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Insulin and gender: An insulin-like gene expressed exclusively in the androgenic gland of the male crayfish

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Abstract

Members of the insulin family of hormones are generally not regarded as gender-specific, although there is sporadic evidence for the possible involvement of insulin pathways in sexual differentiation. In crustaceans, sexual differentiation is controlled by the androgenic gland (AG), an organ unique to males. To date, attempts to identify active AG factors in decapods through either classical purification methods or sequence similarity with isopod AG hormones have proven unsuccessful. In the present study, the first subtractive cDNA library from a decapod AG was constructed from the red-claw crayfish *Cherax quadricarinatus*. During library screening, an AG-specific gene, expressed exclusively in males even at early stages of maturation and termed *Cq-IAG* (*C. quadricarinatus* insulin-like AG factor), was discovered. *In situ* hybridization of *Cq-IAG* confirmed the exclusive localization of its expression to the AG. Following cloning and complete sequencing of the gene, its cDNA was found to contain 1445 nucleotides encoding a deduced translation product of 176 amino acids. The proposed protein sequence encompasses Cys residue and putative cleaved peptide patterns whose linear and 3D organization are similar to those of members of the insulin/insulin-like growth factor/relaxin family and their receptor recognition surface. The identification of *Cq-IAG* is the first report of a pro-insulin-like gene expressed in a decapod crustacean in a gender-specific manner. Its expression in a male-specific endocrine gland controlling sex differentiation supports the notion that insulin may have evolved in the context of regulating sexual differentiation.

Keywords: Androgenic gland; cDNA library; *Cherax quadricarinatus*; Crayfish; Crustacea; Decapoda; Differential expression; *Cq-IAG*; Insulin-like; Suppression subtractive hybridization

1. Introduction

Sexual differentiation and the development of secondary sexual characteristics are controlled by different mechanisms across evolution. In vertebrates and some invertebrate groups, these processes are under the control of sex hormones. Given the recent reconfirmation that insects have no sex hormones (Maas and Dorn, 2005), the agents responsible for the sexual maturation of arthropods remain under debate. Indeed, differentiation of primary and secondary sexual characteristics in insects is thought to be exclusively controlled by the genetic inventory of the individual cell (Baker and Ridge, 1980). Despite their evolutionary proximity to insects, crustaceans surprisingly possess an androgenic gland (AG) which is responsible for male sexual differentiation, most likely acting through sex hormone(s) (Charniaux-Cotton and Payen, 1988; Payen, 1990; Sagi and Khalaila, 2001; Sagi et al., 1997).

In the Australian crayfish *Cherax quadricarinatus*, the AG has been identified (Khalaila et al., 1999) and the wide array of effects for which this gland is responsible were demonstrated by its implantation into immature females

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(Khalaila et al., 2001; Manor et al., 2004). Such AG implantation resulted in the development of male secondary characteristics and inhibition of female secondary characteristics and vitellogenesis. The pivotal role of the AG was further demonstrated by its removal in *C. quadricarinatus* intersex individuals, leading to development of female characteristics, including the onset of vitellogenesis and the regression of male primary and secondary characteristics (Sagi et al., 2002). It was moreover shown that the AG induces male-like reproductive and aggressive behavior (Barki et al., 2003; Karplus et al., 2003).

The effects elicited by the AG are thought to be mediated by the AG hormone (AGH). AGHs, identified and sequenced in isopods such as *Armadillidium vulgare*, show similarity to the proinsulin superfamily of peptides (Martin et al., 1999; Martin et al., 1998; Okuno et al., 1999). Similar AGHs were identified in two other isopod species, i.e., *Porcellio scaber* and *Porcellio dilatatus*, with the amino acid sequence of the mature AGH peptide being highly conserved amongst the three species (Ohira et al., 2003). In decapods, however, no AGH has been thus far identified. Several lipidic substances were suggested to act as AGHs in decapod crustaceans, based on histological evidence in prawns (Veith and Malecha, 1983) and biochemical analysis of a crab AG extract (Berreur-Bonnenfant et al., 1973), later shown to be farnesylacetone (Ferezou et al., 1978). As such, it is surprising that the ultrastructure of the AG in different crustaceans resembles that of a vertebrate protein-producing cell rather than a steroid-producing cell (King, 1964). This, together with recent histological evidence in prawns supporting the idea of a proteinaceous androgenic hormone (Awari and Kiran, 1999; Okumura and Hara, 2004), has given rise to the belief that a proteinaceous androgenic hormone will eventually be purified from the AG of decapod crustaceans.

Nonetheless, attempts to identify and purify a decapod AGH have not been successful, despite extensive effort. As an alternative approach, the search for specifically AG expressed genes through the use of a subtractive cDNA library of *C. quadricarinatus* AGs was suggested, given the power of suppression subtractive hybridization (SSH) in discovering differentially expressed genes (Diatchenko et al., 1999). In mammals, SSH was used to search for novel target genes induced by the sexually dimorphic growth hormone (Gardmo et al., 2002). SSH has also been employed to characterize sex-specific differentially expressed genes. Diatchenko et al., 1999 constructed tissue-specific cDNA libraries of human testis, ovary, and prostate to identify functional sequences with sex-specific expression. In mouse, 28 novel genes were found to have testes-specific expression, with 20 of them potentially involved in spermatogenesis or fertilization (Hong et al., 2005). Comparing differences in transcript levels in adult *Drosophila melanogaster*, Arbeitman et al., 2002 identified sex-specific somatic genes. In a decapod crustacean, the prawn *Macrobrachium rosenbergii*, SSH served to identify a novel gene of unknown function in the epithelial cells of the male reproductive tract (Cao et al., 2006).

