Aquaculture 433 (2014) 325–334

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Molecular identification of a new androgenic gland-specific insulin-like gene from the mud crab, *Scylla paramamosain*

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ARTICLE INFO

Article history:
Received 15 February 2014
Received in revised form 14 June 2014
Accepted 25 June 2014
Available online 1 July 2014

Keywords:
The androgenic gland
Sex differentiation
Scylla paramamosain
Endocrine
Insulin-like gene

ABSTRACT

It is well known that the androgenic gland is a specific endocrine organ of the male crustacean, controlling its primary and secondary sexual characteristics. The androgenic gland hormone is a key peptide hormone involved in sexual differentiation in crustaceans. In this study, a full length cDNA encoding insulin-like gland hormone from *Scylla paramamosain*, named *Sp-IAG*, was identified. The predicted protein had similar molecular organization to other insulin-like androgenic gland factors reported in the Decapoda, encoding a signal peptide, B chain, C peptide and A chain. There were two disulfide bridges between the B chain and A chain, and one disulfide in the B chain. In particular, a full genomic DNA sequence of *Sp-IAG* with a 5′ flanking region of 2988 bp was revealed from which many potential regulatory binding sites were first identified in *S. paramamosain*. The *Sp-IAG* gene was highly expressed in the androgenic gland and also lowly expressed in the seminal vesicle and ejaculatory duct, which was not reported in previous studies. It is noteworthy that Sp-IAG was highly expressed both in the androgenic gland and in the seminal vesicle using western blot analysis, suggesting that Sp-IAG might play a major role in these two organs. In addition, it was quantitatively detected that *Sp-IAG* mRNA expression was up-regulated after mating, which suggested that this protein might have an activity related to mating behavior. Eyestalk ablation can induce a high level of *Sp-IAG* expression, demonstrating that the *Sp-IAG* of *S. paramamosain* was negatively controlled by the X-organ–sinus gland in the eyestalk. These findings provided the basis for elucidating the mechanism of sex differentiation in crustaceans.

1. Introduction

Scylla paramamosain is an important marine aquaculture species, which is distributed in the IndoWest Pacific region. Mature female crabs have a relatively high market value because of the rich yolk in the gonad which is known to be nutritious. Crustacean breeding is now prone to be monosexual for commercial purposes. Monosex aquaculture is now acknowledged and has been widely used in fish farming (Hunter et al., 1983; Jensen and Shelton, 1979). Hormonal induction of sex reversal is the most widely used technique available to mass-produce monosex populations of fishes (Singh, 2012). However, due to the fact that the regulation mechanism of crustacean sexual determination and differentiation is still ambiguous, reports on crustacean monosex breeding are rare. In recent years, study of the androgenic gland (AG) hormone gene has increased the possibility of manual sex control, thus bringing huge commercial interests to aquaculture (Ventura and Sagi, 2012). The latest research on *Macrobrachium rosenbergii* obtained the sex-reversed female by silencing the AGH gene (Panigrahi et al., 2012). Because of this, understanding more concerning IAG will be of great significance in terms of reasonable application in aquaculture in the future.

The AG was first found in *Callinectes sapidus* in 1947 (Cronin, 1947). Charniaux-Cotton (1954) later identified the AG, and suggested that it was involved in sexual differentiation and spermatogenesis. Following this, more work on elucidating the role of the AG in isopods and decapods was carried out, particularly in the Decapoda. Charniaux-Cotton (1954) first successfully removed the AG of male amphipods and implanted it into females. Since then, multiple analogous studies have been conducted, and those experiments revealed that AG-implanted females were masculinized and AG-ablated males feminized (Barki et al., 2006; Katakura, 1960; Khalaila et al., 2001; Malecha et al., 1992; Manor et al., 2004; Nagamine and Knight, 1987; Nagamine et al., 1980; Puckett, 1964; Sagi et al., 1990; Suzuki and Yamasaki, 1997; Taketomi and Nishikawa, 1996). Most AGs are found alongside the distal area of the male ejaculatory ducts (ED), and act via a hormone secretion. These findings support that the AG plays a vital role in male sex differentiation, and maintains individual primary and secondary sex characteristics. They also suggest that AG is associated with spermatogenesis in

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http://dx.doi.org/10.1016/j.aquaculture.2014.06.033
0044-8486/

crustaceans. This provided some clues for further elucidating the mechanisms of sex determination and differentiation in crustaceans.

