Fish & Shellfish Immunology 51 (2016) 77–96

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Responses of three very large inducible GTPases to bacterial and white spot syndrome virus challenges in the giant fresh water prawn *Macrobrachium rosenbergii*

Ying Huang [a], Min Jin [c], Shaowu Yin [a, b], Zhengfeng Ding [a], Wen Wang [a, **], Qian Ren [a, *]

[a] Jiangsu Key Laboratory for Biodiversity & Biotechnology, Jiangsu Key Laboratory for Aquatic Crustacean Diseases, College of Life Sciences, Nanjing Normal University, 1 Wenyuan Road, Nanjing 210046, PR China
[b] Co-Innovation Center for Marine Bio-Industry Technology of Jiangsu Province, Lianyungang, Jiangsu 222005, PR China
[c] MOE Key Laboratory of Aquatic Product Safety/State Key Laboratory of Biocontrol, School of Marine Sciences, Sun Yat-sen University, Guangzhou, PR China

ARTICLE INFO

Article history:
Received 25 September 2015
Received in revised form
28 January 2016
Accepted 31 January 2016
Available online 3 February 2016

Keywords:
Macrobrachium rosenbergii
VLIGs
Innate immunity
Bacterial challenge
WSSV

ABSTRACT

Interferons (IFNs) are cytokines secreted by cells in response to invasion by pathogens, such as viruses, bacteria, parasites, or tumor cells. Very large inducible GTPases (VLIG) are the latest IFN-inducible GTPase family to be discovered and are the largest known GTPases of any species. However, VLIG proteins from invertebrates have yet to be characterized. In this study, three forms of VLIGs designated as *MrVLIG1*, *MrVLIG2*, and *MrVLIG3* were cloned from the giant fresh water prawn *Macrobrachium rosenbergii*. *MrVLIG1* has a 5445 bp open reading frame (ORF) encoding an 1814-amino acid protein. The complete nucleotide sequence of *MrVLIG2* cDNA is 7055 bp long consisting of a 5757 bp ORF encoding a protein with 1918 amino acids. The full length of the *MrVLIG3* gene consists of 5511 bp with a 3909 bp ORF encoding a peptide with 1302 amino acids. BLASTP and phylogenetic tree analyses showed that the three MrVLIGs are clustered into one subgroup and, together with other vertebrate VLIGs, into a branch. Tissue distribution analysis indicated that the mRNAs of the three *MrVLIGs* were widely expressed in almost all detected tissues, including the hemocytes, heart, hepatopancreas, gills, stomach, and intestine, with the highest expression in the hepatopancreas. They were also detected in the intestine but with relatively low expression levels. Quantitative real-time RT-PCR analysis showed that the mRNA transcripts of the *MrVLIGs* in the hepatopancreas were significantly expressed at various time points after infection with *Vibrio parahaemolyticus* and white spot syndrome virus. In summary, the three isoforms of VLIG genes participate in the innate immune response of the shrimps to bacterial and viral infections.

1. Introduction

Interferons (IFNs) are a family of immunomodulatory cytokines secreted by host cells in mammals in response to the presence of pathogens, such as viruses, bacteria, parasites, or tumor cells [1,2]. IFNs exert biological activities by binding to specific cell membrane receptors to trigger a well-characterized intracellular signaling pathway culminating in the transcriptional induction of IFN-stimulated genes [3,4]. More than 20 distinct IFN genes and proteins have been identified in animals, including humans. These genes are typically divided among three classes: type-I (I-IFNs), type-II (II-IFN or IFN-γ), and type-III (alternatively termed interleukin 28A/B, 29 or IFN-λ) [5]. Type I IFNs are further classified into at least six subtypes (-α, -β, -ω, -δ, -κ, and -τ) based on primary structure, serology, and time of evolutionary divergence [6,7]. The ability of IFNs to protect against different facultative organisms is conferred by the complex transcriptional programs they initiate [8]. Type I (α/β) and type II (γ) IFNs regulate partially overlapping sets of several hundred genes at the transcriptional level [9,10]. IFN-γ exerts regulatory functions in various physiological and pathophysiological processes [11,12], and as many as 1300 genes might be enlisted for this purpose [13,14]. Meanwhile, at least 100–300 genes are engaged for the same purpose of IFN-α/β [15]. Only a fraction of these mRNA transcripts encode host proteins with well-characterized antimicrobial activities; these proteins include

