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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.