In the present study, we describe construction of the first crustacean AG cDNA library and report its use for the identification of *C. quadricarinatus* genes uniquely expressed in the AG. One such gene, *Cq-IAG* (*C. quadricarinatus* insulin-like AG factor, data bank accession number DQ851163), was cloned, fully sequenced and shown to be seemingly sex-specific, being expressed only in males. Its deduced amino acid sequence suggests it to be a member of the evolutionarily related insulin and/or insulin-like growth factor/relaxin families. Structurally, all these peptides consist of two polypeptide chains (A and B) linked by two disulfide bonds. All share a conserved arrangement of four Cys residues in the A chain in which the first of these residues is disulfide-linked to the third and the second and fourth Cys residues are linked by interchain disulfide bonds to Cys residues in the B chain. Unlike *Cq-IAG*, members of the insulin family of hormones are generally not regarded as gender-specific, although there is sporadic evidence for the possible involvement of insulin pathways in sexual differentiation (Nef et al., 2003). Our findings thus suggest a novel insulin-like protein specific to male decapod crustaceans, offering support to the notion that insulin may have evolved in the context of sexual differentiation.

2. Materials and methods

2.1. Animals

Mature *C. quadricarinatus* males (40–70 g) were collected from a 5 m^3 tank. Water quality and temperatures ranging between 20 and 30 °C were assured by circulating the entire tank volume through a biofilter. Food comprising shrimp pellets (Rangen Inc., 30% protein) was supplied *ad libitum* three times a week. In some cases, to enable easier identification of the AG, an endocrine manipulation was employed causing hypertrophy of the AG (hAG), as described previously (Khalaila et al., 2001,Khalaila et al., 2002). For detection of *Cq-IAG* expression in juveniles, *C. quadricarinatus* egg-bearing females were transferred to separate compartments where they were monitored for egg development. After releasing all post larvae, the females were removed. Juveniles were collected 8 and 22 days later. Sex was determined by the presence of genital papillae, viewed under a dissecting microscope.

2.2. Construction of a cDNA library of the AG using Suppression Subtractive Hybridization (SSH)

Total RNA was isolated by placing the crayfish on ice for 2–5 min until they were anesthetized. Following the dissection of hAGs and other peripheral glands (a mix of mandibular organs and green glands), RNA from endocrinologically manipulated males was extracted using an EZ-RNA Total RNA Isolation Kit (Biological Industries, Beit Haemek, Israel). cDNA was prepared from 1 μg of total RNA using the Super SMART PCR cDNA Synthesis kit (BD Biosciences). The cDNA was then used to prepare a subtraction library of the AG with the Clontech PCR Select cDNA Subtraction Kit (BD Biosciences), following the manufacturer's instructions, using the cDNA from AG as the tester and the cDNA from other peripheral glands as the driver. After two hybridization cycles, unhybridized cDNAs, representing genes that are expressed in the AG but are absent from the driver, were amplified by two PCRs. The primary (27 cycles) and secondary (20 cycles) PCRs were performed as recommended in the Takara DNA polymerase manual and the PCR products were cloned into the pGEM-Teasy vector (Promega). Clones containing the inserts were isolated and grown overnight. Plasmid DNA was purified (Qiagen Miniprep kit) and the inserts were sequenced.

2.3. RT-PCR

cDNA was prepared by a RT reaction containing 1 μg of total RNA, extracted as above from mature males (AG, hAG, muscle, cuticle, testis, peripheral glands, hepatopancreas, and thoracic ganglia) or mature females (ovary and hepatopancreas), and M-MLV reverse transcriptase H minus (Promega), according to the manufacturer's instructions. The cDNA was then amplified by PCR (one cycle at 94 °C—2 min; 35 cycles at 94 °C—30 s, 55 °C—30 s, 72 °C—2 min; one cycle at 72 °C—10 min), using twenty-specific primers designed on the basis of the analyzed sequences of the AG subtractive cDNA library. To amplify *Cq-IAG* using cDNA from the AG or hAG, the forward and reverse primers *Cq-IAG*-f: 5′-ACTCAGCAGAAACGAG CCTA-3′ (nt 1213–1232, Fig. 5A) and *Cq-IAG*-r: 5′-ATTTAATGGAA GGCGCTGGA-3′ (nt 1389–1408, Fig. 5A), respectively, were employed.

To address the developmental *Cq-IAG* expression pattern, RNA was extracted as above from the base of the fifth walking legs (the aproximate location of the AG in male *C. quadricarinatus*) of juvenile and mature males and females. For each amplification, forward primer *Cq-IAG -DEV*-f: 5′-ACTGTGAACAGTTGGAGGACGGA-3′ (nt 1046–1068, Fig. 5A) and reverse primer *Cq-IAG -DEV*-r: 5′-ATGGAAGGCGCTGGAAAG CCATG-3′ (nt 1381–1403, Fig. 5A) were used. The PCR conditions employed were: 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min, followed by final elongation step of 72 °C for 10 min. As a positive control, forward: 5′-GTCTTCGTGCAGG AGAAACTCG-3′ and reverse: 5′-CGACGAGGGCACCATCAGTTA-3′ primers to elongation factor 2 (EFT-2) were used.

2.4. In situ hybridization

hAGs attached to ~0.5 cm of the terminal ampullae were dissected and prepared as described (Shechter et al., 2005). Digoxygenin (DIG)-labeled oligonucleotides for antisense and sense probes, corresponding to nucleotides 31–1403 of *Cq-IAG* cDNA, were synthesized using SP6 and T7 RNA polymerases. The probes were hydrolysed to reduce their lengths to ~200 b, as described in the DIG Application Manual (Roche Applied Science). Hybridization was carried out as performed previously (Shechter et al., 2005), except that tRNA was used as a tissue blocking reagent instead of sheared salmon sperm DNA.

2.5. Northern blot analysis

Total RNA was isolated as above from the hAG, muscle, hepatopancreas and testis of adult males. Five micrograms of RNA from each organ was electrophoresed through a 1% agarose formaldehyde gel, transferred to a nitrocellulose membrane, and UV-cross-linked. The blot was prehybridized overnight as described (Shechter et al., 2005) and radiolabeled with a ^{32}P probe prepared by adding dCTP, together with a cDNA template isolated from a clone containing a cDNA insert (*Cq-IAG*), to a random priming labeling mix (Biological Industries). The blot was incubated overnight in prehybridization buffer containing the ^{32}P-labeled DNA. The membrane was washed as described (Shechter et al., 2005) and exposed to BioMax MS Kodak film with intensifying screens at −70 °C for 25 min. Ribosomal RNA was visualized with ethidium bromide.

