NOVEL PLAYERS IN ECDYSIS REGULATION: A MOLECULAR CHARACTERIZATION OF CRUSTACEAN HYPERGLYCEMIC HORMONE IN *MANDUCA SEXTA*

Christina C. Harris, Anna Drexler, Megumi Fuse
Department of Biology, San Francisco State University, San Francisco, CA 94132

Abstract

rustacean Hyperglycemic Hormone (CHH), a major neuropeptide family in crustaceans, governs hysiological processes ranging from reproduction to metabolism. CHH was recently identified as a ayer in crab molting (Chung et al, 1998), and has important regulatory functions in other arthropods. Endo et al,1999). We seek to understand CHH involvement in insect ecdysis. Immunocytochemical nalyses in the tobacco hornworm, *Manduca sexta*, were used to localize CHH-like peptides to eurosecretory cells and neurohemal release sites in the subesophageal and thoracic ganglia. A onserved region of the CHH gene was amplified and sequenced using degenerate oligonucleotide rimers drawn from a comparative analysis of crustacean and insect CHH coding sequences. equence data from *Manduca* cDNA shows a high degree of conservation with other insects and a sser degree with crustacean species. Analyses of CHH clones suggest the presence of multiple CHH- e peptides in the tobacco hornworm. Additionally, ICC and molecular studies indicate their presence developmental stages from larvae to pupae. This data will be used to study the influence of the rustacean Hyperglycemic Hormone neuropeptide family in regulating insect ecdysis and add to a rowing database of phylogenetic information on these multifaceted peptide hormones.

Introduction

Crustacean Hyperglycemic Hormone peptide family consists of structurally ted neuropeptides that regulate basic physiological functions across a range of ropods. CHH-like peptides (CHH-LPs) are 69-78 amino acid long peptide hones and are the major neurohormones synthesized in the primary crab ocrine gland, the X-Organ Sinus (Webster et al, 2003). CHH was initially found to ease blood glucose levels in arthropods; since then it has been shown to have a ety of functions (eg. Keller et al, 1989; Webster, 1996). The peptide hormones influence water and electrolyte balance (Macins et al, 1999) and stress (Toullec , 2003). A CHH-like peptide – PO-CHH – regulates cardiac function, but has no erglycemic or ion transport activity (Dirksen et al, 1998). Additionally, CHH-LPs oit ecdysteroid synthesis (Ollivaux et al, 2000) and thus aid in the commencement cdysis, or the discrete stereotyped behaviors that result in expulsion of the rigid cle to allow the progression of growth and development. CHH and blood glucose ls are elevated at the onset of ecdysis in crabs (Chung et a,. 1998). The effect on rgy mobilization may be important in the timing of this developmental event.

diversity of function of CHH in the crab prompts us to ask the question: what are roles of CHH-like peptides in the insect? Preliminary protein localization with two H antibodies from two different species of crab reveals that CHH-like peptides are served in *M. Sexta*. Blocking experiments with CHH peptide (*Carcinus maenis*) al that the antibody is partially blocked by peptide incubations. Additionally, we oit the high conservation of CHH-like peptide sequences across taxa to explore composition of *Manduca* CHH.

results confirm that *Manduca sexta* has the gene for a CHH-like peptide, and that peptide is more similar to insect CHH-LPs than to crustacean CHH-LPs. Also, our ies imply that, like other arthropods, the tobacco hornworm synthesizes multiple Hs and produces the peptides across several developmental stages.

The following studies aim to characterize CHH like peptides in *M. sexta*, and to d light on the larger question of CHH's role in insect molting.

Methods

Primers:
Primer1 (CHH-F): 5'-gagtgtaaaggrgtytwcga-3'
Primer2(CHH-R): 5'-ctygamgaytgttwcaatct-3'.

Polymerase Chain Reaction
n: DNEasy kit (Qiagen). **Tissue:** 5th larval stage *Manduca* CNS. **PCR conditions:** 94C/45sec, 55C/45sec, 72C/45sec, x 35 cycles. Genomic DNA template, CHH-F/CHH-R primers. **2º PCR:** 1º PCR product template (clean, separated fragments), CHH-F/CHH-R primers. **PCR fragment separation**: agarose gel electrophoresis (2.8% agarose, 100V).
mbinations (1º PCR): M13(-20)/M13(rev); M13(-20)/CHH-F; M13(-20)/CHH-R; M13(rev)/CHH-F; M13(rev)/CHH-R; CHH-F/CHH-R.
2º PCR: 1º PCR product template, CHH-F/CHH-R primers. Purification and PCR conditions as listed above.

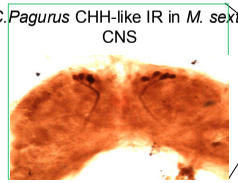
Molecular Cloning
Cloning: Topo 10-kit (Invitrogen).
PCR: M13(-20) and M13rev vector primers.
Cycle Sequence: T7 and T3 vector primers.