It is known that some congenital factors contribute to sex development in crustaceans, including the gonad inhibitory hormone produced in the X-organ–sinus gland (XO–SG) and gonad stimulating hormone in the brain and thoracic ganglion. Both of these hormones are important reproductive regulators (Eastman-Reks and Fingerman, 1984; Ōtsu, 1963). In a few decapods, eyestalk ablation (ESA) leads to hyperplasia of the AG (Chung et al., 2011; Hoffman, 1968), implying that XO–SG is the negative regulator of AG. This is described as the eyestalk–AG–testis endocrine axis, which shows direct inhibition by XO–SG on the AG (Khalaila et al., 2002). However, whether there was also eyestalk–AG–testis axis existed in *S. paramamosain* has not yet been confirmed, much less is known about the reproductive physiology of male *S. paramamosain* at the molecular level.

The first cDNA of the molecular AG hormone (AGH) was cloned from the AG in the isopod *Armadillidium vulgare* (Okuno et al., 1999). At present, several insulin-like AG-specific genes in the Decapoda have been identified, most by construction of an AG cDNA subtractive library (Manor et al., 2007; Sroyraya et al., 2010; Ventura et al., 2009). More recently, an insulin-like AG factor (IAG), *Cas-IAG*, was identified using degenerate PCR (Chung et al., 2011). Two spliced variants of insulin-like AGH gene in *Fenneropenaeus chinensis* were identified, and this is the first report of two distinct variants of IAG transcripts in crustaceans (Li et al., 2012). A newly found AG-specific gene, *Mn-IAG*, is also isolated from a transcriptome library of *Macrobrachium nipponense* (Ma et al., 2013).

In this study, we employed degenerate PCR to identify a novel insulin-like AG-specific gene *Sp-IAG* (GenBank accession no. JQ681748), in the mud crab *S. paramamosain*. The genomic sequence and promoter region were obtained using a genome walking approach, which first revealed the full genomic DNA sequence and many potential regulatory binding sites were identified. Tissue-specific expressed *Sp-IAG* mRNA transcripts implied its importance in gender determination. Also, western blot

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Sp-IAG (JQ681748)