2.6. 5′ rapid amplification of cDNA ends (5′ RACE) and confirmation of the sequence

The sequence of the 5′ end of *Cq-IAG* was obtained by 5′ rapid amplification of cDNA ends (RACE), carried out with the Clontech SMART™ RACE kit (BD Biosciences), following the manufacturer's protocol. PCR was performed using the gene-specific reverse primer

Cq-IAG RACE-r: 5′-TGGAAGGCGCTGGAAAGTCATGATAA-3′ (nt 1377–1402, Fig. 5A) and the UPM (Universal Primers Mix) provided in the kit. The PCR products were cloned and sequenced as described above.

In order to confirm the obtained sequence, a forward primer from the 5′ end was designed based on the 5′ RACE results: *Cq-IAG* begin-f: 5′-TCAGCACCCGTCCAGCACA-3′ (nt 31–49, Fig. 5A). This primer was used for PCR with cDNA from hAG and with the reverse primer *Cq-IAG*-r that was shown above (nt 1389–1408, Fig. 5A) and a product of about 1400 bps was amplified. This product was cloned and sequenced as described above and to ensure the quality of the sequence, each region of the gene was sequenced 3–10 times.

2.7. Bioinformatic analysis

To enhance the quality of the selected expressed sequence tags (ESTs), the obtained cDNA sequences were first stripped of low quality, vector and primer sequences using Sequencher™ software (GeneCodes Corp.). Clustering and assembly of the remaining sequences was performed using CAP3 (Huang and Madan, 1999). The resulting contigs and singlets were unified and their sequences were compared to the Uniprot database (Swiss-Prot + TrEMBL from 18.5.05), using a local installation of NCBI's BLASTx algorithm. Further functional annotation was achieved by assigning a Gene Ontology (GO) term to the best Uniprot BLASTx hit of

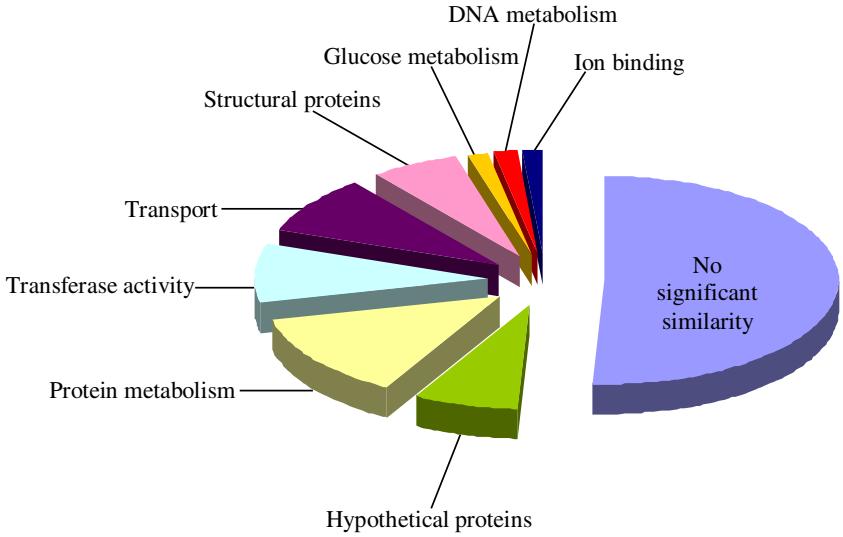

Fig. 1. Sequence similarity analysis of 300 ESTs (66 putative genes) from a *C. quadricarinatus* androgenic gland cDNA subtractive library. More than 50% of the sequences had no significant similarity (*E*-value > 0.01) to any Uniprot protein. 7.4% were similar to hypothetical proteins, and the rest could be related to proteins associated with the indicated GO (Gene Ontology) categories.

each contig/singlet using AmiGO (http://www.godatabase.org/cgi-bin/amigo). The full length of one of the cDNA sequences, *Cq-IAG*, was computationally translated using the ExPASy Proteomics Server (http://ca.expasy.org/tools/dna.html) and the most likely frame was selected (5′3′ Frame 1). The deduced amino acid sequence was further considered by SMART (http://smart.embl-heidelberg.de/smart) and CBS Prediction Servers (http://www.cbs.dtu.dk/services). The multiple sequence alignments of the predicted mature Cq-IAG sequence with representative members of the insulin/insulin-like growth factor/relaxin family and of the predicted pro-Cq-IAG sequence with the three pro-AGHs known in Isopoda (Ohira et al., 2003; Okuno et al., 1997) were performed by ClustalX (Thompson et al., 1997). The 3D model of Cq-IAG was created by the ESyPred3D web server (http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred) (Lambert et al., 2002) and processed by Swiss-PdbViewer software (Guex and Peitsch, 1997).

3. Results

From a cDNA subtractive library constructed from hypertrophied *C. quadricarinatus* AGs [hAG (Khalaila et al., 2002)], 300 DNA sequence fragments were assembled into 56 contigs and 10 singlets, together encompassing 66 putative genes. As shown in Fig. 1, more than 50% of the assembled sequences share no significant similarity (*E* value > 0.01) to any known genes or proteins in the database, while 7% of the assembled sequences share significant similarity with genes encoding hypothetical proteins with no annotated function. The remaining sequences were related to a wide range of functions including protein metabolism, transferase activity, transport, structural proteins, glucose metabolism, DNA metabolism, and ion binding.

One of the genes, termed *Cq-IAG* (*C. quadricarinatus* insulin-like AG factor), was shown by RT-PCR to be exclusively expressed in AG and in hAG tissues of adult crayfish but not in the other tissues examined, i.e., muscle, cuticle, testis, peripheral glands, hepatopancreas, and thoracic ganglia from mature males and ovary and hepatopancreas from mature females (Fig. 2A). *Cq-IAG* expression was not detected in the X organ/sinus gland complex as well (data not shown). The expression of *Cq-IAG* was further analyzed in different parts of the male reproductive system in greater detail and again was found to be expressed uniquely in the AG and not in the sperm duct or testis (Fig. 2B). The expression of *Cq-IAG* was detected in juvenile males 8 and 22 days after their maternal release but not in juvenile females of the same ages (Fig. 2C). To assure that cDNA was present in all samples examined, positive controls were performed using primers for the housekeeping gene, elongation factor 2 (EFT-2); product was amplified in all cases (Figs. 2A–C). Localization of *Cq-IAG* expression by *in situ* RNA hybridization confirmed the specific expression of the gene in the AG and not in adjacent tissues, including the sperm duct (Fig. 3). A strong, specific signal was detected in AG cells using an antisense probe, with slight variability among cells and different areas of the gland. No signal was detected in the negative control, in which the sense-strand probe was employed.