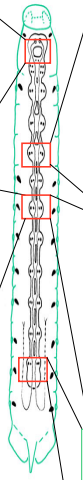
Nucleotide Sequence Analyses
ification: (as above). **Cycle-sequence reactions:** 1.5ul Big Dye pre-mix, 1ul 5x buffer,0.33ul primer, 1-4ul template, H_2O to 12 ul.
Sequence data analyses: Sequencher ™.

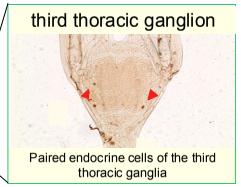
Immunohistochemistry
Primary antibody: Rabbit anti-*Cancer* CHH and Rabbit anti-*Carcinaes* CHH (1:1000)
Antibody: peroxidase-conjugated Donkey anti-Rabbit IgG (1:500). **Color reaction:** Diaminobenzidene (DAB) Tetrahydrochloride.
Blocking: Preabsorption with CHH-peptide from *Cancer meanas* 0.2pmol and *Carcineas pagurus* 0.3pmol.

Fig. 1

C.Pagurus CHH-like IR in *M. sexta* CNS

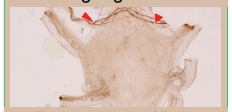




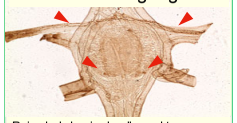


Paired endocrine cells of the third thoracic ganglia

fourth abdominal ganglion

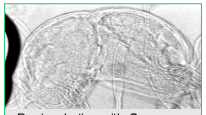


Transverse Nerves of Fourth Abdominal Ganglia



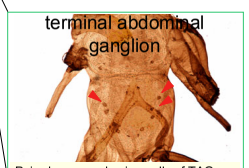
Paired abdominal cells and transverse nerves of the 4th abdominal ganglia

Binding Specificity



Pre-incubation with *C. pagurus* CHH blocks staining with *C. pagurus* CHH antibody in *Manduca sexta* brain.

N=27



Paired neuroendocrine cells of TAG

3/30 preps

	LFBM	AFBM	L5	Wandering
Brain	4/5	8/9	5/13	0/1
SEG	1/9	0/15	1/13	0/2
T1	0/11	0/17	1/14	0/2
T2	0/12	0/15	1/14	0/2
T3	1/11	0/15	0/16	0/2
Ab1	4/7	6/15	3/14	1/2
Aab2	5/7	8/14	7/15	2/2
Ab3	10/11	5/13	7/14	1/2
Ab4	8/9	4/13	7/14	1/2
Ab5	9/12	4/12	6/14	2/2
Ab6	9/12	4/12	6/14	1/2
TAG	4/10	2/10	6/13	0/2

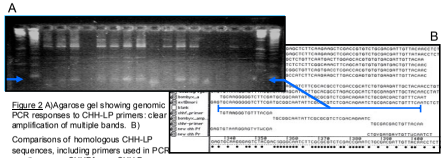
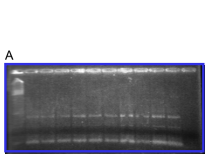
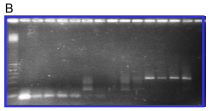
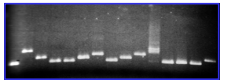


Figure 2 A)Agarose gel showing genomic PCR responses to CHH-LP primers: clear amplification of multiple bands. B) Comparisons of homologous CHH-LP sequences, including primers used in PCR reactions: new CHHPf, new CHH Pr. Arrows point to 65bp band.







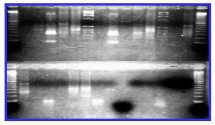
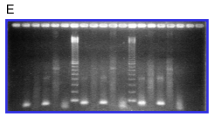
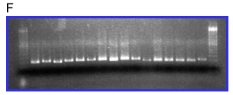


Figure 3: (A) PCR from genomic template used to isolate fragments. (B) Re-amplification of purified fragments. (C) Amplification of clones containing different sized fragments. (D) Nested PCR from cDNA template combination of vector primers and CHH primers. (E) Reamplification of PCR product above, lanes 4,5,9,10,15, with CHH-F/CHH-R primers. (F)Amplification of clones from cDNA PCRs.





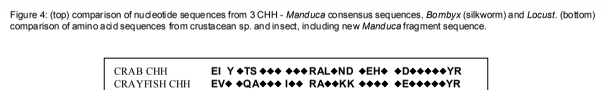


Figure 4: (top) comparison of nucleotide sequences from 3 CHH - *Manduca* consensus sequences, *Bombyx* (silkworm) and *Locust*. (bottom) comparison of amino acid sequences from crustacean sp. and insect, including new *Manduca* fragment sequence.

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