General and Comparative Endocrinology 215 (2015) 76–87

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Discovery of a novel insulin-like peptide and insulin binding proteins in the Eastern rock lobster *Sagmariasus verreauxi*

Jennifer C. Chandler [a], Joseph Aizen [a], Abigail Elizur [a], Lian Hollander-Cohen [b], Stephen C. Battaglene [c], Tomer Ventura [a,*]

[a] GeneCology Research Centre, Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast, 4 Locked Bag, Maroochydore, Queensland 4558, Australia
[b] The Robert H. Smith Faculty of Agriculture, Food and Environment, Department of Animal Sciences, The Hebrew University of Jerusalem, Rehovot 76100, Israel
[c] Institute for Marine and Antarctic Studies, University of Tasmania, Private Bag 49, Hobart, Tasmania 7001, Australia

ARTICLE INFO

Article history:
Available online 9 September 2014

Keywords:
Crustacea
Insulin-like peptide (ILP)
Drosophila ILP7 (DILP7)
Insulin-like growth factor binding protein (IGFBP)
Single insulin-binding domain (SIBD)

ABSTRACT

This study reports, for the first time in any of the commercially important decapod species, the identification of an insulin-like peptide (ILP), distinct from the androgenic gland hormone. Bioinformatics analysis of the *de novo* assembled spiny lobster, (*Sagmariasus verreauxi*) transcriptome, allowed identification of *Sv-ILP1* as well as eight binding proteins. Binding proteins were termed as *Sv-IGFBP*, due to homology with the vertebrate insulin-like growth-factor binding protein and *Sv-SIBD1-7*, single insulin-binding domain protein (SIBD), similar to those identified in other invertebrate species. *Sv-ILP1* was found to be expressed in the eyestalk, gonads and antennal gland of both sexes and to a lesser extent in male muscle, androgenic gland and hepatopancreas. The expression profiles of each binding protein were found to vary across tissues, with *Sv-SIBD5*, *6* and *7* showing higher expression in the gonad, demonstrated by PCR and digital gene expression. Further spatial investigations, using *in-situ* hybridisation, found *Sv-ILP1* to be expressed in the neurosecretory cells of the thoracic ganglia, in keeping with the tissue expression of *Drosophila* ILP7 (DILP7). This correlative tissue expression, considered with the phylogenetic clustering of Sv-ILP1 and DILP7, suggests Sv-ILP1 to be a DILP7 orthologue. The broad expression of *Sv-ILP1* strongly suggests that ILPs have a role beyond that of masculinisation in decapods. The function of these novel peptides may have application in enhancing aquaculture practices in the commercially important decapod species.

1. Introduction

Insulin is the most expansively studied peptide hormone (Wu and Brown, 2006). This year marks 30 years since the identification of the first insulin-like peptide (ILP) in an invertebrate, with the discovery of bombyxin in the silk moth *Bombyx mori* (Nagasawa et al., 1984). In subsequent years there has been an abundance of ILPs identified in invertebrate species, primarily in the arthropods (see Wu and Brown (2006) for a full review), but also the parasitic platyhelminthes (Wang et al., 2013), molluscs (Smit et al., 1998; Floyd et al., 1999), the nematode *Caenorhabditis elegans* (Li and Kim, 2008) and the branchiopod crustacean *Daphnia pulex* (Dircksen et al., 2011), but not in any decapod crustacean species. From an evolutionary perspective, the broad occurrence of ILPs in a wide array of taxonomic phyla is clear evidence of the importance of the insulin-signalling pathway across *Animalia*.

The insulin-like superfamily can be divided into two major subgroups, the insulin-like peptides (ILPs) and the insulin-like growth factors (IGFs); there are also several smaller subgroups, including the vertebrate relaxins that are involved in reproduction (Duret et al., 1998). The ILPs are synthesised as pre-prohormones with an N-terminal (N′) to C-terminal (C′) architecture of a signal peptide, B-chain, C-peptide and A-chain. Post translational modification results in cleavage of the signal and the C-peptide, giving rise to the mature hormone consisting of the A and B-chains. In the majority of cases, the conserved backbone of six cysteine residues results in two inter-chain and one intra-chain (within the A-chain) disulphide bridges (Wu and Brown, 2006); some exceptions exist in the additional disulphide bridges found in several ILPs of *C. elegans* (Duret et al., 1998) and molluscs

Abbreviations: ILP, insulin-like peptide; IAG, insulin-like androgenic gland hormone; IGFBP, insulin-like growth factor binding protein; SIBD, single insulin-binding domain.