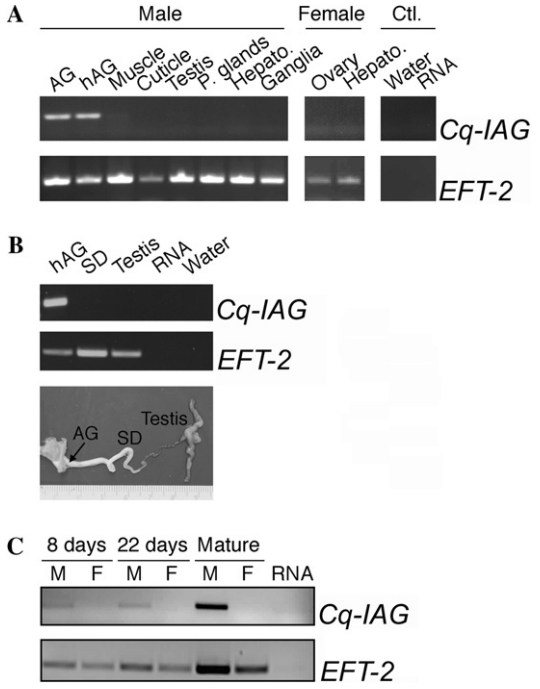

Fig. 2. Agarose gels showing RT-PCR products demonstrating spatial and temporal expression patterns of the *Cq-IAG* gene. (A) Template RNA was extracted from mature males (AG, hAG, muscle, cuticle, testis, peripheral glands, hepatopancreas, and thoracic ganglia) and mature females (ovary and hepatopancreas). RT-PCR was performed using specific primers of the *Cq-IAG* and a housekeeping gene (elongation factor 2 (EFT-2), GenBank Accession No. AI253924). Controls were performed with AG RNA and sterile water in the PCR. Expression of *Cq-IAG* was detected only in AG and in hAG tissues. (B) Template RNA was extracted from different locations of the male reproductive system (hAG, sperm duct (SD), and testis), as shown in the picture on the bottom. RT-PCR was performed as described in (A). Expression of *Cq-IAG* was detected only in the hAG. (C) Template RNA was extracted from juveniles (8 and 22 days post-release) and from the base of the fifth walking legs of mature males and females. RT-PCR was performed as described above. Expression of *Cq-IAG* was detected only in the juvenile males and in the fifth walking legs from mature males.

Northern blot analysis initially showed the *Cq-IAG* transcript size to be approximately 1500 b, with a single band being detected in the hAG but not in muscle, hepatopancreas or testicular tissues, despite rRNA being detected in all samples (Fig. 4). *Cq-IAG* was then fully sequenced and the corresponding cDNA was shown to contain 1445 nucleotides (Fig. 5A). The cDNA includes an open reading frame of 528 b encoding a deduced 176 amino acid translation product, a 555 b 5′UTR and a 361 b 3′UTR ending in a poly A tail. Based on the output of CBS Prediction Servers, the deduced translation product includes a putative 23 amino acid-long signal peptide (Signal peptide probability: 0.963) and two potential *N*-glycosylation sites at Asn 53 (potential = 0.6853) and Asn 137 (potential = 0.5416). The SMART domain search tool predicted the existence of a domain between residues 32–165 similar to insulin (*E* value = 0.059) and to members of the insulin-like growth factor (IGF)/relaxin family (*E* value = 0.08814). The similarity to insulin is mainly reflected in the number and

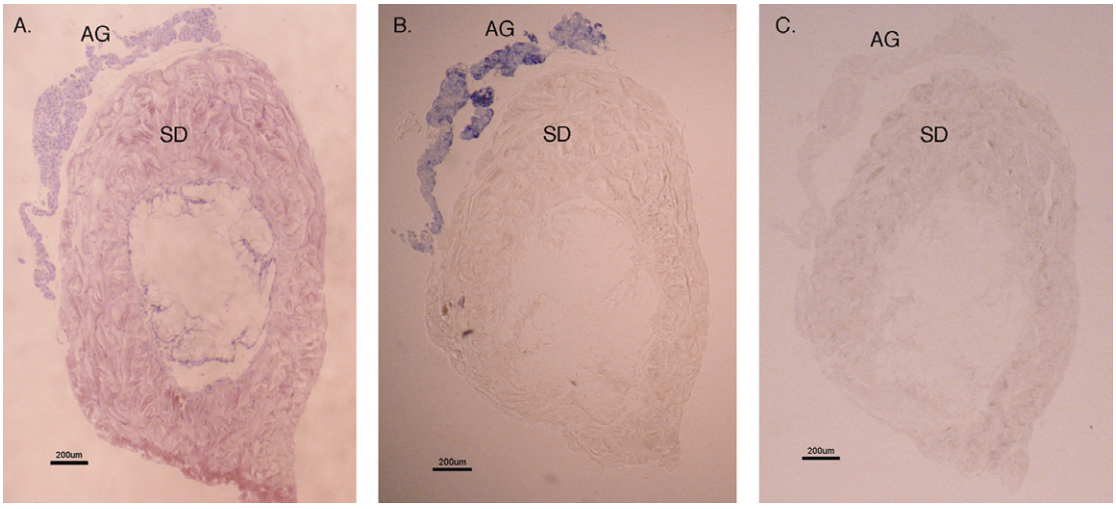

Fig. 3. Localization of the expression of *Cq-IAG* was performed by *in situ* RNA hybridization. Consecutive sections (5 μm) of the proximal part of the sperm duct (SD) and the attached hypertrophied androgenic gland (hAG) were probed with *Cq-IAG* sense and antisense probes. A strong, specific signal in the AG was detected by the antisense probe. No signal was detected using the sense-strand negative control probe. (A) Hematoxylin–eosin staining; (B) antisense probe; (C) sense probe. Bar = 200 μm.

