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Molecular cloning and differential expression pattern of two structural variants of the crustacean hyperglycemic hormone family from the mud crab *Scylla olivacea*

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ABSTRACT

Two full-length cDNA sequences encoding a crustacean hyperglycemic hormone (CHH) precursor were cloned from tissues of the mud crab *Scylla olivacea*. *Sco-CHH* (*S. olivacea* CHH) was cloned from eyestalk ganglia, whereas *Sco-CHH-L* (*S. olivacea* CHH-like peptide) was cloned from extra-eyestalk tissues (pericardial organ and thoracic ganglia). Each conceptually translated precursor is expected to be processed into a signal peptide, a CHH precursor-related peptide (CPRP), and a mature CHH or CHH-like peptide. The two precursors are identical in amino acid sequence through the 40th residue of the mature peptide, but different from each other substantially in the C-terminus. Both CHH variants contain the six highly conserved cysteine residues characteristic of the CHH family peptides, and share higher sequence identities with other brachyuran CHH sequences than with those of other taxonomic groups. As determined by reverse transcription-polymerase chain reaction (RT-PCR), the transcripts of *Sco-CHH* and *Sco-CHH-L* were present in eyestalk ganglia and several extra-eyestalk tissues (the thoracic ganglia, pericardial organ, brain, circumesophageal connectives, and gut). *Sco-CHH* was the predominant form in eyestalk ganglia, while *Sco-CHH-L* was the predominant form in several extra-eyestalk tissues. Neither transcript was expressed in the muscle, hepatopancreas, ovary, testis, heart, or gill. Antisera were raised against synthetic peptides corresponding to a stretch of sequence-specific to the C-terminus of Sco-CHH or Sco-CHH-L. Western blot analyses of tissues expressing *Sco-CHH* and *Sco-CHH-L* detected a Sco-CHH immunoreactive protein in the sinus gland, and a Sco-CHH-L immunoreactive protein in the pericardial organ. Immunohistochemical analyses of the eyestalk ganglia localized both Sco-CHH and Sco-CHH-L immunoreactivity to the sinus gland, and only Sco-CHH immunoreactivity to the X-organ somata; analyses of the pericardial organs also localized both Sco-CHH and Sco-CHH-L immunoreactivity to the anterior and posterior bars, as well as to longitudinal trunks joining the two bars. The combined data provided supporting evidence that Sco-CHH and Sco-CHH-L are co-localized in the same tissue.

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1. Introduction

Crustacean hyperglycemic hormone (CHH) is a peptide hormone originally identified in a crustacean neurosecretory complex, the X-organ/sinus gland complex, located within the eyestalks (Keller, 1992; Soyez, 1997). Sequence analysis of CHHs isolated from various decapod crustaceans shows they are peptides of 72–73 amino acid residues with a considerable degree of sequence similarity (Soyez, 1997). On the basis of sequence homology, CHH is placed in the crustacean hyperglycemic hormone (CHH) family, which also includes molt-inhibiting hormone (MIH), vitellogenesis-inhibiting hormone (VIH) or gonad-inhibiting hormone (GIH), and mandibular organ-inhibiting hormone (MOIH), as well as other peptide members found in non-crustacean species (Keller, 1992; Soyez, 1997; Lacombe et al., 1999; Chen et al., 2005).

Molecular characterizations of CHH precursors indicated that the precursor consists of a signal peptide, a CHH precursor-related peptide (CPRP), and a mature CHH peptide. Based on this and other sequence characteristics, it was proposed that CHH peptides be categorized as members of the type I subgroup of the CHH family, whereas MIH, VIH/GIH, and MOIH (the precursors of which lack CPRP) be categorized as members of the type II subgroup of the CHH family (de Kleijn and van Herp, 1995; Lacombe et al., 1999; Chan et al., 2003; Chen et al., 2005).

An intriguing feature of CHH is the presence of multiple molecular variants of the peptide in the X-organ-sinus gland complex (Soyez, 1997; Chan et al., 2003; Chen et al., 2005; Fanjul-Moles, 2006). For example, CHH variants differing from each other in the

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stereo configuration of the third residue (a phenylalanine) were isolated from the sinus glands of various astacideans (Soyez et al., 1994, 1998; Yasuda et al., 1994; Aguilar et al., 1995; Bulau et al., 2003). It was suggested that D-Phe^3-CHH is post-translationally derived from L-Phe^3-CHH (Ollivaux and Soyez, 2000; Soyez et al., 2000). In addition, multiple CHH variants presumably encoded by separate genes were reported for astacideans and penaeids. Thus, there are two CHH variants for *Homarus americanus* and *Cherax destructor* (Tensen et al., 1991; Bulau et al., 2003), five for *Penaeus monodon* (Davey et al., 2000), and six for *P. japonicus* (Yang et al., 1997; Khayat et al., 1998) that are different to varying extent in the primary sequences. Direct evidence showing the presence of multiple CHH genes is provided by studies of *Metapenaeus ensis* and *Carcinus maenas* (Gu and Chan, 1998; Gu et al., 2000; Dircksen et al., 2001).

The presence of CHH-related peptides in the extra-eyestalk tissues was proposed approximately a decade ago (de Kleijn et al., 1995; Chang et al., 1998). Subsequently, it was reported that a CHH identical to that originally found in the X-organ/sinus gland complex is also expressed in the gut of *C. maenas* (Chung et al., 1999). Further, a novel CHH variant was purified from the pericardial organ (PO) of the same species (Dircksen et al., 2001). This novel CHH, dubbed PO-CHH, and the sinus gland CHH (SG-CHH), share an identical N-terminal sequence (residues 1–40), but differ considerably in the remaining sequence; they are likely to be alternatively spliced products (Dircksen et al., 2001). CHH variants showing similar differences in sequence characteristics have recently been reported in *Macrobrachium rosenbergii*, *Pachygrapsus marmoratus*, and *Potamon ibericum* (Chen et al., 2004; Toullec et al., 2006).

CHH from the sinus gland has been implicated in the regulation of carbohydrate metabolism (see Santos and Keller, 1993) and possibly several other physiological processes, including molting, osmoregulation, and reproduction (Chang et al., 1990; Yasuda et al., 1994; Khayat et al., 1998; Spanings-Pierrot et al., 2000; Serrano et al., 2003). Presumed structural variations as might be expected from differences in the residue chirality or primary sequence appear to change quantitatively or qualitatively the biological activity of CHH. Thus, SG-CHH variants express differential potency in hyperglycemic, molt-inhibiting, osmoregulatory, or vitellogenesis-inhibiting activities (Yasuda et al., 1994; Yang et al., 1997; Khayat et al., 1998; Keller et al., 1999; Serrano et al., 2003). Moreover, assays of biological activity showed the CHH-like peptides of *C. maenas* and *M. rosenbergii* (Cam-PO-CHH and Mar-CHH-L, respectively) lack hyperglycemic activity, or molt-inhibiting activity, or both (Dircksen et al., 2001; Ohira et al., 2006).

