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Non-specific activation of antiviral immunity and induction of RNA interference may engage the same pathway in the Pacific white leg shrimp *Litopenaeus vannamei*☆

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ABSTRACT

Many questions remain unanswered regarding RNAi-based mechanisms and dsRNA-induced antiviral immune responses in penaeid shrimp. In this study, we report the characterization in the white leg shrimp *Litopenaeus vannamei* of RNAi pathway associated proteins Lv-Ago 1 and Lv-Ago 2, two members of the Argonaute family of proteins, as well as Lv-sid 1, the first shrimp homologue of Sid-1, a membrane channel-forming protein implicated in the cellular import of dsRNA. To decipher their functional implication in RNAi-related phenomena, we monitored their relative expression following stimulation by specific and non-specific RNA duplexes of diverse length. The findings show that the length of small RNA duplexes plays a critical role in the activation of both RNAi-related and innate antiviral responses. They also suggest that these two mechanisms of antiviral response may activate the same pathway, requiring Lv-Sid 1 and Lv-Ago 2 induction.

1. Introduction

RNA interference (RNAi) is a nucleic acid-based mechanism widely conserved among all higher eukaryotes studied so far that mediates sequence-specific targeted gene silencing. This process is initiated by double-stranded RNA (dsRNA), which is processed by a member of the Dicer family of RNase-III-like enzymes into small effector RNA duplexes (e.g. short-interfering RNAs or siRNAs, microRNAs or miRNAs, etc.). The siRNAs are incorporated into a multimeric protein complex, the RNA-induced silencing complex (RISC) and related complexes, of which an Argonaute (Ago) family protein forms the catalytic core. The incorporated RNA then directs the targeted sequence-specific degradation, translational repression, and other silencing phenomena by means of complementary base-pairing. The RNAi mechanism is involved in a variety of biological phenomena including developmental processes (Grishok et al., 2001), heterochromatin remodelling (Riddle and Elgin, 2008), suppression of transposon activity (Aravin et al., 2007) and antiviral immunity (Li and Ding, 2005). Because dsRNA or siRNAs can be supplied exogenously to trigger specific gene silencing, RNAi has rapidly become the most widely used gene-silencing tool in a broad variety of eukaryotic organisms (Campbell and Choy, 2005).

In penaeid shrimp, exploiting this process is becoming increasingly important as an experimental tool to unravel gene function *in vivo*. This is exemplified by the increasing number of studies which have recently resolved gene functions involved in molting (Hui et al., 2008), osmo-regulation (Tiu et al., 2007), reproduction (Treerattrakool et al., 2008), glucose metabolism (Lugo et al., 2006) or immune responses (de la Vega et al., 2008; Shockey et al., 2009) in shrimp by using gene-specific dsRNA technology. RNAi-based applications have also offered new opportunities for experimental blockade of viral infections in shrimp by injecting animals with

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virus-specific RNA duplexes (as first described by Robalino et al., 2005), providing thus a potential approach for virus control in the shrimp farming industry (Shekhar and Lu, 2009). An additional promising avenue of this technology is the observed partial protection from viral infection induced by dsRNA of diverse length, sequence, and base composition (Robalino et al., 2004). Development of methods for induction of this dsRNA-induced innate immunity could be of great interest for aquaculture.

The existence of an intact RNAi machinery in shrimp was first supported by the identification of RNAi pathway homologues such as Pem-AGO in the black tiger shrimp *P. monodon* (Dechklar et al., 2008) and Pm-Ago, another isoform of the Argonaute protein family (Unajak et al., 2006), as well as Pm Dcr1, a member of the Dicer family (Su et al., 2008). However, although a substantially impaired RNAi was observed in *Pem-ago*-depleted cells, suggesting its functional involvement in the silencing process (Dechklar et al., 2008), many questions remain unanswered regarding RNAi-based mechanism in penaeids. First, the core of this machinery and its regulation remain poorly understood. Second, it is still not known whether cross-talk and/or interactions occur between RNAi and the sequence-independent antiviral immunity observed following dsRNA injections. Finally, important efficiency discrepancies to promote gene silencing have been reported following siRNA injections (Li et al., 2007; Robalino et al., 2005; Westenberg et al., 2005; Wu et al., 2007; Wu et al., 2008). These inconsistencies need to be explored to improve efficacy in RNAi-based applications in shrimp aquaculture.