* Corresponding author.
E-mail address: tventura@usc.edu.au (T. Ventura).

http://dx.doi.org/10.1016/j.ygcen.2014.08.018
0016-6480/

(Smit et al., 1998; Floyd et al., 1999). An additional intra-chain bridge has also been noted in decapod crustaceans (Ventura et al., 2011). The mature IGFs on the other hand, have a truncated C-peptide that remains un-cleaved and have an additional D-domain at their C′ (the C′ E-domain is cleaved from the prohormone) (Brogiolo et al., 2001; Mizoguchi and Okamoto, 2013). Additional C′ D-domains have been noted in some ILPs, such as the mollusc species, *Aplysia californica*, but unlike IGFs, are cleaved during processing (Floyd et al., 1999).

The functionality of the insulin-like family is diverse, encompassing metabolic, growth, immune and even reproductive roles under endocrine, paracrine and autocrine mediation (Brogiolo et al., 2001; Mizoguchi and Okamoto, 2013; Wang et al., 2013; Wu and Brown, 2006; Yang et al., 2008; Antonova et al., 2012). Such diversity is even apparent within species, where multiple ILPs occur, each with distinct spatial and temporal expression pattern. Examples in the arthropods include the seven ILPs of *Drosophila melanogastar* (*dilps1-7*) (Brogiolo et al., 2001; Grönke et al., 2010) and five ILPs of *Anopheles* species (Krieger et al., 2004; Marquez et al., 2011); in other species, such as the nematode, *C. elegans*, as many as 40 distinct ILP genes have been identified (Li and Kim, 2008). In the case of the branchiopod crustacean *D. pulex*, four ILPs have been identified (Colbourne et al., 2011).

More recently, the functional significance of the ILP family expanded with the discovery of the insulin-like androgenic gland hormone (IAG) in *Crustacea* (Martin et al., 1999; Okuno et al., 1999; Manor et al., 2007). This male specific hormone, produced by and secreted from the male-specific, androgenic gland (AG) was found to be the key regulator of masculine differentiation and maintenance of the male phenotype (Okuno et al., 2002; Rosen et al., 2010; Ventura et al., 2009). The commercial importance of this discovery became apparent a few years later when the suppression of IAG, using *RNAi*, was shown to be a commercially feasible tool to induce full sex-reversal, allowing the production of monosex populations (Ventura et al., 2012). This IAG-mediated technique holds significant benefit to the farming of this group of commercially valuable *Crustacea* (Ventura and Sagi, 2012).

However, since the discovery of IAG, whose expression is limited to the AG, no other ILP has been discovered in the commercially valuable group of decapod crustaceans. Considering the abundance of ILPs with broader expression and functionality that have been identified across other invertebrate taxa, the lack of such ILPs in decapods suggests a significant gap in knowledge. As it stands, there is clear evidence of the glycostatic and anabolic effects of ectopic insulin and IGF-I administered to decapod crustaceans: including the lobster, *Homarus americanus* (Sanders, 1983a); crayfish *Cherax quadricarinatus* (Chaulet et al., 2012; Richardson et al., 1997); and shrimp *Litopenaeus vannamei* (Gutiérrez et al., 2007). Furthermore, the presence of putative ILPs has been demonstrated *via* insulin immunoreactivity in *H. americanus* (Sanders, 1983b) and the isolation of a 6 kDa protein with metabolic effects in the hepatopancreas of *Panulirus argus* (Gallardo et al., 2003).