In the present study, cDNAs encoding two CHH precursors were cloned from tissues of the mud crab, *Scylla olivacea*. *Sco-CHH* (*S. olivacea* CHH) was cloned from eyestalk ganglia; *Sco-CHH-L* (*S. olivacea* CHH-like peptide) was cloned from extra-eyestalk tissues. *Sco-CHH* and *Sco-CHH-L* are widely co-expressed among tissues as determined by RT-PCR, with *Sco-CHH* being predominantly expressed in the eyestalk ganglia and *Sco-CHH-L* in extra-eyestalk tissues. Western blot analyses, using antisera raised against CHH variant-specific synthetic peptides, indicated that Sco-CHH and Sco-CHH-L immunoreactive proteins are present in the sinus gland and pericardial organ, respectively. Co-localization of Sco-CHH and Sco-CHH-L in the eyestalk ganglia and pericardial organs was confirmed by immunostaining analyses.

2. Materials and methods

2.1. Animals

Mature mud crabs (*S. olivacea* Herbst 1796) were purchased from local suppliers, transported to the laboratory, and immediately sacrificed for tissue dissection.

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

Tissues (eyestalk ganglia, thoracic ganglia, and pericardial organ) were dissected from ice-anesthetized animals, and promptly placed in wells containing RNA*later*™ solution (Ambion). Total RNA was extracted from individual tissues using a Purescript® RNA Isolation Kit (Gentra), treated with RQ1 RNase-free DNase (Promega), and reverse transcribed (M 3682, Promega) as described previously (Chen et al., 2007).

PCR contained 0.5 μl of the reverse transcription (RT) reaction, 0.2 mM each of dNTPs, 0.2 μM each of primers, 2.5 U of *Taq* DNA polymerase (Promega), 3 mM $MgCl_2$, and 1× reaction buffer (10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$). The final volume was adjusted to 25 μl with sterile distilled water. PCR was performed in a DNA Thermal Cycler (MJ Research) under the following parameters: an initial denaturation (5 min, 95 °C), 35 cycles of denaturation (1 min, 95 °C), annealing (1 min, 53 °C), and extension (1 min, 72 °C), followed by a final extension (10 min, 72 °C).

For the amplification of eyestalk ganglia-derived cDNA, the primers were sCHHf (5′-GGRCGVATGGAKARGHTWYTGGC-3′) and sCHHr (5′-CATYADDARVAGGTCHTCCATGCA-3′). For the amplification of pericardial organ- or thoracic ganglia-derived samples, the primers were all-Sco-F (5′-CGCAGAGGAAAGACATACCT-3′) and all-Sco-R (5′-GCGCCTATGGTGAATCTTTA-3′, see Fig. 2 for the location of the primers).

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

The 3′- and 5′-cDNA ends of the PCR-amplified fragments were obtained by the rapid amplification of cDNA ends (RACE) method as described by Chen et al. (2007). Poly(A)$^+$ RNA isolated from the tissues (Micro-FastTrack™, Invitrogen) was reversed transcribed using reagents and a protocol provided by the Smart™ RACE cDNA Amplification Kit (Clontech).

Gene-specific primers (GSPs) for RACE reactions were designed based on the sequences of the PCR products generated using methods described in Section 2.2. For amplification of the 5′-cDNA end of the eyestalk ganglia-derived PCR product, the GSP was 5-Sco-R (5′-GCTCTATCGTAAACGCCCTTGCACG-3′), and for amplification of the 3′-cDNA end, the GSP was 3-Sco-F (5′-CAGAA CTTCCCATGTCGCTAGTGGGT-3′). For amplification of the 5′-cDNA end of the pericardial organ- or thoracic ganglia-derived PCR products, the GSP was 5-TPC-R (5′-CCTCCTCCGGATTGTGCAGCAG-3′), and for amplification of the 3′-cDNA end, the GSP was 3-TPC-F (5′-GCTTATGAGAGACGCCATCAGGG-3′).

Complementary DNA samples (0.5 μl) were amplified with one of the above-mentioned GSPs and a universal primer mix (UPM) supplied with the RACE kit. RACE PCRs were carried out in a 25-μl reaction containing the template, 0.2 mM each of dNTPs, 0.2 μM each of the primers, 10 U of Titanium™ *Taq* DNA polymerase (Clontech), and 1× reaction buffer (40 mM Tricine–KOH, pH 8.7, 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, 3.75 μg/ml BSA, 0.005% Tween 20, 0.005% Nonidet-P40). Both 3′- and 5′-RACE PCR was performed under the following conditions: an initial denaturation (5 min, 95 °C), 30 cycles of denaturation (30 s, 95 °C), annealing (30 s, 63 °C), and extension (3 min, 68 °C), followed by a final extension (3 min, 68 °C).

2.4. Gel purification, cloning, sequencing and sequence analysis

After PCR amplification, an aliquot of the reaction was separated on a 1.2% agarose gel and visualized with GelStar® (Cambrex). Reagents and procedures used for purification, cloning, and auto-sequencing of the PCR products, as well as software resources for sequence analyses, were as described by Chen et al. (2007).

2.5. Tissue distribution of Sco-CHH and Sco-CHH-L transcripts

Equal amounts (1 μg) of total RNA, extracted separately from the eyestalk ganglia, pericardial organ, thoracic ganglia, brain, circumesophageal connectives, gut, muscle, heart, gill, hepatopancreas, ovary, and testis, were DNase-treated and reverse transcribed as described above in Section 2.2. PCRs were carried out in a 25-μl reaction containing the template, 0.2 mM each of dNTPs, 0.2 μM each of primers, 2.5 U of *Taq* DNA polymerase (Promega), 3 mM $MgCl_2$, and 1× reaction buffer (10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$). For contamination controls, tissue total RNA or water (instead of cDNA) were used as template in PCR. The primers used were allchh-F (5′-CGGCGCACCCCTTAGAAA AAAGAC-3′) and allchh-R (5′-CCCTTTAACCCTTGCTAAGCTGCC-3′) for amplification of both *Sco-CHH* and *Sco-CHH-L*, SG-CHH-F2 (5′-GTGGGTGCAGATCTAACTGCTACAG-3′) and all-Sco-R (5′-GCG CCTATGGTGAATCTTTA-3′) for amplification of *Sco-CHH* (see Fig. 2), or actin-F (5′-AAGCTTTGCTACATCGCCCTTGAC-3′) and actin-B (5′-CCTTCTGCAAGCGATCAGCAATAC-3′, Accession No. D14612) for amplification of actin.

All PCRs were performed under the following conditions: an initial denaturation (5 min, 95 °C), 30 cycles of denaturation (30 s, 95 °C), annealing (1 min, 55 °C), and extension (1 min, 72 °C), followed by a final extension (7 min, 72 °C).

2.6. Production of polyclonal antibodies

Based on analyses of sequence diversity and predicted antigenicity (Hoop and Woods, 1981), the region of the CHH variants corresponding to residues 59–71 was selected for production of antipeptide antibodies. The 13-mer peptides (MDNFEEIARKIQM in the case of Sco-CHH; HNPEEVLLMRDAI in the case of Sco-CHH-L) were commercially synthesized, and synthetic multiple antigen peptides (MAP) built by conjugating the appropriate peptide to a peptidyl core of eight radially branched lysine residues using the method as described by Posnett et al. (1988) were custom-produced by GlycoNex Inc.