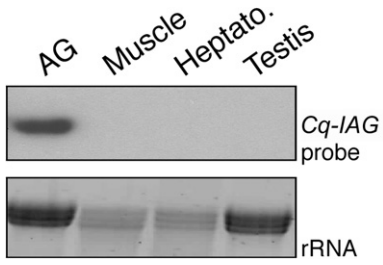

Fig. 4. Northern blot analysis of tissue-specific expression of *Cq-IAG* mRNA. Each slot was loaded with 5 μg of total RNA purified from mature male tissues (hAG, muscle, hepatopancreas, and testis). A band of approximately 1500 b appeared exclusively in the lane containing RNA from the hAG. rRNA was visualized with ethidium bromide.

location of Cys residues (six), the location of putative cleavage sites, and the higher degree of similarity to insulin found in the A and B chains of Cq-IAG as compared to the signal and C peptide regions. The similarity of mature Cq-IAG to representative insulin-like family members, as revealed by sequence alignment, is presented in Fig. 5B.

Based on its similarity to members of the insulin-like family, a linear model for the organization of Cq-IAG can be proposed (Fig. 6A). The linear model includes a signal sequence (aa 1–23), B chain (aa 24–59), C peptide (aa 60–130) and A chain (aa 131–176). The model assumes that the C peptide is excised by the actions of Arg C proteinase at ^{56}RAVR^{59} and ^{127}RRRR^{130}, leaving a mature peptide comprising the A and B chains interconnected through two disulfide bridges (Cys 35–Cys 148 and Cys 46–Cys 165) and one intra disulfide bridge within the A chain (Cys 147–Cys 156). The molecular weight of the predicted pro-peptide is 19925.5 Da, while the molecular weight of the predicted mature peptide is 9882.79 Da. Based on this model and the solved NMR structure of a close insulin-like protein family member (Fig. 5B), i.e., bombyxin (Nagata et al., 1995b), a 3D structure of mature Cq-IAG is proposed (Fig. 6B). This 3D model of Cq-IAG includes the entire classical Cys-containing region in both chains (encompassing residues 24–54 of the B chain and 142–165 of the A chain). Regions beyond this core region were not included in the model since the additional Cq-IAG residues (namely residues 55–59 from the C terminal of the B chain and residues 131–141 and 166–176 from the A chain N and C termini, respectively) are not found in the shorter mature bombyxin protein (48 aa). The regions in the B and A chains on which the 3D model of Cq-IAG is based, were found to have high similarity with insulin family members and especially with bombyxin (53.8% identity for the region between the two Cys residues in the B chain, and 37.5% identity for the entire region of the A chain). In bombyxin, it was demonstrated that A chain N terminal residues (Gly A1, Val A3), the Cys A20–Cys B19 disulfide and the central part of the B-chain are important for protein activity (Nagata et al., 1995a). The 3D model suggested here for Cq-IAG shares a high degree of similarity with these domains of bombyxin, found to be essential for receptor recognition. Of these, the most important features shared by the Cq-IAG and bombyxin structures include the conserved Val in the A chain (Val 143 in Cq-IAG and Val A3 in bombyxin), the disulfide bond between Cys 46 from the B chain and Cys 165 from the A chain in Cq-IAG (Fig. 6A) and Cys A20–Cys B19 in bombyxin, as well as a central region of the B chain from Cys 35 to Cys 46 in Cq-IAG, corresponding to Cys B7 to Cys B18 in bombyxin. Functionally, the high similarity of Cq-IAG to bombyxin, particularly to its receptor-recognition surface, suggests that the core of the encoded molecule is indeed the receptor-recognition domain of Cq-IAG.

