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Identification and developmental expression of mRNAs encoding putative insect cuticle hardening hormone, bursicon in the green shore crab *Carcinus maenas*

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Abstract

Bursicon is the ultimate hormone in insect ecdysis, which is involved in cuticle hardening. Here we show that mRNAs encoding the heterodimeric cystine knot protein bursicon (Burs α, β), are present in crustaceans, suggesting ubiquity of this hormone in arthropods. We firstly report the cloning, sequencing of mRNAs encoding subunits from the water flea, *Daphnia arenata* and the CNS of the crab, *Carcinus maenas*, in comparison with insect bursicon subunits. Expression patterns of α and β *burs* mRNAs were examined by *in-situ* hybridisation (ISH) and quantitative RT-PCR. In the thoracic ganglion, *burs* α and β mRNAs were completely colocalised in neurones expressing crustacean cardioactive peptide (CCAP). However, in the brain and eyestalk, bursicon transcripts were never observed, despite a complex expression pattern of CCAP interneurones. Patterns of expression of *burs* α and β mRNAs were constitutive during the moult cycle of adult crabs, in stark contrast to the situation in insects. Whilst copy numbers of *burs* β transcripts closely matched those of *CCAP*, those of *burs* α mRNA were around 3-fold higher than *burs β.* This pattern was apparent during embryogenesis, where bursicon transcripts were first observed at around 50% development—the same time as first expression of CCAP mRNA. Transcript ratios (*burs α: β*) increased during development. Our studies have shown, for the first time, that bursicon mRNAs are expressed in identified neurones in the nervous system of crustaceans. These findings will now promote further investigation into the functions of bursicon during the moult cycle and development of crustaceans.

Keywords: Arthropods; Bursicon; Crustaceans; Developmental expression; Ecdysis; *In-situ* hybridisation; Neurohormones; Quantitative RT-PCR

1. Introduction

All arthropods periodically replace their exoskeletons in order to grow and metamorphose; it has long been known that this moulting is controlled by several hormones-current research in insects has suggested considerable and unforeseen complexity. At the very least, six or more different hormones are involved: notwithstanding the well known roles of ecdysteroids in directing the synthesis of the new cuticle (review by Riddiford, 1989) and the role of prothoracicotropic hormone (PTTH)[1] in stimulating ecdysteroid secretion by the prothoracic glands (reviews by Gilbert et al., 1996, 2002), a series of neuropeptide hormones are involved in the insect ecdysis programme. These act in a precisely timed series during the penultimate stages of premoult, which are characterised by a precipitous

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[1] *Abbreviations used:* AK, arginine kinase; AMV-RT, avian myeloblastosis virus-reverse transcriptase; CCAP, crustacean cardioactive peptide; CNS, central nervous system; CG, cerebral ganglion; DIG-11-UTP, digoxygenin-11-uridine-5′ triphosphate; ES, eyestalk; EST, expressed sequence tag; ISH, *in-situ* hybridisation; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase PCR; TG, thoracic ganglion; UTR, untranslated region.

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decline in ecdysteroid levels (reviews by Ewer and Reynolds, 2002; Mesce and Fahrbach, 2002). Until recently, most studies used larvae of the tobacco hornworm, *Manduca sexta*, as a model. Several hormones, including pre-ecdysis triggering hormone (PETH), ecdysis triggering hormone (ETH), eclosion hormone (EH), crustacean cardioactive peptide (CCAP), and finally the ultimate hormone involved in this cascade- bursicon, are now known to be involved in the ecdysis programme (review by Truman, 2005). Not unexpectedly, the rich genetic resources available in *Drosophila* by way of mutants, targeted ablations via knock outs and transgenic technologies have now added considerably to the lepidopteran models, and such techniques have expedited tremendous progress in pursuit of previously intractable questions; these have recently been elegantly reviewed (Ewer, 2005).

