

Short Communication

Cloning of an insulin-like androgenic gland factor (IAG) from the blue crab, *Callinectes sapidus*: Implications for eyestalk regulation of IAG expressionJ. Sook Chung^{a,*}, R. Manor^b, A. Sagi^b^a University of Maryland Center for Environmental Science, Institute of Marine and Environmental Technology, 701 East Pratt Street, Columbus Center, Suite 236, Baltimore, MD, United States^b Department of Life Sciences and the National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, Beer-Sheva, Israel

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ABSTRACT

In malacostracan crustaceans, sex differentiation is uniquely regulated by a hormone secreted by the male-specific androgenic gland (AG). An isopod AG hormone was the first to be structurally elucidated and was found to belong to the insulin superfamily of proteins. Recently, it has been found that the AGs of several decapod crustaceans express insulin-like androgenic gland factors (IAGs), whose function is believed to be similar to that of the isopod AG hormone. Here we report the isolation from the blue crab *Callinectes sapidus* of the full-length cDNA encoding a candidate insulin-like AG hormone, termed *Cas-IAG*. The predicted protein *Cas-IAG* was encoded as a precursor consisting of a signal peptide, the B chain, the C peptide, and the A chain in that order. While the AG was the main source of *Cas-IAG* expression, as found in other decapod species, the hepatopancreas of male *Callinectes sapidus* crabs displayed minor *Cas-IAG* expression. Eyestalk ablation confirmed the presence of a possible endocrine axis between the eyestalk ganglia and the AG, implying that *Cas-IAG* expression is negatively regulated by (a) substance(s) present in the eyestalk ganglia.

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1. Introduction

Most crustacean species are gonochoristic and exhibit sex dimorphism whose expression may be hormonally controlled (unlike insects, in which genetic input alone is believed to determine sex differentiation [27]). In malacostracan crustaceans, sex differentiation and secondary sex characteristics are thought to be controlled largely by a hormone secreted by the androgenic gland (AG) [5], an organ unique to this family of animals. The presence of the AG, a male-specific endocrine gland, was first described some 60 years ago in the crab *Callinectes sapidus* [11]. Thereafter, it was also discovered in other crustacean species, including several isopod and decapod species [5,38].

Studies showing the AG to be the key regulator of male sex differentiation were based largely on AG manipulations, including AG implantation and injection of AG extracts into females and AG removal from males. AG implantation and injection of AG extracts in female isopod and amphipod species caused masculinization [1,6,17,20,40] and in decapod females led to the inhibition of vitellogenesis, the development of secondary male sex characteristics and the regression of ovarian development [12,23,26,31,32]. In

contrast, AG removal led to feminization in amphipods and decapods [6,32,37]. The data from the above studies suggested the presence of a putative AG factor and gonad plasticity, even in mature crustaceans. Although the AG factor was determined to be proteinaceous in nature [18,25], studies conducted over several decades with the aim of isolating and characterizing this elusive factor have, to date, revealed very little information, as is reviewed briefly below.

The primary amino acid sequence with three disulfide bridges and the full-length cDNA of the first AG hormone (AGH) to be described were characterized from the isopod *Armadillidium vulgare* [30,36]. This AGH was shown to be a heterodimeric glycoprotein, linked by three disulfide bridges – two between the B and A chains and one in the A chain – which shared structural similarity with vertebrate insulin [30,35]. A few years later, using suppression subtractive hybridization (SSH), our laboratory identified two AG-specifically expressed genes, which were found to encode insulin-like androgenic gland factors (IAGs), one in *Cherax quadricarinatus* [29] and the other in *Macrobrachium rosenbergii* [42], designated *Cq-IAG* and *Mr-IAG*, respectively. Thereafter, several other genes that encode IAGs in decapods were identified in *Portunus pelagicus* (*Pp-IAG*; [39]), *Penaeus monodon* (*Pem-IAG*; GU208677), and *Cherax destructor* (*Cd-IAG*; EU718788).

As is the case of other peripheral glands in crustaceans, the AG is thought to be negatively regulated by the X-organ sinus gland (XO-SG) complex residing in the eyestalk. It is believed that an

Abbreviations: IAG, insulin-like androgenic gland factors; AG, androgenic gland; AGH, AG hormone.

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endocrine interaction between the eyestalk ganglia and the AG controls male reproduction [19,24]. The activity of the AG is generally known to be down-regulated by a substance secreted from the eyestalk ganglia, as eyestalk ablation caused the hypertrophy of the AG and stimulation of spermatogenesis [24]. However, the hormonal status of IAG or AGH, i.e., its concentration in hemolymph in relation to male sex maturity, has not yet been confirmed in any crustacean species.