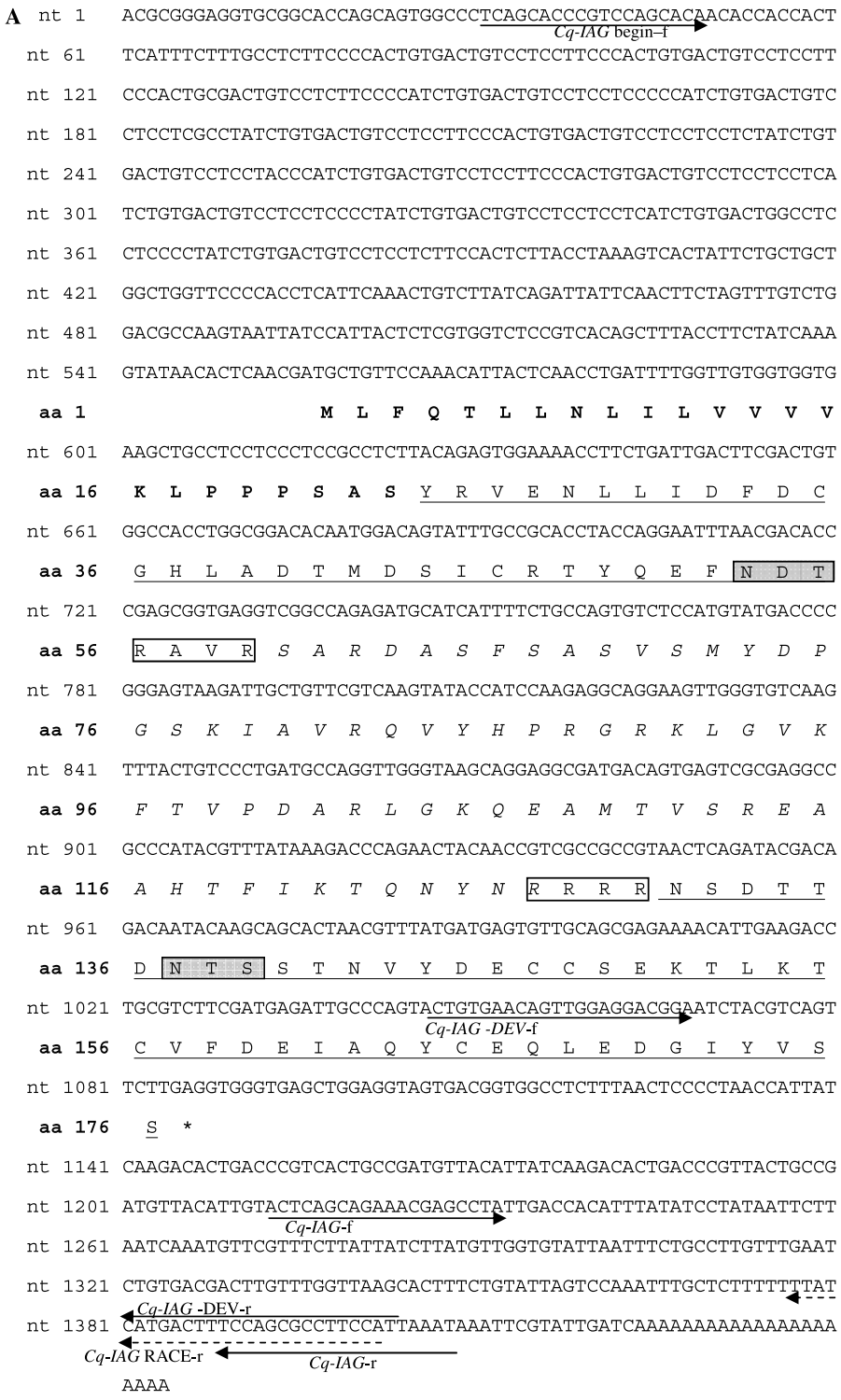

Fig. 5. The sequence of *Cq-IAG*, a gene encoding an insulin-like factor from the AG of *C. quadricarinatus*. (A) Complete cDNA and deduced amino acid sequence of *Cq-IAG*. The putative signal peptide sequence is marked in bold, the putative A and B chains are underlined and the putative C peptide is in italics. The gray colored boxes indicate the predicted *N*-glycosylated sites while the open boxes indicate the predicted cleavage sites of Arg C proteinase. The asterisk indicates the position of the stop codon in the cDNA. The primers used are marked by arrows. The *Cq-IAG* RACE-r primer is marked in dashed arrow. (B) Multiple sequence alignment of the putative mature Cq-IAG with representative members of the insulin/insulin-like growth factor/relaxin family, calculated by ClustalX. The sequences shown include: mouse insulin [INS1_MOUSE, (Wentworth et al., 1986)], insulin-like growth factor I of bovine [IGF1_BOVIN, (Fotsis et al., 1990)], human relaxin H1 [REL1_HUMAN, (Hudson et al., 1984)], bombyxin C-1 of *B. mori* [Silk moth, BXA6_BOMMO, (Nagasawa et al., 1986)], *D. melanogaster* insulin-like protein (Q4V6B2_DROME) and AGH of *A. vulgare* [AGH_ARMVU, (Okuno et al., 1999)]. Conserved Cys residues are shown in boxes while the (−) indicates gaps introduced into the amino acid sequence to allow for the maximal degree of identity in the alignment.

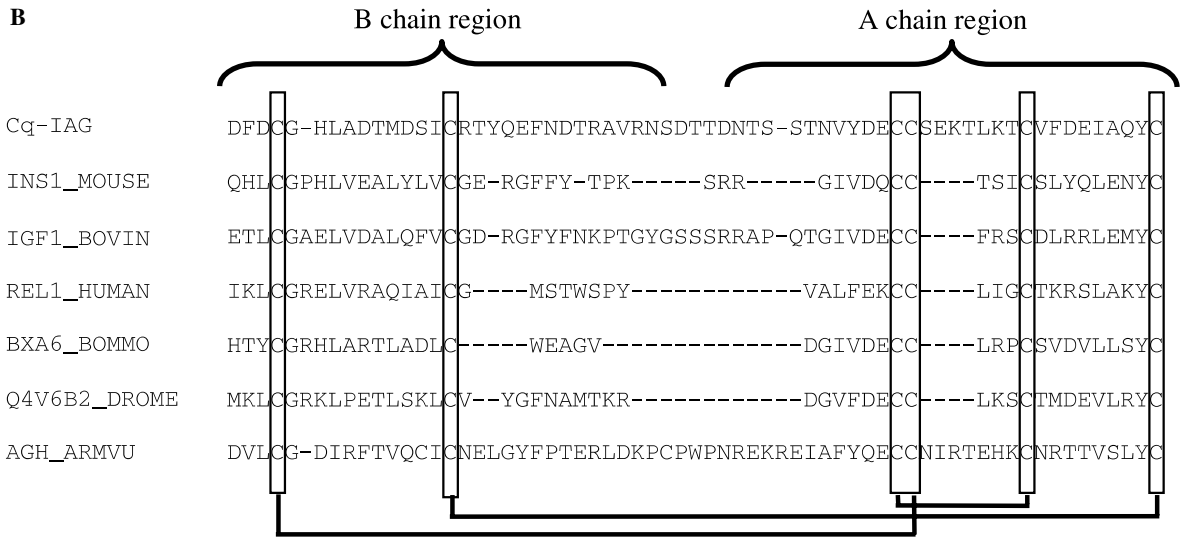

Fig. 5 (*continued*)

4. Discussion

Our previous studies in *C. quadricarinatus* showed that the AG wields a tremendous impact in regulating a large array of sex-related phenomena. Other than secondary characters, male-like aggressive and reproductive behavioral patterns (Barki et al., 2003; Karplus et al., 2003), these regulatory roles also extend to growth rate, a trait which is dimorphic in the sense that *C. quadricarinatus* males grow significantly faster than females (Manor et al., 2001). Accordingly, implantation of AG into females produced similar growth promotion as in males (Manor et al., 2004). This AG-mediated growth enhancement could be the result of a direct growth factor or via an indirect effect, such as a shift of energy resources from female reproduction to growth. In fact, the wide morphological, physiological, and behavioral effects of the gland (Sagi and Khalaila, 2001) have raised the question of whether a single factor could account for all the above-described effects in higher crustaceans. Our hypothesis was that these sex-specific phenomena, including differential growth, are mediated by genes uniquely expressed in the AG. *Cq-IAG*, the insulin-like gene found in the present study, might be such a gene. The similarity of *Cq-IAG* to insulin-like proteins is in agreement with our previous findings (Manor et al., 2004) on the AG being involved in growth enhancement, with the relation of this gland to crustacean dimorphic growth (Manor et al., 2004), and with members of the insulin family, such as insulin-like growth factors, having been shown to serve as potent growth enhancers in other systems (Lelbach et al., 2005). Indeed, insulin-like growth factors (IGF-I/-II) are not only the endocrine mediators of growth hormone-induced metabolic and anabolic actions but have also been shown to act in paracrine and autocrine manners to regulate cell growth, differentiation, apoptosis and transformation (Lelbach et al., 2005). However, further experiments are required in order to examine *Cq-IAG* activity.