As alluded to above, the ultimate hormone in the ecdysis cascade in insects has long been known to be bursicon, which was identified as a key hormone involved in tanning and melanisation of the insect cuticle more than 40 years ago (Fraenkel and Hsiao, 1962; Cottrell, 1962; Fraenkel and Hsiao, 1963, 1965; Fraenkel et al., 1966). At that time, the classical bioassay for this hormone involved injection of newly-eclosed, neck-ligated flies (*Sarcophaga bullata*) with extracts of the CNS, and subsequent recording of tanning (melanisation and sclerotisation) of the cuticle (review by Seligman, 1980). Despite numerous attempts at purification and characterisation of bursicon (Kaltenhauser et al., 1995; Kostron et al., 1995, 1999; Honegger et al., 2002), the complete determination of the structure of bursicon remained elusive. However, using sequence information obtained from 2 D-gel electrophoresis and tryptic digests of cockroach (*Periplaneta americana*) bursicon containing extracts (Honegger et al., 2002), and *in silico* data mining, bursicon has recently been identified in *Drosophila*, as a heterodimeric cysteine knot protein comprising the product of CG13419 (*burs* or *burs* α) and CG15284 (*pburs*, or partner of burs, *burs* β) (Luo et al., 2005; Mendive et al., 2005). Subsequently, heterodimeric bursicon molecules have been identified from a number of model insects, from conceptual translation of cDNA and gDNA sequences (Van Loy et al., 2007). The cognate receptor of bursicon, DLGR2 (a GPCR) has also been identified in *Drosophila* (Baker and Truman, 2002; Luo et al., 2005; Mendive et al., 2005), for which loss of function mutations in DLGR2 display a phenotype (*rickets* $rk^{-/-}$) which is similar to that induced by bursicon deficiency resulting from neck ligation.

Bursicon would perhaps be firstly considered as a prototype insect hormone, but since it has been known for some years that it is present in crustacean cardioactive peptide (CCAP) expressing neurones in the insect CNS (Kostron et al., 1996; Honegger et al., 2002; Davis et al., 2003; Dewey et al., 2004) and because these neurones have a surprisingly conserved architecture in all arthropods (review by Dircksen, 1998), it seemed possible that crustaceans might also possess bursicon-like molecules. Furthermore, in view of a report that extracts of abdominal ganglia from lobsters (*Homarus americanus*) showed bursicon activity in the *Sarcophaga* bioassay (Kostron et al., 1995), we reasoned an attractive hypothesis would be that bursicon-like molecules might have a wider distribution than previously suspected in the arthropods. This hypothesis also had tentative grounding from our previous observations showing that CCAP release in crustaceans was associated with stereotyped ecdysis behaviour in crayfish and crabs (Phlippen et al., 2000). However, our most germane observation was that an early interrogation of the *Daphnia arenata* EST database revealed a single EST (Wfes0000858) with a remarkable similarity to *Drosophila burs* α.

Here we report the cloning and sequencing of mRNAs encoding bursicon subunits (*burs* α, *burs* β) in the model crustacean *Daphnia,* followed by cloning, sequencing and expression profiles of both subunit mRNAs in our crab model, *Carcinus maenas* during the moult cycle of adults and during embryonic development. We also report the distribution of these transcripts in the CNS of *Carcinus* by whole mount *in-situ* hybridization. We show that in the thoracic ganglion expression of bursicon mRNAs occurs exclusively in CCAP neurones, and that expression of *burs* α, *burs* β mRNA in the CNS of *Carcinus* is constitutive during the moult cycle, in contrast to the situation in insects, perhaps suggesting new roles for this hormone in crustaceans.

2. Materials and methods

2.1. Animals, tissue preparation and RNA extraction

Adult *C. maenas* were collected using baited traps (Menai Strait, Anglesey, UK) and maintained in a recirculating seawater aquarium system under ambient conditions with *ad libitum* feeding. Specimens of *D. arenata* (Log $_{50}$ clone) were a generous gift from Prof. J. Colbourne, University of Indiana, Bloomington, IA, USA. *Daphnia* were cultured in glass jars containing filtered pond water and a mixed phytoplankton culture in direct sunlight.

Nervous systems (thoracic ganglia, brain, eyestalk) were dissected from ice-anaesthetised, moult staged *Carcinus*, and immediately placed in RNAlater (Ambion, Texas) (4 °C overnight, followed by storage at −80 °C), or for tissues used in for *in-situ* hybridisation (ISH), fixed immediately in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). After overnight fixation at room temperature (RT), tissues were briefly washed in PBS then dehydrated in a graded methanol/PBS series. Tissues were stored in 100% methanol at RT for a maximum of 3 days before ISH. Embryos were removed in batches of 100 from ovigerous female *Carcinus*, developmentally staged (Chung and Webster, 2004), and stored in RNAlater. For *Daphnia* samples, quantities (*ca* 200) of adults were staged (according to ovarian development/presence of embryos or ephippia in the brood chamber), sampled by individually picking out with a mounted insect pin and placed directly into liquid nitrogen.

Total RNA was prepared using TRIzol (Invitrogen, Carlsbad, CA, USA), followed by treatment with 2 units of DNase 1 (37 °C, 1 h), followed by clean up with DNA-*free* (Ambion) and quantification (ND-1000, NanoDrop Technologies, Wilmington DE, USA). For embryos, and RNA from thoracic ganglia subsequently used in RACE procedures, mRNA was isolated using Dynabeads (Dynal, Oslo, Norway), and stored in 10 mM Tris at −80 °C.