Pre-immune and anti-peptide immune sera were produced in New Zealand white rabbits according to Catty and Raykundalia (1988). Prior to immunization, blood was withdrawn from ear veins for preparation of pre-immune sera. The synthetic MAP conjugate (1 mg) was mixed 1:1 with Freund's complete adjuvant (Sigma) and injected subcutaneously into the rabbit. Two weeks after the first immunization, rabbits were then boost injected twice with a 2-week interval with the MAP mixed with Freund's incomplete adjuvant. Two weeks after the second boost injection, blood was withdrawn, allowed to clot (37 °C for 1 h, then 4 °C overnight), and centrifuged (10,000*g*, 10 min). Blood withdrawn prior to immunization was similarly processed to obtain pre-immune sera. Both pre-immune and immune sera (anti-Sco-CHH and anti-Sco-CHH-L) were aliquoted and stored at −20 °C.

2.7. Tricine–sodium dodecyl sulphate–polyacrylamide gel electrophoresis (Tricine–SDS–PAGE) and Western blotting

Tissues (sinus gland, thoracic ganglia, pericardial organ, brain, circumesophageal connective, and gut) were separately homogenized in 0.1 M HEPES-buffered Pantin's saline (pH 7.6) (Pantin, 1934), then centrifuged at 16,000*g* for 30 min at 4 °C. Supernatants were recovered and separated by Tricine–SDS–PAGE according to Schagger and Von Jagow (1987) on a 16.5% T, 3% C running gel overlaid by a 10% T, 3% C spacer gel and a 4% T, 3% C stacking gel. After electrophoresis, gels were stained with Coomassie blue R 250 (C7900, US Biological). For Western blotting, tissues proteins were electrophoretically separated as described above and then blotted (400 mA, 1 h) onto PVDF membranes (0.45 μm, IPVH00010, Millipore) using an electroblotter (TE 22, Hoefer) and Towbin buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) with 20% methanol (Towbin et al., 1979). After overnight incubation at 4 °C with 2% bovine serum albumin in 0.01 M PBS (as the blocking buffer), the blots were incubated for 2 h with the primary antiserum (anti-Sco-CHH or anti-Sco-CHH-L, 1:500 dilution in blocking buffer), washed three times with washing buffer (0.01 M PBS with 0.1% Tween 20 and 0.02% sodium azide), and incubated for 2 h with a goat anti-rabbit IgG alkaline phosphatase conjugate (1:2000 dilution in blocking buffer). After three washes, blots were developed with BCIP/NBT (B-1911, Sigma). As controls, Western blot analyses were performed with the primary antiserum (anti-Sco-CHH or anti-Sco-CHH-L) being replaced by pre-adsorbed antiserum, which was prepared by diluting each primary antiserum with blocking buffer (1:500 dilution) and incubating at 4 °C overnight with the respective synthetic MAP conjugate (10 or 100 μg/ml diluted antiserum) that was used for antiserum production. Blotting analyses of tissue proteins were also performed using, instead of the primary antiserum, pre-immune serum, which did not detect any immunoreactive protein in the analyzed tissues (data not shown).

2.8. Immunostaining analyses

Eyestalk ganglia and pericardial organs were dissected out from ice-anesthetized animals, fixed in 0.1 M phosphate-buffered saline (PBS, pH 7.2) containing 4% paraformaldehyde (4 °C, overnight). Fixed eyestalk ganglia were cryoprotected in 15% sucrose in 0.1 M PBS (4 °C, overnight), embedded in Tissue-Tek® O.C.T. compound (Sakura Finetechnical Co., Ltd.), and sectioned (10 μm) longitudinally at −20 °C using a Leica CM 1850 cryostat. Tissue sections of eyestalk ganglia were washed with 0.1 M PBS, and incubated at room temperature with blocking buffer (2%BSA, 5% normal goat serum, 0.5% Triton X-100 in 0.01 M PBS) for 1 h, then with anti-Sco-CHH or anti-Sco-CHH-L (1:320 in blocking buffer) for 1 h. After three washes (10 min each) with washing solution (0.05% Tween 20 in 0.01 M PBS), tissue sections were incubated for 1 h with a goat anti-rabbit IgG-FITC conjugated secondary antibody (AP132F, Chemicon) diluted 1:400 in blocking buffer, followed by three washes with washing solution. After three washes, sections were coverslipped (DakoCytomation Fluorescent Mounting Medium). For whole-mount staining of pericardial organs, fixed tissues were washed with 0.1 M PBS (6 h), and similarly stained as described for tissue sections of eyestalk ganglia, with changes in incubation time and temperature for the steps of blocking (4 °C, overnight), primary antiserum incubation (4 °C, 72 h), and secondary antiserum incubation (4 °C, overnight). Extensive washing (room temperature, 6 h) was carried out between successive incubations. Fluorescent images were collected using a Zeiss microscope system (Axio Imager A1).

As controls, the primary antiserum (anti-Sco-CHH or anti-Sco-CHH-L) was replaced by (1) pre-immune serum, or (2) pre-adsorbed antiserum, prepared as described above. No immunoreactivity was detected when pre-immune serum was used instead of the primary antiserum (data not shown).

3. Results

Using a PCR-based cloning strategy (RT-PCR followed by 3′- and 5′-RACE), two full-length cDNAs encoding crustacean hyperglycemic hormone (CHH) precursors were cloned from tissues of the mud crab *S. olivacea*. *Sco-CHH* (*S. olivacea* CHH) was cloned from eyestalk ganglia; *Sco-CHH-L* (*S. olivacea* CHH-like peptide) from extra-eyestalk tissues (pericardial organ and thoracic ganglia) (Fig. 1). The 1665-base pair (bp) *Sco-CHH* contains a 426-bp open reading frame (ORF) that predicts a precursor of 141 residues. The