The blue crab *Callinectes sapidus* displays clear sexual dimorphism: the abdomen is semi-circular in adult females and T-shaped in males [43], and the chelae are orange-red in females and blueish in males [10]. Despite extensive progress in the understanding of endocrine regulation of vitellogenesis in female crustaceans, including *Callinectes sapidus* [46–48], much less is known about the reproductive physiology of male *Callinectes sapidus*, despite the fact that the AG was first discovered in this species six decades ago.

The objectives of this study were thus to isolate the full-length cDNA of *Cas-IAG* from adult male *Callinectes sapidus* by using homology-based cloning and to examine a putative endocrine axis between the eyestalk ganglia and the AG. First, we isolated the full-length cDNA of the insulin-like androgenic gland factor of *Callinectes sapidus*, designated *Cas-IAG*, by using molecular cloning in combination with degenerate PCR and 5' and 3' rapid amplification of cDNA ends (RACE). We also employed a qRT-PCR assay to determine the regulatory role of the eyestalk ganglia on the AG. To this end, we measured the regulatory effect of the eyestalk on the expression levels of *Cas-IAG*.

2. Materials and methods

2.1. Animals

Juvenile males were obtained from the blue crab hatchery [Institute of Marine and Environmental Technology (IMET), Maryland] and reared to adulthood under the same conditions as those described previously [21]. Adult male *Callinectes sapidus* at intermolt (carapace width 120–140 mm) were bilaterally eyestalk-ablated three and seven days prior to the AG collection. On days 3 and 7, intact and ablated animals were anesthetized on ice for 10 min before being dissected.

2.2. cDNA synthesis for 5' and 3' rapid amplification of cDNA ends (RACE)

Total RNA from different tissues was extracted using TRIzol[®] Reagent (Invitrogen) and quantified with a NanoDrop spectrometer (Thermo Scientific). Total RNA from androgenic glands (1–3 µg) treated with DNase I (Fermentas) was subjected to cDNA synthesis [7]. The cloning of cDNA *Cas-IAG*, 5' and 3' RACE cDNA were performed using a SMART cDNA Amplification kit (BD Bioscience) according to the manufacturer's protocol.

2.3. PCR with degenerate primers

The degenerate primers listed in Table 1 were produced (IDT Technology) based on the conserved amino acids found from the multiple alignment Clustal W (www.genome.ad.jp) of deduced amino acid sequences of IAGs of *Cherax quadricarinatus* (GenBank DQ851163) and *Macrobrachium rosenbergii* (GenBank FJ409645). The first touch-down PCR was carried out using the dF1 primer (Table 1) and universal primer (BD Biosciences) under the following PCR conditions: 94 °C, 2.5 min; 8 cycles at 94 °C, 30 s, annealing temperature decreasing 1 °C/cycle from 47 to 40 °C, 30 s, 72 °C,

Table 1

Primer sequences for isolating the full length cDNA of *Cas-IAG* from the AG of *Callinectes sapidus*.

	Primer sequences (5', 3')
dF1	GAYTTYGAYTYGGNSAYYT
dR2	ACTGCGGCSACMTSGSCGACA
Cas-IAG-3F1	ATCCTTCTCCTCCGTCTGCCTTAC
Cas-IAG-3F2(=QF)	CAGATACAATGGGAGTGACTTCCTTTGA
Cas-IAG-5R1	GTTACAGTATCGGGAGGATCTGC
Cas-IAG-5R2	CCTTCTCCTGCCACTGAGTCTTG
Cas-IAG-5R3(=QR)	CTTGAAGAGTTGGAAGCGCTTTCT

'd' represents degenerate primers. Forward primers of Cas-IAG-3F1 and 3F2 were used for 3' RACE and Cas-IAG-5R1, 2, and 3 were used for 5' RACE. Cas-IAG-3F2 and 5R3 primers were used for qRT-PCR analysis.

1.5 min, 25 cycles at 94 °C, 30 s, 48 °C, 30 s, 72 °C, 1.5 min and the final extension at 72 °C for 7 min.