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1     gcggtgcatcatcaggagctgctcttggcacttgacacctcggcacggcacggcacggca      60
61    caccttccgcccgccacgcccttccgccacctcttccttttctagtcagcagcaactact     120
121   tctcctggcctgtactacgttttctttggcctcccttccagcctgcattcaattcgccct     180
181   tgtctggtttgcctcctccagcttgggtcctccacataactttcagcccgtgtacgccac     240
241   cgccaagaATGTGTCCGCGTGTGATCTTAATCCTGGTGCTGCTGACGGCGACGCAGACG      300
1                M  C  P  R  V  I  L  I  L  V  L  L  T  A  T  Q  T      17
301   AAGGCGGATCTTATTAGCGACTTCTCCGTGGACTGTGGTAACCTACTGAGATCCTTTTCC     360
18    K  A  D  L  I  S  D  F  S  V  D  C  G  N  L  L  R  S  F  S      37
361   TCCGTTTGCCTCACCTATAAACAACCTCTCAGCGAAAGATATAAACGAGGCACAGAAACA     420
38    S  V  C  L  T  Y  K  Q  P  L  S  E  R  Y  K  R  G  T  E  T      57
421   AAGGGCGCGGCTTCCTTTGACGATGCTACCACTGAATTCCGTCCCCGTCCGCTTCACGTT     480
58    K  G  A  A  S  F  D  D  A  T  T  E  F  R  P  R  P  L  H  V      77
481   CTCCTCGCGGAACAAGACGAAGACCCGATGCTGCCCCCAGAAGACGCCTTCCAACTCGTC     540
78    L  L  A  E  Q  D  E  D  P  M  L  P  P  E  D  A  F  Q  L  V      97
541   AAGACTCATTGGACAAGAGAAAGGTTCCGCAGGTCCCACCGGGACGTGAATGGCTATGAC     600
98    K  T  H  W  T  R  E  R  F  R  R  S  H  R  D  V  N  G  Y  D     117
601   GAGTGCTGCCCGCAGTCCACCAAGAACTGCACTGTGTATGAGGTGGCCGAGTACTGTAAC     660
118   E  C  C  P  Q  S  T  K  N  C  T  V  Y  E  V  A  E  Y  C  N     137
661   AGCCTCAGGCCCCCGTATAGGGAACTCTTAGCTTCCAGAAAGAGGCAGTAAaagtaggagg     720
138   S  L  R  P  P  Y  R  E  L  L  A  S  R  K  R  Q  *               153
721   aggacaacgcggccactgctgctccgaacaatttaagtcagttcacttcgcagtctcgtc     780
781   attttcgtccgtatcttccttccaaatgaggtactaaaggaatatgtttacaaaattatc     840
841   acttcacatccttacaatttttttttttttcggtacgggttagaaaaggtaaaagtaat     900
901   tgcaaaacctgaaaatttccatccgtctcctccctctagaaggcactattgaaggatttg     960
961   tgtttgcagaattcttacctcacgcctccacttgtttcatctttccaaaaacaccagagg    1020
1021  aagatcaacatcaagatcaacaagttatcgcttcagaacctgacaattttcattccatgc    1080
1081  ggtctaaaaaagttcgtaaaagttcatgctaacaaattaaagcttcataacctaataatt    1140
1141  ttgcatcccttccatgcccctccccactcataaaaaaaaaaaaaaaactaaactaacattt    1200
1201  ttaacaaatcaataatttgcaaactgaaactgttcatccattccaatctctctttcaca     1260
1261  aaaaaaaaaaaaaaaaaaaaaaaaaaaaa                                   1290
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Fig. 1. A. Sequences of the full-length cDNA and deduced amino acids of Sp-IAG. The start of the ORF (ATG) is shown in bold, and a stop codon (TAA) is shown in bold and marked with an '*'. The predicted signal peptide is shown in bold italics. The amino acid sequence of the C peptide is flanked between the B and A chains and is underlined in bold. The predicted cleavage sites are marked with squares. A putative N-linked glycosylated site is shaded. B. A sketch map of B and A chains of disulfide bridges in Sp-IAG. C. Neighbor-joining phylogenetic tree of Sp-IAG amino acid sequences from different species. One thousand bootstrap trials were run using the neighbor-joining algorithm using the MEGA 5.0 software. The species and their GenBank accession numbers are given in Supplementary Figure S1. D. The structure of *Scylla paramamosain Sp-IAG* genomic DNA and cDNA. Exons are indicated by boxes and introns are indicated. Length of the introns and exons are labeled on the diagram and expressed in terms of base pairs. E. Deduced promoter region of the *Sp-IAG* 5′ flanking sequence. Putative HSF, Sox-5, cap, C/EBPb, ER, GR, and PR binding sites are underlined. Putative Skn-1, GATA-1, Dl, GC-box, NKx-2, and GATA-3 binding sites are shaded. Putative CREB, ADR-1, Dfd, Hb, GATA-2, PBx-1, and MZF1 binding sites are in bold. Putative SRY, CRE-BP, P300, AML-1, AP4, NIT2, and CdxA binding sites are indicated with an arrow.