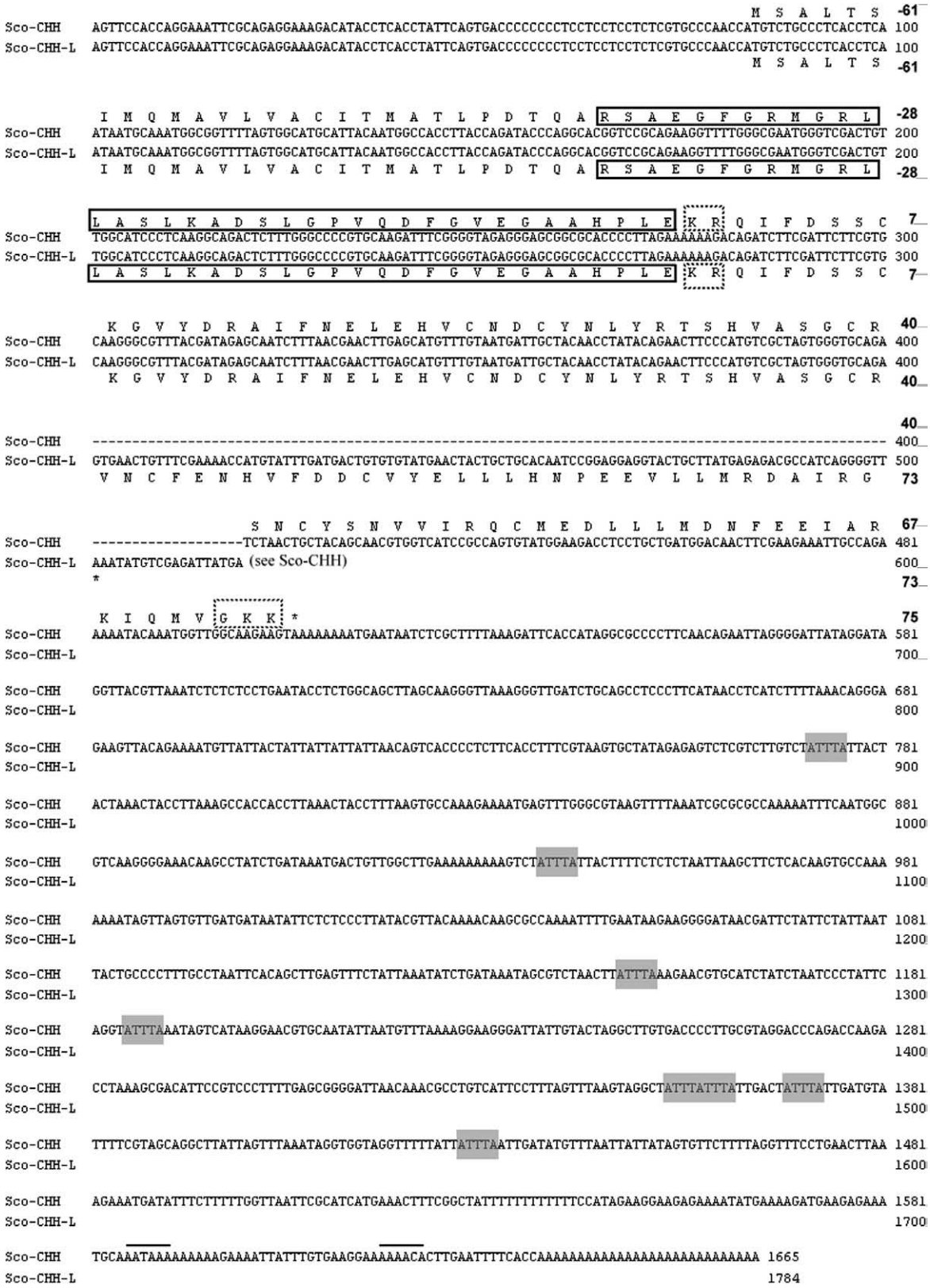

Fig. 1. The cDNA and deduced amino acid sequences of *Sco-CHH* and *Sco-CHH-L*. cDNA sequences are numbered from the first nucleotide. Deduced amino acid sequences are numbered relative to the first residue of the mature CHH peptide, and shown in single-letter code above (*Sco-CHH*) or below (*Sco-CHH-L*) the respective nucleotide sequence. Nucleotide sequence of *Sco-CHH-L* after nucleotide 519 is omitted (see *Sco-CHH*). CHH-precursor-related peptides (CPRP) are boxed by solid lines, which are preceded by signal peptides, and followed by CHH or CHH-like peptides. Putative dibasic processing site (KR) and C-terminal amidation signal sequence (GKK) are enclosed by dashed lines. Stop codons are indicated with asterisk (∗), degradative processing recognition sites grayed, and polyadenylation signals (AATAAA, AAAACA) overlined. Nucleotide sequence data have been submitted to GenBank database under the Accession Nos. AY372181 and EF530127 for *Sco-CHH* and *Sco-CHH-L*, respectively.

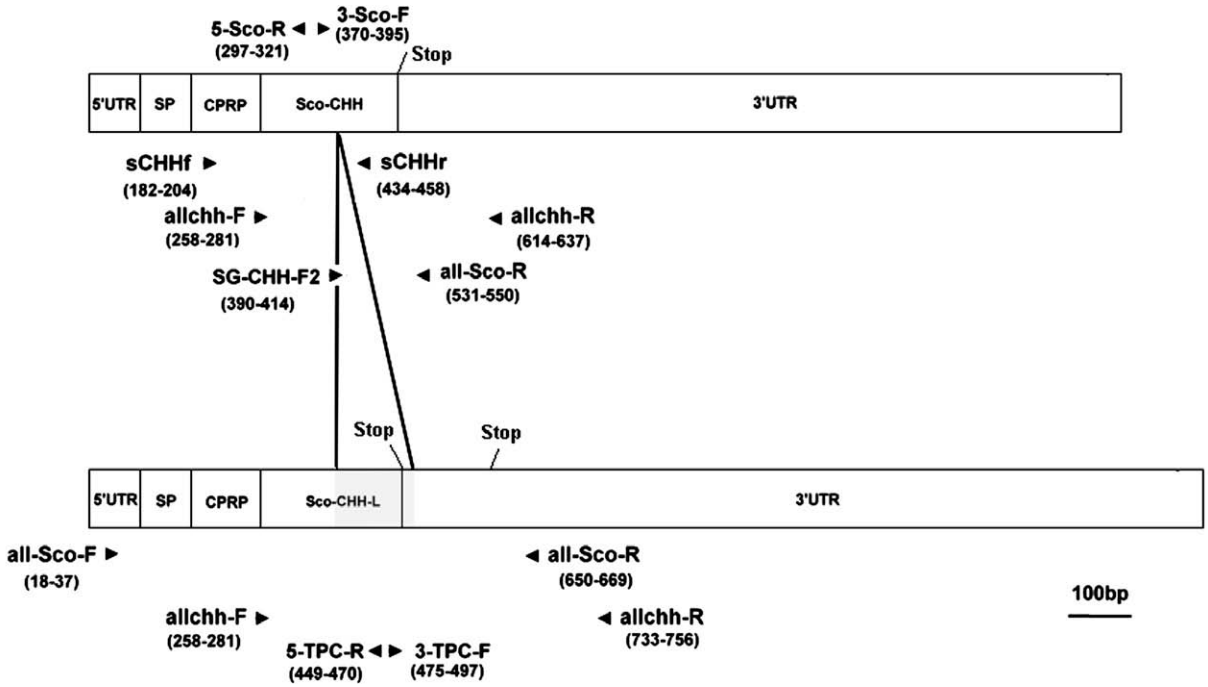

Fig. 2. Schematic presentation of *Sco-CHH* (upper) and *Sco-CHH-L* (lower). Peptides encoded are indicated as SP (signal peptide), CPRP (crustacean hyperglycemic hormone precursor-related peptide), and Sco-CHH or Sco-CHH-L. 5′UTR and 3′UTR: 5′- and 3′-untranslated regions, respectively. Stop: stop codon. The stretch of nucleotide sequence (nucleotides 401–519) unique to *Sco-CHH-L*, but not present in *Sco-CHH*, is grayed. Names and direction (indicated by arrowheads) of the primers used are given. Numbers in the parenthesis indicate the positions of the primers on the corresponding cDNA sequence.

ORF, preceded by a 82-bp 5′-untranslated region (UTR), begins with an initiator methionine at nucleotides 83–85 (ATG), and ends with a TAA stop codon at nucleotides 506–508 that is followed by a 1157-bp 3′-UTR. The 1784-bp *Sco-CHH-L* contains a 420-bp ORF that predicts a precursor of 139 residues. The ORF, preceded by a 82-bp 5′-UTR, begins with an initiator methionine at nucleotides 83–85 (ATG), and ends with a TAA stop codon at nucleotides 500–502 that is followed by a 1282-bp 3′-UTR. *Sco-CHH* and *Sco-CHH-L* are identical except for the presence in *Sco-CHH-L* of a 119 bp stretch (nucleotides 401–519) that is absent from *Sco-CHH* (Figs. 1 and 2).