The first PCR product served as a template for a semi-nested PCR with a combination of dF1 and dR2 (Table 1) at the PCR conditions of 94 °C, 2.5 min; 40 cycles at 94 °C, 30 s, 46 °C, 30 s, 72 °C, 30 s and the final extension at 72 °C for 7 min. The semi-nested PCR products were analyzed on a 1.8% agarose gel. The band with the expected size of ~280 bp was excised for cloning. The remainder of the cloning and sequencing procedures were the same as those described previously [9]. Based on the initial sequence of *Cas-IAG* obtained, gene-specific primers (Table 1) were generated for characterizing the full-length cDNA encoding *Cas-IAG*.

2.4. 5' and 3' RACEs of *Cas-IAG*

The first touch-down PCR for 5' and 3' RACE was carried out as above except for the primers used – 5' RACE with the 5R1 primer and 3' RACE with 3F1 primer (Table 1) and the initial annealing temperature starting from 57 to 50 °C for 8 cycles and 27 cycles at 58 °C. One microliter of the first touch-down PCR reaction was amplified with NUP (BD Biosciences) and 5R2 primer for 5' RACE and 3F2 primer (Table 1) for 3' RACE. Bands were excised for cloning into a pGEM[®]-T Easy vector (Promega) and sequencing as above.

2.5. Spatial distribution of *Cas-IAG* expression in an adult male *Callinectes sapidus*

Tissues were collected from an adult male crab. Total RNAs were treated with DNase I (Fermentas) prior to cDNA synthesis using MMLV reverse transcriptase and random hexamers (Fermentas). The expression pattern of *Cas-IAG* was determined using 25 ng of total RNA equivalent to each cDNA of these tissues, with *Cas-IAG-3F1* and *Cas-IAG-5R1* (Table 1). Amplification of the *arginine kinase* gene (*AK*) was carried out as a reference gene [9]. PCR conditions were similar to those stated above except for annealing at 58 °C for 30 s and 33 cycles.

2.6. Effect of eyestalk ablation on *Cas-IAG* expression in androgenic glands

Adult males at the intermolt stage were subjected to bilateral eyestalk ablation three and seven days prior to AG collection with the aim to examine whether a neuropeptide(s) present in the eyestalk ganglia have an effect on the level of *Cas-IAG* expression. The controls collected on days 3 and 7 were pooled, since there was no difference between the two. The tissues were processed for total RNA extraction and cDNA synthesis. The expression level of *Cas-IAG* was determined using qRT-PCR analysis (Applied Biosystems 7500). Each sample (20 ng of total RNA equivalent of cDNA) was assayed in a duplicate using Power SYBR Green (Applied

Biosystems) with the following primers: Cas-IAG-3F2(=QF) and Cas-IAG-5R2(=QR) (Table 1). The data were calculated into number of copies/ μ g total RNA.

2.7. Statistical analysis

The data obtained from qRT-PCR analysis were analyzed for statistical significance using Graph-Pad InStat (GraphPad Software Inc.). The significance at $P < 0.05$ was analyzed using one-way ANOVA and Tukey–Kramer multiple comparisons tests.

3. Results

3.1. Molecular cloning of the full-length cDNA encoding the putative *Callinectes sapidus* Cas-IAG, multiple sequence alignment of IAGs of several crustacean species, and phylogenetic analysis

The full-length cDNA (1126 bp) of *Cas-IAG* (GenBank HM594946) was isolated from an AG by using PCR with degenerate primers, followed by 5' and 3' RACE. It was found that *Cas-IAG* consists of a putative coding region (462 bp) is flanked by a short 5' UTR (128 bp) and a long 3' UTR (536 bp) containing a putative polyadenylation site (AATAAA, underlined in Fig. 1).

The open reading frame (ORF) of *Cas-IAG* cDNA was predicted by the ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The signal peptide of 19 amino acids (aa), MCLRVLILVLTATQTKA (italicized; Fig. 1), was predicted by SignalP (<http://www.cbs.dtu.dk/services/SignalP/>) with a P -value of 0.999. The two putative cleavage sites, RYKR and RFRR, which flanked the C peptide, were joined to the B and A chains, respectively.

The predicted ORF of *Cas-IAG* encodes a preprohormone in the order of a signal peptide, the B chain, the C peptide, and the A chain and a schematic diagram of *Cas-proIAG* is shown in Fig. 2A. The B and A chains of *Cas-IAG* were configured by two putative inter-chain disulfide bridges formed between the Cys₁₀ and Cys₂₁ residues located in the B chain and Cys₁₂ and Cys₂₈ located in the A chain, respectively. Two Cys residues, Cys₁₁ and Cys₁₉, located in A chain, form an intrachain disulfide bridge.

