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Short Communication

Cloning of an insulin-like androgenic gland factor (*IAG*) from the blue crab, *Callinectes sapidus*: Implications for eyestalk regulation of *IAG* expression

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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.

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 vulgae* [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 vitellogensis 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, 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.

Table 1
Primer sequences for isolating the full length cDNA of *Cas-IAG* from the AG of *Callinectes sapidus*.

	Primer sequences (5′, 3′)
dF1	GAYTTYGAYTGYGGNSAYYT
dR2	ACTGCGGCSACMTSGSCGACA
Cas-IAG-3F1	ATCCTTCTCCTCCGTCTGCCTTAC
Cas-IAG-3F2(=QF)	CAGATACAATGGGAGTGACTTCCTTTGA
Cas-IAG-5R1	GTTCACGTATCGGGAGGATCTGC
Cas-IAG-5R2	CCTTCCTCCTGCCCACTGAGTCTTG
Cas-IAG-5R3(=QR)	CTTGAAGAGTTGGAAGGCGTTTTCT

'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.

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% argarose 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