Sequence analysis predicts the 141-residue Sco-CHH precursor consists of a 27-residue signal peptide (SP), a 37-residue crustacean hyperglycemic hormone precursor-related peptide (CPRP), a dibasic processing site (KR), a 72-residue mature CHH peptide, and a putative C-terminal amidation signal sequence (GKK). The 139-residue Sco-CHH-L precursor consists of a 27-residue SP, a 37-residue CPRP, a dibasic processing site (KR), and a 73-residue mature CHH-like peptide (Fig. 1). The deduced amino acid sequences of Sco-CHH and Sco-CHH-L precursors are identical in SP, CPRP, and the first 40 residues of the mature peptide, but differ in the remainder of the C-terminus (Figs. 1

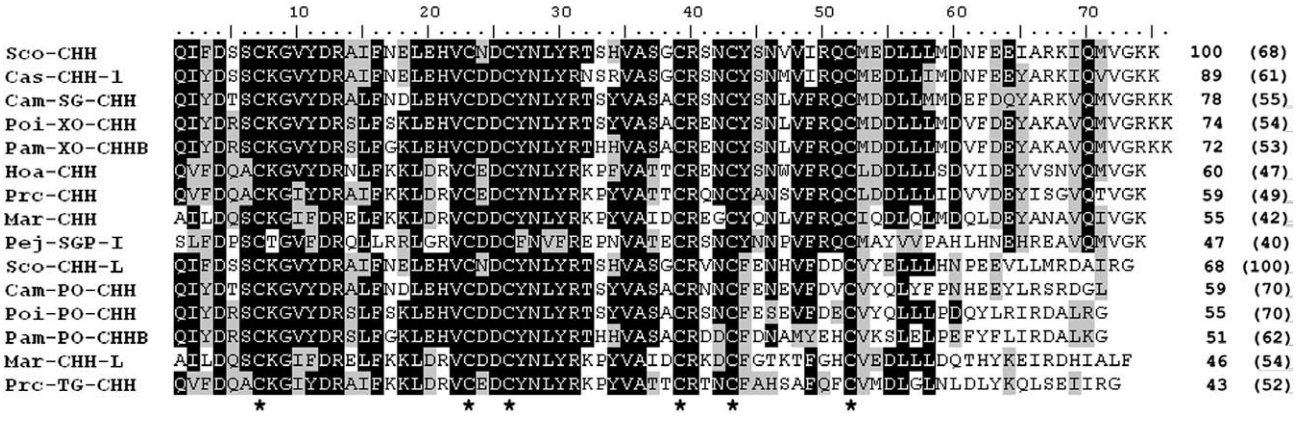

Fig. 3. Multiple alignments of Sco-CHH and Sco-CHH-L with representative CHH-related sequences. Conserved cysteine residues characteristic of CHH family peptides are indicated by an asterisk (∗). Numbers after each sequence represent % identity relative to Sco-CHH (not parenthesized) or Sco-CHH-L (parenthesized). Sequences originally identified in the eyestalks are from the following sources, Sco-CHH: *Scylla olivacea*, AY372181 (present study); Cas-CHH-1: *Callinectes sapidus*, AY536012 (Cheol et al., 2006); Cam-SG-CHH: *Carcinus maenas*, X17596 (Weidemann et al., 1989); Poi-XO-CHH: *Potamon ibericum*, DQ176431 (Toullec et al., 2006); Pam-XO-CHHB: *Pachygrapsus marmoratus*, AY180334 (Toullec et al., 2006); Hoa-CHH: *Homarus americanus*, S76846 (de Kleijn et al., 1995); Prc-CHH: *Procambarus clarkii*, AB027291 (Yasuda et al., 1994); Mar-CHH: *Macrobrachium rosenbergii*, AF219382 (Chen et al., 2004); Pej-SGP-I: *Marsupenaeus japonicus*, AB007507. Those originally identified in extra-eyestalk tissues are from the following sources, Sco-CHH-L: *S. olivacea*, EF530127 (present study); Cam-PO-CHH: *C. maenas*, AF286084 (Dircksen et al., 2001); Poi-PO-CHH: *P. ibericum*, DQ176432 (Toullec et al., 2006); Pam-PO-CHHB: *P. marmoratus*, AY180335 (Toullec et al., 2006); Mar-CHH-L: *M. rosenbergii*, AF372657 (Chen et al., 2004); Prc-TG-CHH: *P. clarkii*, AF474408.

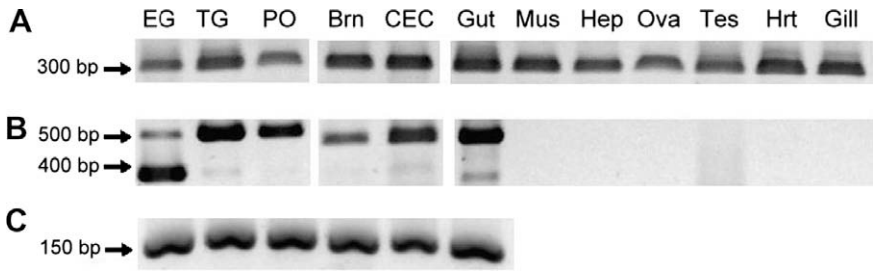

Fig. 4. Tissues distribution of *Sco-CHH* and *Sco-CHH-L* transcripts. Total RNA prepared from the tissues as indicated was reverse transcribed and amplified with primers as specified in Section 2. The expected size for the amplicons was actin, 304 bp (A); *Sco-CHH*, 380 bp; *Sco-CHH-L*, 499 bp (B); *Sco-CHH*, 161 bp (C). Arrows indicate positions of the size markers. EG, eyestalk ganglia; TG, thoracic ganglia; PO, pericardial organ; Brn, brain; CEC, circumesophageal connective; Mus, muscle; Hep, hepatopancreas; Ova, ovary; Tes, testis; and Hrt, heart.

and 2). Differences in the C-terminal sequence are accounted for by the presence (or absence) of the 119-bp stretch of nucleotide sequence mentioned above. This additional stretch of nucleotide sequence (with a stop codon at nucleotides 500–502 of *Sco-CHH-L*) encodes the C-terminal sequence (residues 41–73) of Sco-CHH-L. The corresponding region of Sco-CHH (residues 41–72), whose cDNA sequence lacks the additional 119-bp insert, is encoded by another stretch of sequence (nucleotides 401–505 of *Sco-CHH*) (Figs. 1 and 2).

Alignment of Sco-CHH and Sco-CHH-L with representative CHH sequences is presented in Fig. 3. All six cysteine residues characteristic of CHH family peptides are present in Sco-CHH and Sco-CHH-L. Sco-CHH and Sco-CHH-L share higher identities with brachyuran sequences (Sco-CHH: 51–89%; Sco-CHH-L: 53–70%) than with astacuran, penaeid, and palaemonid sequences (Sco-CHH: 43–60%; Sco-CHH-L: 40–54%) (Fig. 3). When only brachyuran sequences were compared, Sco-CHH shares higher identities with sequences originally identified in the eyestalk ganglia (72–89%) than those in the pericardial organ or thoracic ganglia (51–68%), whereas Sco-CHH-L shares only slightly higher identities with sequences originally identified in the extra-eyestalk tissues (62–70%) than those in the eyestalk ganglia (53–68%) (Fig. 3).

