE, mRNA samples were reverse transcribed (50 °C, 50 min) using SuperScript III RT (Invitrogen, Carlsbad, CA, USA) and primed with the Gene Racer 3′oligo(dT) adapter primer (Invitrogen) according to the manufacturer's instructions. For 5′ RACE, mRNA was dephosphorylated, decapped, ligated to a 5′ RACE RNA oligo (Invitrogen) and reverse transcribed using SuperScript III with random hexamers according to the manufacturer's instructions. Samples were then treated with 2 U RNase H (37 °C, 20 min). For degenerate PCR, mRNA was reverse transcribed (50 °C, 50 min) using SuperScript III RT and primed with random hexamers.

For qRT-PCR total RNA was reverse transcribed using a Taqman High Capacity cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). Briefly, 5 ng of total RNA was reverse transcribed using random hexamer primers in 20 μl reaction volumes according to the manufacturer's instructions. qRT-PCR cRNA standards were reverse transcribed simultaneously with the RNA samples.

2.3. Primers

A complete list of primers and their identifying abbreviations is provided in Table 1.

2.4. Degenerate PCR of cDNA encoding burs α

Degenerate primers previously used to identify cDNA encoding *burs α* in *C. maenas* (Wilcockson and Webster, 2008), were used to identify cDNA fragments encoding *burs α* of *H. gammarus*. PCRs were performed using the following conditions: 12.5 μl AmpliTaq Gold Master Mix (Applied Biosystems), 9 μl water, 1.25 μl (100 μmol l^{-1}) forward and reverse primers (4F GCVPKPIP, 11R MCRPCTSIE; 1 μl cDNA template. Amplification conditions were: 1 cycle 94 °C 9 min; 5 cycles of 94 °C 30 s, 63 °C 30 s; 5 cycles of 94 °C 30 s, 60 °C 30 s; 25 cycles of 94 °C 30 s, 57 °C 30 s, 72 °C 45 s and final extension at 72 °C for 10 min. A second PCR was performed using fully nested primers (8F ERSCMCCQE, 9R CMCRPCTSI) using the following conditions: 1 cycle 94 °C 4 min, 35 cycles of 94 °C 30 s, 58 °C 30 s, 72 °C 45 s, final extension at 72 °C for 7 min. One microlitre of first round PCR was used as template. PCR products were electrophoresed on 2% agarose gels and bands of the expected size excised and extracted using a gel purification kit (Perfectprep Gel Cleanup, Eppendorf AG, Hamburg, Germany).

2.5. Rapid amplification of cDNA ends (RACE)

Using sequence information obtained from cloning and sequencing degenerate PCR products, and using available sequence

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