Multiple sequence alignment of three isopod AGHs and six decapod IAGs are shown in Fig. 2B. The positions of only nine amino acids, including six cysteine residues, were conserved in all crustacean species: two cysteines in the B chain (marked with a dotted line), one R residue at the cleavage site, and six residues in the A chain (marked with a solid line). A cladogram, generated by using neighbor-joining methods, separated decapod IAGs from isopod AGHs (Fig. 2C). *Cas-IAG* was subgrouped with Mr-IAG, while the other four decapod IAGs belonged to a separate subgroup.

3.2. *Cas-IAG* tissue specificity in *Callinectes sapidus* adult males

The expression of *Cas-IAG* examined by an end point RT-PCR revealed that the AG was the major site of *IAG* expression (Fig. 3, lane no. 10). In *Callinectes sapidus*, however, the hepatopancreas of male animals also expressed *IAG*, although the level was low, as indicated by a very faint band. The sequence of hepatopancreas *IAG* was confirmed to be identical to that of the *IAG* of the AG (seq. data not shown). Genomic DNA contamination in the hepatopancreas was ruled out because no *IAG* amplification was observed when testing RNAs of AG and hepatopancreas as templates (lanes 11 and 12, respectively).

>*CsIAG* (HM594946)

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1  ACGACACTCCTTCTGCCGCTACGCCCTTCTCCACCGCCTTCTCCTTCCCTAGTACCAG
2  CAGCTACTTTTCGTGTATACTACGTGTTCCCTTCGCCTCCCTCCCAACCAGCCTGCCTTCA
122 CCAAGAATGTTGTCTCCGCGTGATCTTAATCCTGGTGCTGGTGACGGCAACGCAGACTAAG
      M C L R V I L I L V L V T A T Q T K
182 GCAGATCTCATCAGCGACTTCTCCGTGGACTGTGGTAACCTGCTAAGATCCTTCTCCTCC
      A D L I S D F S V D C G N L L R S F S S
242 GTCTGCCTTACTTACAAACAACCTCTCAGCGAAAAGATATAAAACGAGGGACAGATACAATG
      V C L T Y K Q P L S E R Y K R G T D T M
302 GGAGTGACTTCCCTTTGACGACGCTAACACTGACTTCCGTCCCAGCCTTTCACGCTCTC
      G V T S F D A N T D F R P Q P L H A L
362 TCCGTGGAACAAGGCGAAGACCCAATGCTGCCCCAGAAAACGCCTTCCAACCTTCAAG
      S V E Q G E D P M L P P E N A F Q L F K
422 ACTCAGTGGGCAGGAGGAAGGTTCCGCAGATCCTCCCGATACGTGAATGGTTATGACGAG
      T Q W A G G R F R R S S R Y V N G Y D E
482 TGCTGCCACAGTCCACCAAGAGCTGCTCTGTGTATGAGGTGGCCGAGTACTGCGACACT
      C C P Q S T K S C S V Y E V A E Y C D T
542 CTCAGGCCCCCGTATAGGGAACCTCTTGCCTCCCGAAATAGTCAGTAAAGGAAGAGGAA
      L R A P P Y R E L L A S R N S Q *
602 GACGAAGAGGAGGAGAAGGAGGAGGAGGAGGAGAAGAGGACGCTAACCATTTGGTACT
662 TTAAAAAAC TCAAATTTCTCAGTTCAAGTTCTCAACTCATCGACGCTCAAATTTCCCGT
722 CGGATTTTCTTCCAAATATGGTATTAATGGAATGCGTTTTACAAAATTAGCATCACATCC
782 TTATTTTTTTCTTCCGGTGGGGATGAAAAAACAACAACGAAAAACAAC TACAAAACCTTA
842 ACATTTCAACCCCTTTCTTCCCTGTAGAAGATATTACTAGGAGATTTTTGTTTCCAAAAG
902 TCATACATTAACCCCTCACTTGTTTAATCTTTCCTGACACCAAAAATAGAAAATTTCCA
962 AAAAGTTATTGCTTCAAATTTGACAATGTTTATTATATACACTTTTAAAGGTTATTAA
1022 AAGTTTACGTTGACAAATCAAGGCTTCTTAAGTATCATTTTGTATTTCTTCCATGTCCTT
1082 CCCTAATAAAAAATAGCAACTAGAAAAAAAAAAAAAAAAAAAAA

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Fig. 1. Sequences of the full-length cDNA and deduced amino acids of a prepro*Cas-IAG*. The start of the ORF (ATG) is shown in bold, and a stop codon (TAA) is shown in bold and marked with '*'. The signal peptide is shown in bold italics. The amino acid sequence of the C peptide is flanked between the B and A chains underlined in bold. The predicted cleavage sites are marked with squares. A putative polyadenylation site (AATAAA) is underlined.