The discovery of non AG-specific, single insulin-binding domain proteins (SIBDs) in the shrimp (Castellanos et al., 2008) and crab (Gai et al., 2010) adds to the hypothesis that ILPs have a more diverse function in decapods. In vertebrates, insulin-like growth factor binding proteins (IGFBPs) encompass highly conserved N′ and C′ domains. The N′, insulin binding domain (which constitutes the SIBD in decapods) is thought to be most critical for IGF binding, with the C′ dictating specificity and affinity (Castellanos et al., 2008; Hwa et al., 1999). More recently, a complete IGFBP was identified in male *C. quadricarinatus*, structurally similar to those described in vertebrates (Rosen et al., 2013). A binding assay with human insulin, IGF-I and *C. quadricarinatus* AG homogenate, found Cq-IGFBP to specifically bind Cq-IAG. However, expression of Cq-IGFBP was not AG specific, but expressed across multiple tissues (Rosen et al., 2013), suggesting that Cq-IAG may not be the primary binding partner.

Our analyses of the spiny lobster (*S. verreauxi*) transcriptome is, to the best of our knowledge, the first to isolate a non-IAG, ILP in decapods; termed here Sv-ILP1. We found *Sv-ILP1* to be expressed across several tissues of both males and females. In addition we identified an IGFBP (*Sv-IGFBP*) that is broadly expressed in both sexes. Alongside Sv-IGFBP, seven distinct SIBDs (*Sv-SIBD1-7*) were discovered, each with a distinct expression pattern; three of which appear to be highly expressed in the gonad. Taken together, the broader tissue expression of *Sv-ILP1* with *Sv-IGFBP* and the *Sv-SIBDs*, suggests a functional association, warranting further investigation regarding their binding affinities relative to Sv-IAG. Such investigations are a starting point to gain a better functional understanding of this novel ILP and its putative partners. Considering the commercial application of IAG in aquaculture, the discovery of Sv-ILP1 and binding proteins may offer the potential of novel and distinct applications.

2. Materials and methods

2.1. Sample preparation and sequencing

Spiny lobster, *S. verreauxi*, samples were obtained from wild-caught and cultured animals supplied from IMAS aquarium laboratories in Hobart. Lobsters were reared as described in (Fitzgibbon and Battaglene, 2012). Total RNA was isolated from the testis (TS), ovary (OV), male eyestalk (M_ES) and female eyestalk (F_ES) of two mature *S. verreauxi* males and two mature females (intermolt, 2.76–3.11 kg) using Trizol® Reagent (Invitrogen), according to manufacturer's instructions. Next generation sequencing was conducted by BGI (Hong Kong Co. Ltd) as by Illumina protocol (HighSeq 2000, Illumina, San Diego, CA); performing 90 base paired (bp) paired-end sequencing in a single lane. The sequence reads generated for each tissue were stored as FASTQ files.

2.2. Bioinformatics analyses

In order to create the reference transcriptome, raw reads were first cleaned by BGI (using unpublished algorithms). Reads were then *de novo* assembled using the Trinity assembling programme, generating an average of 99,980 Contigs per library, of mean length 384 nt and 65,135 Unigenes, of mean length 647 nt across all four tissues. To ensure accurate assembly 1 million pairs of reads were converted to BAM files and mapped to each tissue assembly. Eyestalk and gonadal transcripts were then annotated by BGI alongside those isolated from other tissues, retrieving annotations with an *e*-value of <0.00001. Databases included: the non-redundant database (NR) and nucleotide database (NT) maintained by the National Centre for Biotechnology Information (NCBI), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups of proteins (COG) and Gene Ontology (GO).

Data mining started with annotation scanning, using key words such as "insulin" to highlight target annotations. Target hits were then computationally translated *via* the ExPASy Proteomic Server (http://web.expasy.org/translate/). The deduced amino acid sequences were analysed further using SMART (http://smart.embl.de/) to predict domain architecture and NCBI (http://www.ncbi.nlm.nih.gov/) as a comparative reference of defined orthologues. Together, this allowed putative sequence structure to be inferred with regard to sequence domains. With regard to the multiple SIBD sequences, Clustal2W alignment programme (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used to ensure that homologues were distinct. Raw sequences of all transcripts were