Similar structures of peptides with homodimeric or heterodimeric peptide chains linked together by disulfide bonds, have been found, such as the Anti Mullerian Hormone which is involved in male sexual differentiation signaling pathways (Chang et al., 2002; Rey, 2005) and is a member of the TGF-β superfamily (Chang et al., 2002).

Insulin-like peptides in insects are encoded by multigene families that are expressed in brain and other tissues. Once secreted, these peptides likely serve as hormones, neurotransmitters, and growth factors (Wu and Brown, 2006). In *Bombyx mori* (Insecta), a prothoracicotropic hormone (bombyxin) was identified as an insulin-like peptide (Ishizaki and Suzuki, 1994) and shown to stimulate the prothoracic glands of the heterologous moth *Samia cynthia ricini* to secrete the molting hormone, ecdysteroid (Nagasawa et al., 1984). The effects of bombyxin on cell proliferation (Goto et al., 2005; Nakahara et al., 2006) and growth (Nijhout and Grunert, 2002) were also reported, while its involvement in the regulation of ovarian development has been postulated (Maniere et al., 2004). In *D. melanogaster*, molecular genetic studies have revealed elements of a conserved insulin signaling pathway that also appears to play a key role in metabolism, growth, reproduction, and aging (Wu and Brown, 2006). In isopods, a presumed pro-AGH, analogous to the proinsulin superfamily of peptides, was identified (Martin et al., 1999; Martin et al., 1998; Okuno et al., 1999). The amino acid sequence of the A and B chains which comprise the mature AGH peptide were highly conserved among three isopod species (Ohira et al., 2003), however, its similarity to Cq-IAG was found to be low. Furthermore, its backbone includes 8 Cys residues with an additional disulphide bond rather than 6 Cys residues, as found in the classical insulin–relaxin family members. In addition to these AGHs, there are some insulin members with 8 Cys residues with a suggested additional disulphide bond such as molluscan insulin-related peptide VII from *Lymneta*