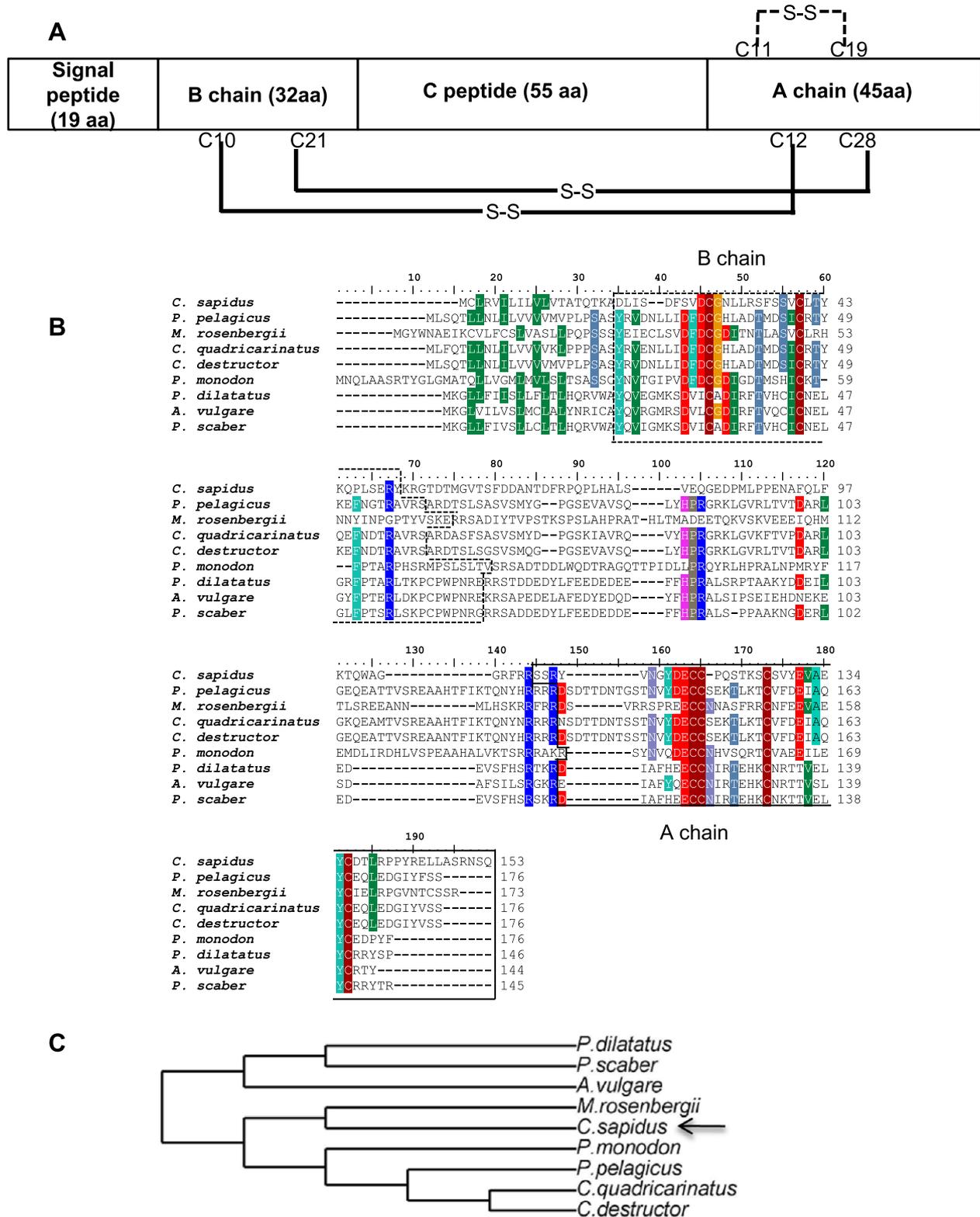


Fig. 2. (A) Schematic diagram of proCas-IAG containing a signal peptide, B chain, C chain, and A chain. Two interdisulfide bridges are connected with solid lines; the interdisulfide bridge is shown with a dotted line. (B) Multiple sequence alignment of deduced primary amino acid sequences of ORFs of decapod IAGs and isopod AGHs was generated using Clustal W [16]: *Callinectes sapidus*: HM594945; *Cherax quadricarinatus*: DQ851163; *Cherax destructor*: EU718788; *Macrobrachium rosenbergii*: FJ409645; *Portunus pelagicus*: HM459854; *Penaeus monodon*: GU208677; *Armadillidium vulgare*: AB029615; *Porcellio dilatatus*: AB089811; *Porcellio scaber*: AB089810. Conserved amino acids are indicated in different in colors. B and A chains are marked with dotted and solid lines, respectively. Amino acid numbers are listed on the right side of the alignment. (C) A cladogram of AGHs and IAGs, constructed by using neighbor-joining methods with the sequence of AGHs of three isopod species and the IAGs of six decapod species and was presented as a cladogram [13] (<http://www.phylogeny.fr>).

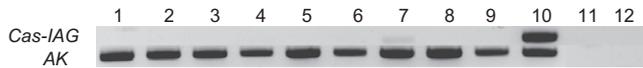


Fig. 3. Spatial distribution of *Cas-IAG* expression in the tissues of an adult male *Callinectes sapidus* crab. Tissue cDNA containing 25 ng of total RNA equivalent was amplified with *Cas-IAG*-3F1 and-5R1 (listed in Table 1). *Arginine kinase* (*AK*) expression as a reference gene is shown in the same tissues. Lanes: 1: brain; 2: eyestalk; 3: thoracic ganglion; 4: pericardial organ; 5: mandibular organ; 6: Y-organ; 7: hepatopancreas; 8: antennal gland; 9: testis; 10: androgenic gland; 11 and 12: RT-controls of AG and hepatopancreas, respectively.

3.3. Effect of eyestalk ablation on *Cas-IAG* expression in the AG and hepatopancreas