* Corresponding author.
** Corresponding author.
E-mail addresses: njnuwang@263.net, wenwang@njnu.edu.cn (W. Wang), renqian0402@126.com (Q. Ren).

http://dx.doi.org/10.1016/j.fsi.2016.01.037
1050-4648/

inducible RNA-dependent protein kinase, phagocyte oxidase, nitric oxide synthase 2, indoleamine-2,3-dioxygenase, and natural-resistance associated macrophage protein 1 [16]. However, little is known about other proteins. Prominent within this largely uncharacterized group are guanosine 5′ triphosphatase (GTPase) families, which include immunity-related GTPase (IRG, formerly called p47 GTPases) proteins, guanylate-binding proteins, myxovirus-resistant proteins (Mx), and very large inducible GTPases (VLIGs) [17,18]. Several studies have demonstrated the impact of these proteins on vertebrate host defense and their potential for pathogen specificity from zebrafish to humans [19–23].

Studies over the past few years explored how some IFN-inducible GTPases function as part of a potent host defense program in several vertebrate species. IRGs are a family of large GTPases that mediate innate immune responses and may bridge upstream sensory events with downstream microbicidal activities to help decode the language of vacuolar pathogen recognition [18,24]. GBPs target both vacuolar and cytosolic pathogens, including bacteria, protozoa, and viruses; this superfamily is also capable of self-assembly [21,25,26]. Different from IRGs and GBPs, Mx proteins, which are strongly induced in all cell types by type I IFNs [27], exert antimicrobial activities almost exclusively toward viruses [28,29]. However, few reports are available about the functions of VLIGs. VLIGs with a molecular mass of ~280 kDa are the latest IFN-inducible GTPase family to be discovered [17] and the largest known GTPases in any species [30]. At least six similar VLIG genes encoded on chromosome 7 are evident in mice, with one putative gene found in humans; these genes share ~70% interspecies identity [18]. Homologous expressed sequence tags (ESTs) are also present in rats, pigs, cows, frogs, and fish [17]. However, VLIGs from invertebrates have yet to be characterized.

In the present study, three VLIGs of IFN-inducible GTPases were discovered and characterized from the economically important giant freshwater prawn *Macrobrachium rosenbergii*. Furthermore, a white spot syndrome virus (WSSV) and *Vibrio* infection model was established. This study aims to understand the biology of the three newly discovered IFN-inducible GTPases within the context of IFN-mediated resistance to virus and bacterial infection.

2. Materials and methods

2.1. Tissue collection and immune challenge of M. rosenbergii

Adult *M. rosenbergii* (approximately 15 g each) were purchased from an aquaculture market in Nanjing, Jiangsu Province, China, and acclimatized under laboratory conditions in freshwater tanks at room temperature (25 °C) for a week before processing. Various tissues (hemocytes, heart, hepatopancreas, gills, stomach, and intestine from five healthy prawns as parallel samples) were collected to determine the tissue distributions of *MrVLIG1–MrVLIG3* transcripts. Hemolymph was collected from untreated prawns by mixing 1/2 vol anticoagulant buffer (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, and 10 mM EDTA, pH 4.6) [31]. Samples were immediately centrifuged at 800× *g* for 15 min at 4 °C to harvest the hemocytes.

Seventy prawns were divided into a control group and two experimental groups, namely, *Vibrio parahaemolyticus*- and WSSV-challenged groups. In the *V. parahaemolyticus* group (25 prawns), approximately 3×10^7 cells were injected into the abdominal segment of *M. rosenbergii* by using a 1 mL syringe. In the WSSV-challenged group (25 prawns), approximately 3.2×10^5 copies of WSSV particles were injected into each prawn. The viral inoculums were prepared and quantified as previously described [32]. The hepatopancreases of prawns challenged with *V. parahaemolyticus* or WSSV at 2, 6, 12, 24, and 48 h (five prawns in each time) were also collected and stored at −80 °C for subsequent RNA extraction.