The spatial pattern of *Sco-CHH* and *Sco-CHH-L* gene expression was initially assessed by RT-PCR using a pair of primers capable of amplifying both precursor genes, but yielding an amplicon of 380 bp for *Sco-CHH* and an amplicon of 499 bp for *Sco-CHH-L*. The results indicated that both *Sco-CHH* and *Sco-CHH-L* transcripts are present in eyestalk ganglia, thoracic ganglia, pericardial organ, brain, circumesophageal connectives, and gut, but neither transcript is present in the muscle, hepatopancreas, ovary, testis, heart, or gill (Fig. 4B). The control transcript (actin) was detected in all tissues (Fig. 4A). Although both CHH transcripts are present in each positive tissue, the results suggest the predominantly expressed form in eyestalk ganglia is *Sco-CHH*, whereas the predominant form in other CHH-expressing tissues is *Sco-CHH-L* (Fig. 4B). Because the *Sco-CHH* amplicon in some extra-eyestalk tissues (*i.e.*, pericardial organ, brain, and circumesophageal connective) was barely discernible on gels (Fig. 4B), RT-PCR assays using a *Sco-CHH*-specific primer pair were performed. The results confirmed the expression of *Sco-CHH* in all extra-eyestalk tissues tested (Fig. 4C). Concurrent controls using water or tissue total RNA (instead of cDNA) as templates for PCR produced negative results indicating the reactions were free of contamination (data not shown).

Polyclonal antisera (anti-Sco-CHH and anti-Sco-CHH-L) were raised against sequence-specific synthetic peptides corresponding to a stretch of amino acid sequence (residues 59–71) located at the C-terminus of Sco-CHH or Sco-CHH-L. Western blot analyses of tissue proteins indicated that anti-Sco-CHH recognized a protein band in the homogenate sample of the sinus gland, but not in other tissue samples probed (Fig. 5A), whereas anti-Sco-CHH-L

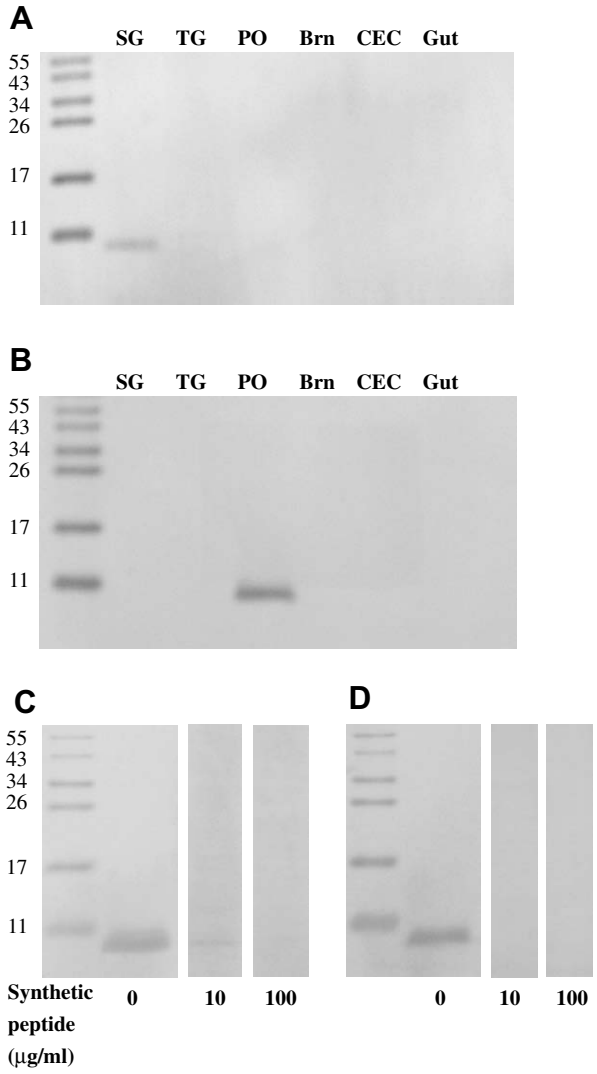

Fig. 5. Western blot analyses of tissue proteins. Tissue proteins (10 μg/lane) were electrophoresed, blotted, and probed as described in Section 2. The primary antiserum used was anti-Sco-CHH (A) or anti-Sco-CHH-L (B). Note that a Sco-CHH immunoreactive protein band was detected in the sinus gland, and a Sco-CHH-L immunoreactive protein band was detected in the pericardial organ. As controls, sinus gland (C) and pericardial organ (D) proteins were electrophoresed, blotted, but probed respectively by anti-Sco-CHH and anti-Sco-CHH-L pre-adsorbed with various amounts of synthetic peptides (0, 10, 100 μg/ml). Numbers are the sizes of the markers (kDa); positions of the immunoreactive proteins are indicated by arrowheads. SG, sinus gland; other abbreviations are the same as those mentioned in Fig. 4.