Using the qRT-PCR assay, we examined the possible involvement of eyestalk neuropeptides in *Cas-IAG* expression in a time-course eyestalk ablation experiment. The expression of *Cas-IAG* in the AG responded to eyestalk removal in a time-course-dependent manner (Fig. 4). While on day 3 after ablation, the level of *Cas-IAG* expression ($10.5 \pm 1.9 \times 10^7$ copies/ μg total RNA, $n = 6$) was similar to that of intact animals ($8.9 \pm 0.3 \times 10^7$ copies/ μg total RNA, $n = 8$), but with slight hypertrophy of the AG, on day 7 significant

hypertrophy of the AG was observed. The total amount of RNA/AG on day 7 was higher than that on day 3 and that in intact animals, a finding that could be attributed solely to elevation in *Cas-IAG* levels: *Cas-IAG* expression was significantly elevated ~six fold ($56.8 \pm 12.9 \times 10^7$ copies/ μg total RNA of AG, $n = 8$), while *AK* expression in the same AG samples remained the same in all three groups (intact; $3.9 \pm 0.5 \times 10^5$; three days after ablation: $4.1 \pm 0.9 \times 10^5$; and seven days after ablation: $5.0 \pm 1.2 \times 10^5$ copies/ μg total RNA).

Similar to the result shown in Fig. 3, the level of *IAG* expression in hepatopancreas *IAG* (*Cas-IAG*_{hep}) of intact animals was ~0.2% of that of the AG: $1.9 \pm 0.5 \times 10^4$ copies/ μg total RNA ($n = 8$). *Cas-IAG*_{hep} expression, as shown in Fig. 4B, responded to eyestalk ablation but showed a modest but significant increase by 50% only on day 7 after ablation ($3.1 \pm 0.4 \times 10^4$ copies/ μg total RNA, $n = 8$, $P < 0.05$), while the level remained similar to the control at day 3 ($1.45 \pm 0.14 \times 10^4$ copies/ μg total RNA, $n = 6$). The expression level of *Cas-AK*_{hep}, like that of *Cas-AK*_{ag}, did not change significantly in comparison with intact animals (intact: $1.7 \pm 0.6 \times 10^6$; on day 3 after ablation: $1.2 \pm 0.4 \times 10^6$; on day 7 after ablation: $2.4 \pm 0.8 \times 10^6$ copies/ μg total RNA).