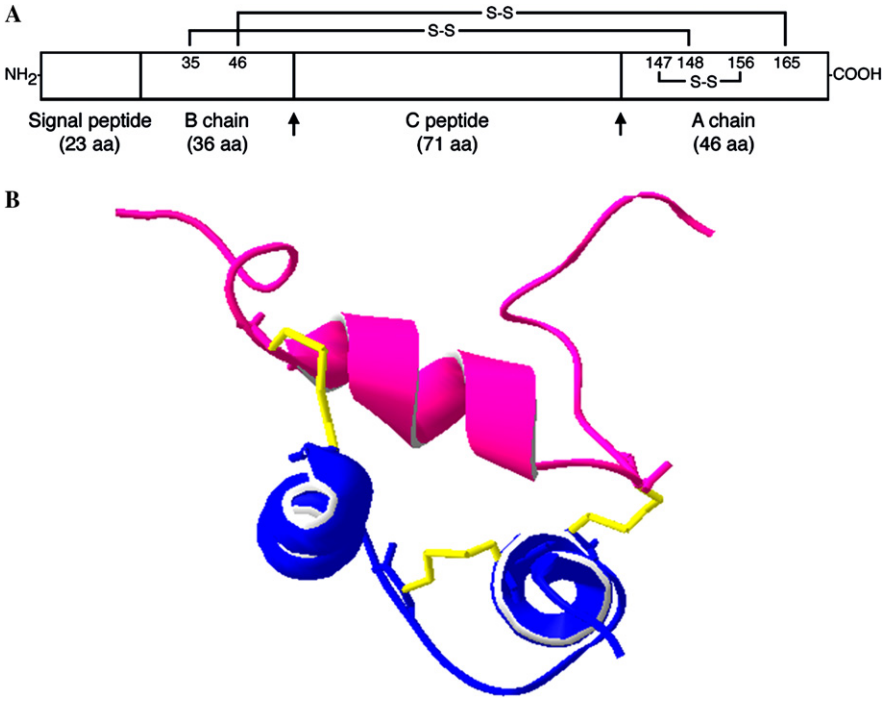

Fig. 6. Linear and 3D models of pro and mature Cq-IAG support its classical insulin-like structure. (A) Linear model for pro-Cq-IAG. The pro-Cq-IAG contains a signal peptide, B chain, C peptide and A chain. This model suggests cleavage of the C peptide by the endonuclease Arg C proteinase after RAVR (aa 56–59) and RRRR (aa 127–130) (see arrows), leaving a mature peptide comprising the A and B chains, interconnected through two disulfide bridges and one intra disulfide bridge within the A chain. (B) 3D model of mature Cq-IAG, based on its organizational similarity to bombyxin, an insulin-like peptide from the silk moth *B. mori* (PDB 1BON). The 3D model was constructed by EsyPred3D and edited by Swiss-PdbViewer. The peptide backbones of B chain amino acids 24–54 (in purple) and A chain amino acids 142–165 (in blue) are presented in the ribbon diagram. Residues 55–59 (found at the C terminal of the B chain) and residues 131–141 and 166–176 (found at the N and C terminals of A chain, respectively) were not included in the model because of the differences in length between mature Cq-IAG (82 aa) and bombyxin (48 aa), for which a NMR structure is available (Nagata et al., 1995b). The side chains of Cys residues containing disulfide bridges are displayed in yellow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stagnalis (Smit et al., 1996) and type β-insulins from *Caenorthabditis elegans* (Duret et al., 1998; Pierce et al., 2001). Multiple sequence alignment of Cq-IAG predicted precursor, with the three pro-AGHs of isopods (Fig. 7) showed that Cq-IAG has some sequence similarity (between 16 and 19% identity) to the three already known isopod AGHs (Ohira et al., 2003; Okuno et al., 1999). The six Cys residues in *Cq-IAG* were located at identical positions with those in the three isopod AGHs and the organization of the predicted pro-*Cq-IAG* seems to be the same, consisted of the signal peptide, B chain, C peptide and A chain (Fig. 7). It seems that an insertion of 22 amino acids occurred in the predicted C peptide of Cq-IAG (aa 63–84) similar to insertions found in other insulin family members [for example in REL1_HUMAN, (Hudson et al., 1984)]. Similar to the three isopod AGHs, the predicted Cq-IAG contains a typical proteolytic cleavage motifs (R-X-X-R) between B chain and C peptide, and between C peptide and A chain. The A chains of the three AGHs possessed a putative *N*-linked glycosylation motif (N-X-S/T) while in Cq-IAG there are 2 putative *N*-linked glycosylation motifs, one in the B chain and one in the A chain. As far as we know, other insulin family members generally do not possess *N*-linked glycosylation motifs. The positions of the proteolytic cleavage motifs and the *N*-linked glycosylation motif were identical among the three isopoda AGHs and different in Cq-IAG (Fig. 7). These differences could explain why no insulin-like proteins have emanated from extensive searches for decapod sequences similar to those of known isopod AGH. Nonetheless, prior to the first identification of a gene encoding an insulin-like peptide in decapods presented here, evidence for the existence of insulins in decapods had been suggested through immunoreactivity in lobsters [*Panulirus argus* (Gallardo et al., 2003) and *Homarus americanus* (Sanders, 1983a)]. In addition, reports on putative insulin receptors (Chuang and Wang, 1994; Lin et al., 1993) and insulin-related metabolic activity (Kucharski et al., 2002; Richardson et al., 1997; Sanders, 1983b) in decapods have appeared.

Insulin and members of the insulin family of hormones are generally not regarded as gender-specific, although scattered evidence for possible involvement of insulin pathways in sexual differentiation has been presented (Nef et al., 2003). Here, we report the rare case of Cq-IAG, an insulin/insulin-like growth factor family member which is expressed in a gender-specific manner, exclusively in males. In some organisms, sexual dimorphism can be manifested by different levels

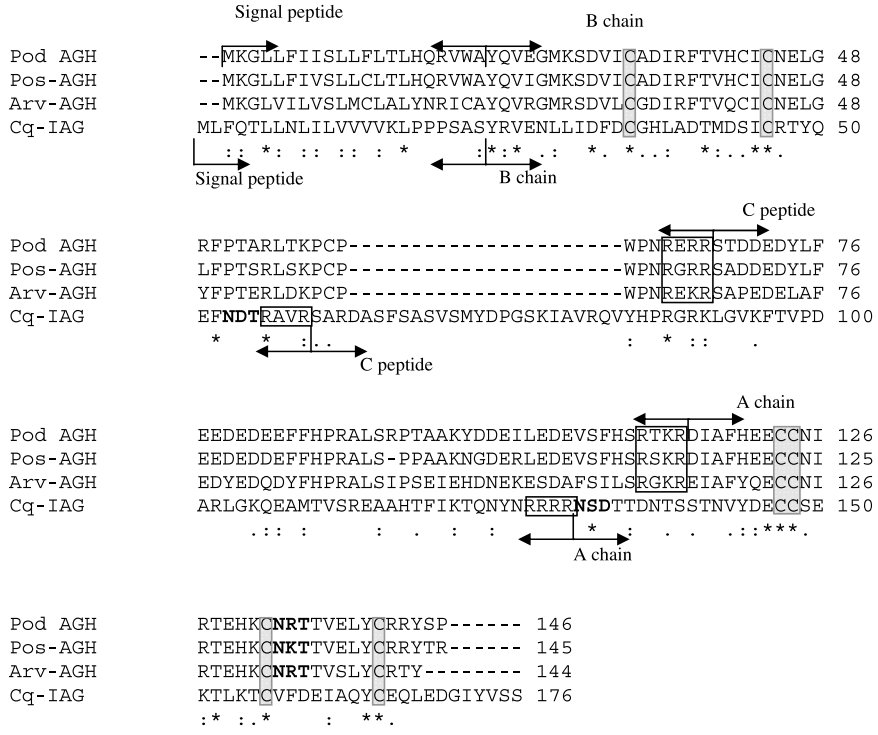

Fig. 7. Alignment of AGH precursors from the isopods *P. dilatatus* (Pod, Ohira et al., 2003), *P. scaber* (Pos, Ohira et al., 2003) and *A. vulgare* (Arv, Okuno et al., 1999) in comparison to *Cq-IAG* predicted precursor. The sequences from the isopods and from *C. quadricarinatus* are aligned using CLUSTAL X. Gray boxes indicate the positions of conserved Cys residues. The typical proteolytic cleavage motifs (R-X-X-R) and the putative *N*-linked glycosylation motif (N-X-S/T) are indicated by open boxes and bold letters, respectively.

of expression of insulin-like growth factors in each sex (Baeza et al., 2001; Garnett et al., 2004; Geary et al., 2003). Such was shown to be the case with bombyxin, suggesting this peptide as being responsible for the regulation of physiological changes underlying sexually distinct activities of adult moths (Satake et al., 1999). In *D. melanogaster*, it was suggested that insulin, which is expressed in both sexes, controls sex-specific differences in the locomotor activity of adults (Belgacem and Martin, 2006). Sex-specific expression of non-insulin-like genes related to the insulin-like growth factors axis was also reported in mammals (Amador-Noguez et al., 2005; Gardmo et al., 2002; Laz et al., 2004; Rosenfeld, 2004). Recently, it was shown that the insulin receptor tyrosine kinase family is required for the appearance of male gonads, and thus for male sexual differentiation, in mice (Nef et al., 2003). Thus, *Cq-IAG* represents a unique case of an insulin-like gene expressed exclusively in males. The discovery of a gender-specific insulin-like family member suggests, therefore, that insulins may have evolved in the context of regulating sexual differentiation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygcen.2006.09.006.

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