2.2. Total RNA isolation and cDNA synthesis

Total RNA was extracted from the above mentioned tissues in accordance with the manufacturer's instructions (Spin-column, BioTeke, Beijing, China). First-strand cDNA was synthesized using the PrimeScript® 1st Strand cDNA synthesis kit with Oligo dT Primer from TaKaRa (Dalian, China). The cDNA mix was diluted to 1:10 with PCR-grade water and then stored at −80 °C for subsequent SYBR Green fluorescent quantitative real-time PCR (RT-PCR). To obtain the full lengths of these GTPases, 5′ and 3′ cDNA sequences for the rapid amplification of cDNA ends (RACE) were synthesized using the total RNA of the hepatopancreas as a template. The first-strand cDNA (5′ and 3′ cDNAs) was synthesized using the Clontech SMARTer™ RACE cDNA Amplification kit from TaKaRa (Dalian, China) in accordance with the manufacturer's instructions. The primers used were 5′-CDS primer A (5′-T_{25}VN-3′) SMARTer IIA oligo (5′-AAGCAGTGGTATCAACGCAGAGTACXXXXX-3′) and 3′-CDS primer (5′-AAGCAGTGGTATCAACGCAGAGTAC(T)30VN-3′).

2.3. Cloning of full-length cDNAs of MrVLIG1–MrVLIG3

Three ESTs in *M. rosenbergii* similar to very large GTPase genes were obtained from the transcriptome data in the hepatopancreas (data unpublished). On the basis of the obtained cDNA sequences of *MrVLIG1–MrVLIG3*, the following three pairs of gene-specific primers were designed to clone the full-length sequences of *MrVLIG1–MrVLIG3*, respectively: (*MrVLIG1*-F: 5′-GCACAGACGAGAGATGAGAGGACACGAT-3′, *MrVLIG1*-R: 5′-CAATGTTGTTGGAATCCCCTGTGGC-3′; *MrVLIG2*-F: 5′-TCACAGGCATGTGGACAATACAGATGGACG-3′, *MrVLIG2*-R: 5′-CGCCCACTCCTTTGCCTCAGTCATTAT-3′; and *MrVLIG3*-F: 5′-CAGAGGGGTGCGCCTACACTTTAATTGATC-3′, *MrVLIG3*-R: 5′-GACCTGTCTGAATCTGGTGTAGTCTGGGGA-3′). The Advantage 2 PCR Kit from TaKaRa (Dalian, China) was used for gene cloning. PCR reaction was conducted under the following conditions: five cycles at 94 °C for 30 s and 72 °C for 2 min; five cycles at 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 2 min; and 20 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 2 min. Full lengths of *MrVLIGs* were obtained by overlapping the ESTs and 5′ and 3′ fragments.

2.4. Sequence analysis

The cDNA sequences and deduced amino acid sequences were analyzed using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Expert Protein Analysis System (http://web.expasy.org/translate/). The protein domains were predicted with the simple modular architecture research tool (SMART) (http://smart.embl-heidelberg.de/). The calculated molecular weight (MW) and predicted isoelectric point (pI) of the genes were determined through ExPASy (http://web.expasy.org/compute_pi/). The neighbor-joining (NJ) method was used for phylogenetic analysis with MEGA 5.05 software [33]. MEGA 5.05 and GENDOC software were also used to create multiple sequence alignments.

2.5. Tissue distribution and expression pattern analysis of MrVLIG1–MrVLIG3

Quantitative real-time PCR was performed using 2 × SYBR real-time PCR premixture (TaKaRa, Dalian, China) with a real-time thermal cycler following the manufacturer's instructions. Fragments of *MrVLIG1*, *MrVLIG2*, and *MrVLIG3* were cloned using the following specific primers, respectively: *MrVLIG1*-RT-F: 5′-