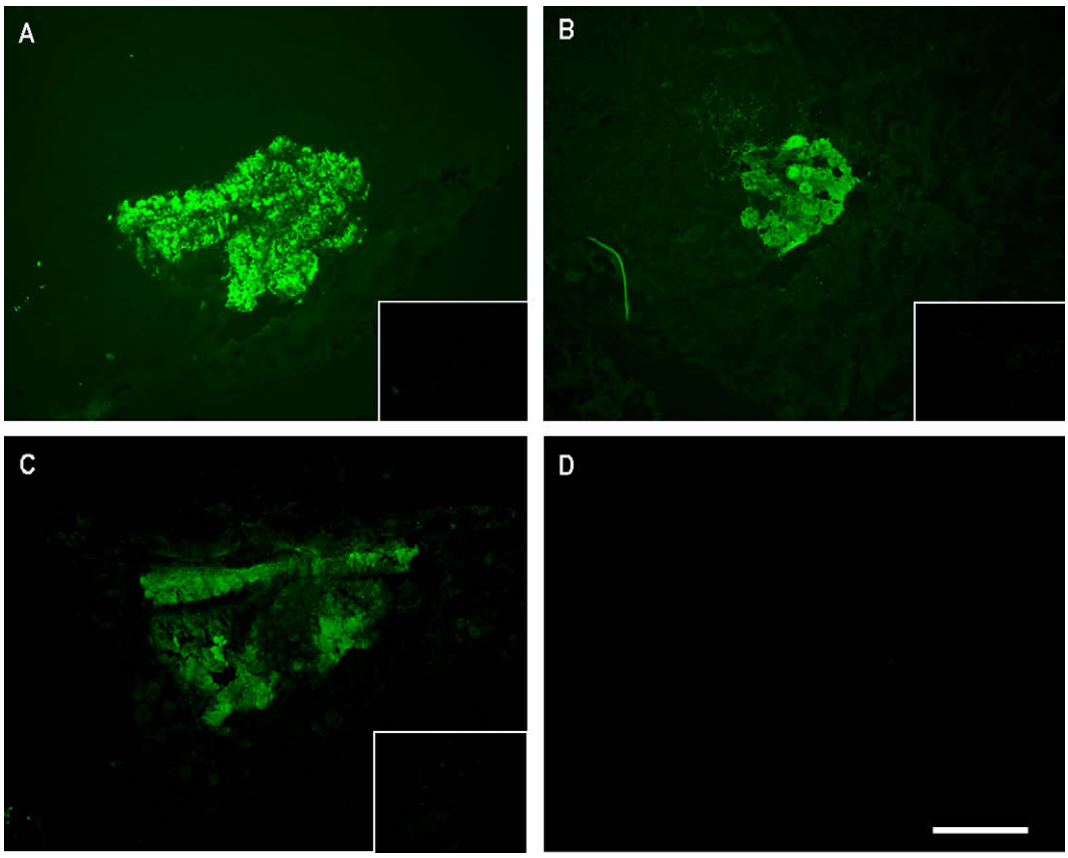

Fig. 6. Immunohistochemical staining of the eyestalk ganglia. Tissue sections were treated as described in Section 2. The primary antiserum used was anti-Sco-CHH (A and B) or anti-Sco-CHH-L (C and D). Note that Sco-CHH (A) and Sco-CHH-L (C) immunoreactivity was detected in the sinus gland; Sco-CHH (B), but not Sco-CHH-L (D), immunoreactivity was detected in the X-organ somata. Insets show a consecutive section probed by antiserum pre-adsorbed with 40 μg/ml of respective synthetic peptide. Scale bar = 200 μm for A–D, and 700 μm for insets.

recognized a protein band in the homogenate sample of the pericardial organ, but not in other tissue samples (Fig. 5B). The blotting analyses were repeated three times, each time with tissue proteins extracted from a different individual crab; the staining pattern shown in Fig. 5A and B was observed in each trial. Further, the intensity of the Sco-CHH immunoreactive band in the sinus gland sample was greatly diminished when probed by anti-Sco-CHH pre-adsorbed with 10 μg/ml of the synthetic peptide that was used for production of anti-Sco-CHH, and completely abolished with 100 μg/ml of synthetic peptide (Fig. 5C). Similarly, the intensity of the Sco-CHH-L immunoreactive band in the pericardial organ sample was completely abolished when probed by anti-Sco-CHH-L pre-adsorbed with 10 or 100 μg/ml of synthetic peptide that was used for production of anti-Sco-CHH-L (Fig. 5D).

Immunostaining analyses using either anti-Sco-CHH or anti-Sco-CHH-L as the primary antibody were carried out on the eyestalk ganglia and pericardial organs. The analyses were repeated three times, each time with tissues prepared from a different individual animal. Immunopositive staining was detected in sinus glands by either anti-Sco-CHH (Fig. 6A) or anti-Sco-CHH-L (Fig. 6C). By contrast, immunopositive staining was detected in X-organ somata by anti-Sco-CHH (Fig. 6B), but was not detected by anti-Sco-CHH-L (Fig. 6D). Sco-CHH immunoreactivity in the sinus gland and the X-organ was abolished when anti-Sco-CHH was pre-adsorbed with 40 μg/ml of synthetic peptide (insets in Fig. 6A and B). Similarly, Sco-CHH-L immunoreactivity in the sinus gland was abolished when anti-Sco-CHH-L was pre-adsorbed with 40 μg/ml synthetic peptide (inset in Fig. 6C). Results of whole-mount immunostaining of the pericardial organs are presented in Fig. 7. Immunopositive somata were predominantly detected in the anterior and posterior bar regions when probed by anti-Sco-CHH-L (Fig. 7A and B) or anti-Sco-CHH (Fig. 7D). Both Sco-CHH-L and Sco-CHH immunoreactivities were also observed in somata and nerve fibers located in longitudinal trunks joining the anterior and posterior bars (Fig. 7C and E). Sco-CHH-L and Sco-CHH immunoreactivities were abolished when the antiserum was pre-adsorbed with 40 μg/ml of respective synthetic peptide (insets in Fig. 7A and D).

4. Discussion

In the present study we report the cloning of two cDNAs, each encoding a CHH precursor, from tissues of the mud crab, *S. olivacea*. *Sco-CHH* (*S. olivacea* CHH) was cloned from eyestalk ganglia, and *Sco-CHH-L* (*S. olivacea* CHH-like peptide) from two extra-eyestalk tissues (pericardial organ and thoracic ganglia). The sequence characteristics of both the precursors and the mature peptides indicate they belong to the type I subgroup of the CHH family of neuropeptides (de Kleijn and van Herp, 1995; Lacombe et al., 1999; Chan et al., 2003; Chen et al., 2005).

The deduced sequence of the Sco-CHH and Sco-CHH-L precursors is identical through the 40th residue of the mature peptide, but differs considerably in the remainder of the C-terminus. The occurrence of similar structural variants among members of the CHH family has also been reported for *C. maenas* (Cam-SG-CHH and Cam-PO-CHH; Dircksen et al., 2001), *M. rosenbergii* (Mar-CHH and Mar-CHH-L; Chen et al., 2004), *P. marmoratus* (Pam-XO-CHHB and Pam-PO-CHHB; Toullec et al., 2006), and *P. ibericum* (Poi-XO-CHH and Poi-PO-CHH; Toullec et al., 2006).

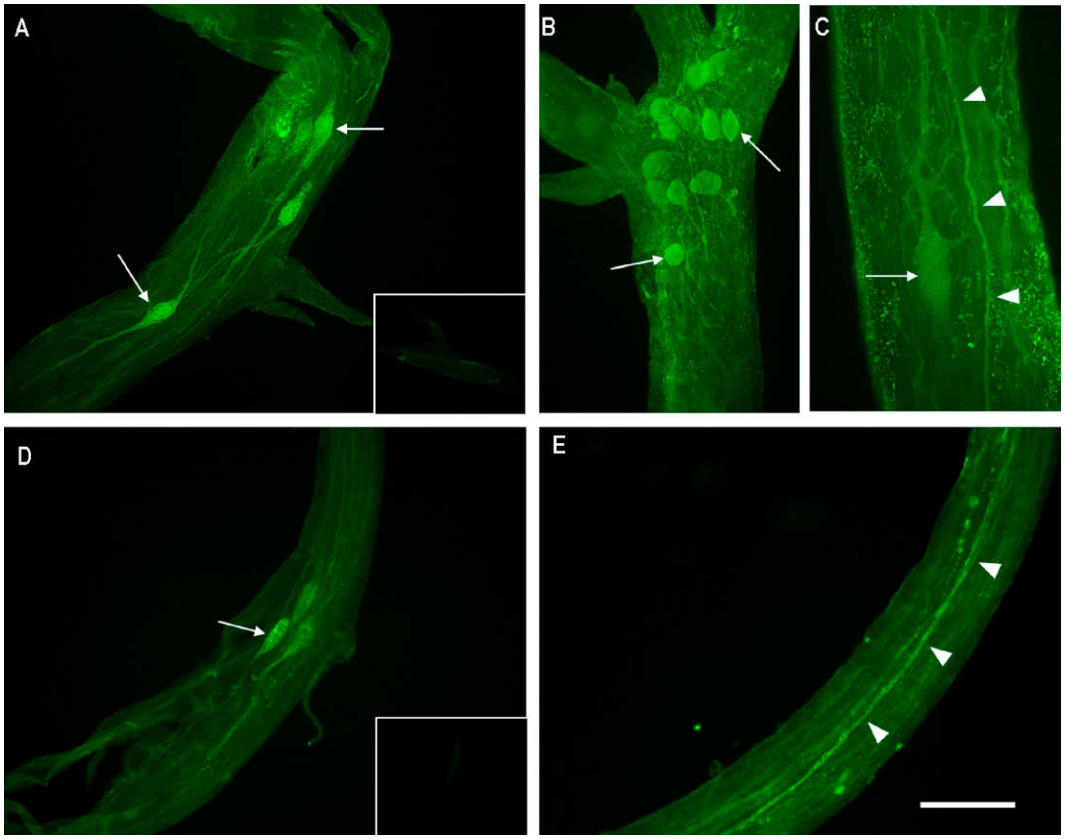

Fig. 7. Whole-mount immunostaining of the pericardial organs. Tissues were treated as described in Section 2. The primary antiserum used was anti-Sco-CHH-L (A–C) or anti-Sco-CHH (D and E). Immunoreactive somata (arrow) were primarily detected in the anterior (A and D) and posterior (B) bars. Longitudinal trunks (C and E) also contained immunoreactive fibers (arrow head) and somata (arrow). Insets show tissues probed by antiserum pre-adsorbed with 40 μg/ml of respective synthetic peptide. Scale bar = 200 μm for A–E, 100 μm for C, and 700 μm for insets.