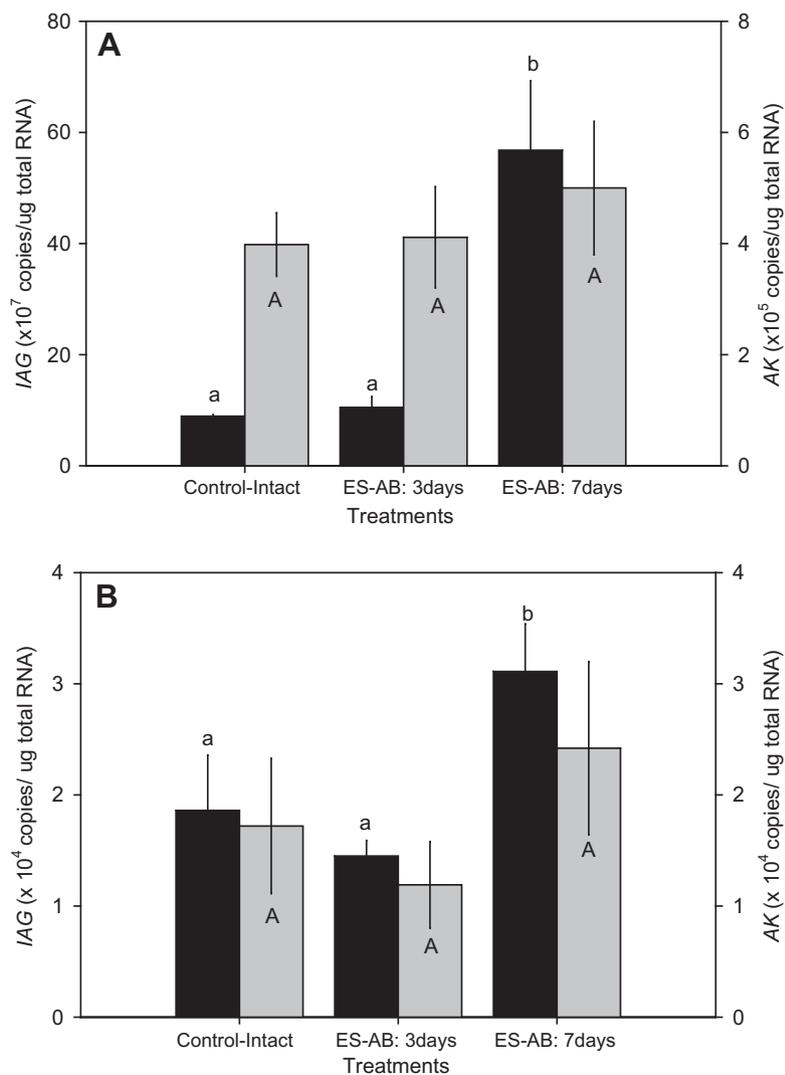


Fig. 4. Effect of eyestalk ablation on *Cas-IAG* expression in the AG (A) and the hepatopancreas (B). Significance at $P < 0.05$ was analyzed using one way ANOVA and Tukey–Kramer multiple comparison tests, significant differences are indicated by different lower case letters for *Cas-IAG* expression (black bars) and capital letters for *AK* expression (gray bars). Data are means \pm SE ($n = 6–8$).

4. Discussion

In the present study, we characterized an insulin-like gene – *Cas-IAG* – from the androgenic gland of the blue crab *Callinectes sapidus*. The expression of *Cas-IAG* found in the AG and hepatopancreas was up-regulated after eyestalk ablation. This finding is in accordance with those of previous studies in decapod crustaceans, which suggested that the eyestalk AG is part of an endocrine axis [24].

Cas-IAG cDNA (1126 bp) is shorter than *Cq-IAG* (1444 bp), [29] and *Mr-IAG* (1824 bp) [42], but longer than *Pp-IAG* (923 bp) [39]. The predicted ORF of *Cas-IAG* was similar to that of other IAGs found in malacostracan crustaceans [29,33,36,39,42]. Two cleavage patterns (RXKR/RXRR) in *Cas-IAG* appear to be similar to the patterns in other decapods (Fig. 1) and isopods [30,33,35]. In contrast to other decapod IAGs, the 3' UTRs of *Cq-IAG* and *Mr-IAG* contain tandem repeats that may play an important role in the regulation of translation of IAG [29,42]. It is not clear how the transcription or translation of IAG is regulated in any of the above decapod species, but it is reasonable to suggest that such regulation may be species-specific in light of the low sequence similarity of the different IAGs together with the different lengths of the UTRs.