Structural analyses indicate the CHH genes of *C. maenas* and *M. rosenbergii* are organized in a 4 exon/3 intron manner (Dircksen et al., 2001; Chen et al., 2004). It was suggested that the two CHH structural variants identified in each species are separately encoded by alternatively spliced transcripts (Dircksen et al., 2001; Chen et al., 2004). One of the transcripts, consisting of exon I, II, III, and IV, encodes CHH precursors that were originally identified in extra-eyestalk tissues (*C. maenas*: Cam-PO-CHH precursor; *M. rosenbergii*: Mar-CHH-L precursor), whereas the other, consisting of exon I, II, and IV, encodes CHH precursors originally identified in the eyestalks (*C. maenas*: Cam-SG-CHH precursor; *M. rosenbergii*: Mar-CHH precursor). These two transcript forms give rise to the two CHH precursors that are different from each other in the C-terminus of the mature peptides. Although relevant data are not yet available, it is likely that Sco-CHH and Sco-CHH-L precursors are generated from a similar mechanism of alternative splicing.

Regarding spatial expression of CHH genes, it has been shown in *M. rosenbergii* by RT-PCR assay that *Mar-CHH* is expressed only in the eyestalk ganglia, and *Mar-CHH-L* only in several extra-eyestalk tissues (Chen et al., 2004). This differs from our observation that while *Sco-CHH* is the predominant CHH transcript in eyestalk ganglia and *Sco-CHH-L* the predominant CHH transcript in extra-eyestalk tissues, both are detectable by RT-PCR in all CHH transcript-positive tissues (Fig. 4). Our findings are consistent with previous reports that transcripts of both *Cam-SG-CHH* and *Cam-PO-CHH* are detectable by RT-PCR in tissues. Thus, *Cam-SG-CHH* was also found to be expressed, though at relatively lower levels, in the pericardial organ and gut (Chung et al., 1999; Dircksen et al., 2001), while *Cam-PO-CHH* was found to be expressed in the eyestalk ganglia, also at relatively lower levels (Dircksen et al., 2001).

It has been common practice to name CHH and CHH-related peptides based on the tissue where they were first identified (*e.g.*, PO-CHH, SG-CHH). Given the current and previous results showing co-expression of two CHH transcript forms in a given tissue, we think the tissue-based nomenclature is not ideal. Thus, we prefer the nomenclature used by Chen et al. (2004) to describe CHH isoforms in the giant prawn, *M. rosenbergii* (the CHH peptide was dubbed Mar-CHH and the CHH-like peptide Mar-CHH-L). This same convention has also been used to describe ion transport peptide (ITP) and ITP-like peptide (also CHH family peptides) in the locust, *Schistocerca gregaria* (thus ScgITP and ScgITP-L; Meredith et al., 1996; Ring et al., 1998). Aside from circumventing inaccuracy regarding the tissue source of the peptide, this convention also distinguishes peptides with known biological functions (Mar-CHH, Sco-CHH, Scg-ITP) from those without any established function (Mar-CHH-L, Sco-CHH-L, ScgITP-L) (Ring et al., 1998; Ohira et al., 2006; Tsai et al., unpublished data).

Western blot analyses show that Sco-CHH immunoreactive protein is present in the sinus gland, but not in extra-eyestalk tissues examined. Conversely, Sco-CHH-L immunoreactive protein is detectable in the pericardial organ, but not in the sinus gland or other extra-eyestalk tissues examined. By contrast, the *Sco-CHH* and *Sco-CHH-L* transcripts are widely co-expressed as determined by RT-PCR. The observation that data derived from RT-PCR assays and Western blot analyses are not in total agreement may reflect variation among tissues in the rates of peptide synthesis, degradation, or release. We hypothesize that Sco-CHH and Sco-CHH-L are co-localized in these tissues, but their steady-state levels in many tissues are below the detection limit of our Western blot assay. Interestingly, the *ScgITP-L* transcript was found by RT-PCR to be present

in a wide variety of tissues (Meredith et al., 1996), but ScgITP-L immunoreactive protein was not detectable by Western blot in the same tissues (Macins et al., 1999). A recent study in several insects (*Manduca sexta*, *Bombyx mori*, and *S. americana*) using immunohistochemical and *in situ* hybridization techniques revealed that ITP-L peptide and transcript are indeed widely expressed in the central and peripheral nervous systems (Dai et al., 2007).

Immunostaining analyses provided supporting evidence that Sco-CHH and Sco-CHH-L are co-localized in the eyestalk ganglia (*i.e.*, the sinus gland) and the pericardial organs. Sco-CHH-L immunoreactivity in the sinus gland was, however, significantly lower than Sco-CHH immunoreactivity, which may explain the apparent absence of Sco-CHH-L immunoreactive protein from the sinus gland samples on the blots. We anticipate that comprehensive tissue screening with immunohistochemical methods using specific antisera will provide a more thorough understanding of the spatial expression pattern of Sco-CHH and Sco-CHH-L.

The wide tissue distribution of Sco-CHH and Sco-CHH-L as suggested by the present study raises a question as to the nature of their regulatory functions. Whether the extra-eyestalk Sco-CHH mediates a localized regulation of glucose metabolism or other functions not related to glucose metabolism, as has been previously suggested (Chang, 2001), awaits further investigation. It was suggested that the gut-derived Cam-SG-CHH is involved in regulating ecdysis-associated water uptake (Chung et al., 1999; Webster et al., 2000). No functional data are yet available for any CHH-like peptides, except that Cam-PO-CHH and Mar-CHH-L have no significant activity in inducing hyperglycemia, or suppressing ecdysteroid synthesis, or both (Dircksen et al., 2001; Ohira et al., 2006).

In summary, the present studies yielded two cDNA clones (*Sco-CHH* and *Sco-CHH-L*), from tissues of the mud crab, *S. olivacea*. Each cDNA encodes a CHH precursor. Sco-CHH and Sco-CHH-L precursors are likely derived from a common gene via an alternative splicing mechanism. The combined results suggest that *Sco-CHH* and *Sco-CHH-L* are co-expressed in a variety of tissues, although transcript and peptide levels may differ among tissues. These results provide a foundation for additional studies of the temporal and spatial expression patterns of the two CHH variants. We expect those studies will contribute to an understanding of the functional significance of the structural differences among CHH peptides.

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