Crustacean AGHs and IAGs both form functional heterodimers. Decapod IAGs consist of A and B chains of similar size, i.e., 31–46 aa and 36–45 aa, respectively. In contrast, in isopod AGHs, the B chain (46 aa) tends to be larger than the A chain (29–31 aa). Overall, putative decapod IAGs and isopod AGHs are larger than vertebrate insulins (52 aa). In vertebrate insulins, the B chain (31 aa) is larger than the A chain (21 aa), as in isopod AG hormones. The positions of the six cysteine (C) residues, as shown in Fig. 2B, are conserved in all crustaceans [29,33,36,39,42]. Based on the disulfide assignments reported in isopod AGH [30,35], there are two interdisulfide bridges between the B and A chains and one intradisulfide bridge in the A chain (Fig. 2A).

Overall, in contrast to the high sequence homology among other crustacean hyperglycemic hormone neuropeptide family members [3,4], the similarity among mature IAGs characterized in decapod crustaceans was found to be lower than 25%. Fig. 2B shows nine amino acid residues that are conserved in the entire coding region of crustacean AGHs and IAGs. The majority of these conserved amino acid residues is located in the A chain, suggesting that the B chain is likely to be more species-specific.

Of interest is the low sequence identity of ~28% between the IAGs of *Callinectes sapidus* and *Portunus pelagicus*, despite the fact that both species belong to the Portunidae family. In contrast, the isopod AGH obtained from three different species belonging to two different families (Armadillidae and Porcellionidae) share >70% sequence identity [33,36]. Of note is the high sequence identity of 96.6% between Cd-IAG (Parastacidae) and Pp-IAG (Portunidae), whereas, Cq-IAG and Cd-IAG, which are from the same genus, surprisingly share a lower sequence identity of 86.9%.

In *Cherax quadricarinatus* and *Macrobrachium rosenbergii*, the IAGs were found to be expressed exclusively in the AG [28,42], while *Cas-IAG* was found to be expressed also in the hepatopancreas as well as the AG. This is the first report of IAG or IAG-like expression to be found in a tissue other than the AG in crustaceans. The function and significance of IAG hepatopancreatic expression is not known. However, in other species it is not unusual to find an insulin-like growth factor (IGF) in more than one tissue, as is often the case in vertebrates and insects in which insulin or IGF may be found in various tissues [2,44].

The specific elevation of IAG expression following eyestalk removal may be due to an increase in cell numbers, as was recently reported in studies involving *Portunus pelagicus*, in which only bilateral (vs. unilateral) eyestalk ablation led to increases in the

numbers of particular types of cell [39]. Although IAG expression in the hepatopancreas was only 0.2% of that in the AG, this expression level may, in fact, be equivalent to that of the AG, since the size of the AG (~30 mg) is 0.2% of that of hepatopancreas (~15 g).

Cas-IAGhep may play the same functional role in spermatogenesis, sex differentiation, and masculinization as *Cas-IAGag*. It is also possible that *Cas-IAGhep* may be a product of a paralog gene of the IAG gene present in the AG and that its function may differ from that of the IAGag. Similar cases have been reported in the localization and function of hormones and receptors of decapod crustaceans [8,14,15,22,34,41,45,46]. For example, the structure of crustacean hyperglycemic hormone (CHH) identified in eyestalk ganglia is the same as that of gut-endocrine cells but there are differences in the expression patterns of the two: the expression pattern of the former is molt-stage independent but that of the latter occurs only during the premolt stage [8,22]. Molt-inhibiting hormones (MIH) A and B were found to have different expression patterns and functions in tissues of *Metapenaeus ensis* [14,15,41] and of *Marsupenaeus japonicus* [34,45]. MIH binding sites were located both in the Y organs (YO) and the hepatopancreas of adult female *Callinectes sapidus* [46]. In vitellogenic females, specific binding sites for MIH were found in the hepatopancreas in large numbers but with low affinity (playing a role in stimulating vitellogenesis) while those in the YO were fewer in number with higher affinity (suppress ecdysteroidogenesis) [48].

Overall, AGHs and IAGs previously found in crustacean species have been shown to play a primary role in spermatogenesis, sexual differentiation, and masculinization. Our data that eyestalk ablation specifically induces the up-regulation of *Cas-IAG* (and not AK) expression both in the AG and the hepatopancreas provide the first molecular evidence for an endocrine axis of eyestalk ganglia and the AG. This implies that a substance present in the eyestalk ganglia may suppress the AG activity.

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