This study was therefore aimed at characterizing RNAi-pathway associated components in the white leg shrimp *L. vannamei*. Specifically, the present work was designed (i) to decipher the functional implication of these components in both innate and RNAi-related phenomena by monitoring their relative expression following stimulation by small RNA duplexes and (ii) to determine the effect of dsRNA length on targeted genetic interference and general antiviral protection.

2. Materials and methods

2.1. Animals and RNA extractions

L. vannamei shrimp from specific pathogen-free (SPF) lines were used for all experiments. Gills were collected in RNA *later* reagent (Ambion) and stored at −20 °C until use. Total RNA was extracted using RNeasy columns (Qiagen) according to the manufacturer's instructions. RNA quantity, purity and integrity were verified spectrophotometrically (A_{260}/A_{280}) and by electrophoresis on 1% agarose gels.

2.2. Cloning of Lv-ago 1, Lv-ago 2 and Lv-sid 1 full-length cDNAs

To isolate a cDNA of *Lv-ago 1*, specific primers (G-3524 and G-3525, Table 1) were designed on a consensus sequence of Argonaute proteins and used for PCR amplification from gill cDNA. PCR was performed as follows: initial denaturation at 95 °C for 5 min; followed by 30 cycles at 95 °C for 30 s; 52 °C for 1 min; 72 °C for 1 min. The full-length cDNA of *Lv-ago 1* was obtained by performing 5′- and 3′-RACE-PCR with the SMART™ RACE cDNA Amplification kit (Clontech/BD Biosciences) using the supplied universal primer mix in combination with either 5′-RACE-primer G-3597 or 3′-RACE-primer G-3595 (Table 1). Amplification profiles for RACE-PCR consisted of 30 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 3 min.

Based on *L. vannamei* expressed sequence tag (EST) sequences homologous to Argonaute and Sid-1 proteins, available at www.marinegenomics.org (O'Leary et al., 2006), 5′-RACE-primer G-3816 and 5′-RACE-primer G-3780 were designed to obtain the 5′ end cDNA sequences of Lv-Sid 1 and Lv-Ago 2 proteins by RACE-PCR, as described above. Finally, the full-length cDNAs of *Lv-ago 1*, *Lv-ago 2* and *Lv-sid-1* were amplified by PCR using specific primers (G-3608/G-3609, G-3822/G-3823 and G-3853/G-3854, respectively) designed at 5′ and 3′ extremities (Table 1) with the BD Advantage 2 polymerase mix (Clontech).

2.3. Phylogenetic tree constructions

A list of known sequences of the members of Argonaute and Sid-1 proteins was obtained from GenBank and EMBL databases using the BLAST (Basic Local Alignment Search Tool) program (Altschul et al., 1997). Trees were built using the MEGA 4 software (Molecular Evolutionary Genetics Analysis, version 4.0) applied to the Neighbor-Joining method (Tamura et al., 2007). Multiple alignments of amino acid sequences were created using the Piwi and multiple transmembrane conserved domains for Argonaute and Sid-1-like proteins, respectively. Bootstrap values (%) of 10,000 replicates were calculated for each node of the consensus tree obtained.

2.4. Preparation of dsRNA

Double-stranded RNAs (dsRNA) were generated as previously described (Robalino et al., 2004). The DNA templates used for *in vitro* transcription were pCR4 vectors (Invitrogen) hosting different size fragments (50, 100, 150 and 200 bp) amplified either from the full-length CDP (CUB domain protein) cDNA (GenBank acc. no. AY907539) or from a 309-bp portion of the immunoglobulin υ (Igυ) cDNA from the duck, *Anas platyrhynchos* (AJ312200), using specific primers indicated in Table 1. Synthetic siRNAs with UU 3′ overhangs specific for duck Igν (Igν siRNA, GGGTTGCCCATGAGGTTCA) and for CDP (CDP siRNA 1, ACTCACCTGGCTGATGTTC; CDP siRNA 2, CACAACCAAGGAACTGATC; CDP siRNA 3, ATTCCACAGCAACAGTGCT) were purchased from Ambion. Finally, siRNA and dsRNAs were diluted to a final concentration of 250 ng/μl in sterile saline solution (10 mM Tris–HCl pH 7.5, 400 mM NaCl) and stored at −80 °C.

2.5. Preparation of viral inoculum and experimental infection

The bioassay system, experimental animals, and white spot syndrome virus (WSSV) inoculum used here have been previously described (Prior et al., 2003; Robalino et al., 2004). Briefly, 1.0–1.5 g SPF *L. vannamei* shrimp (30 shrimp/treatment) were intramuscularly injected with 5 μg (20 μl volume) of either siRNA, dsRNA or sterile saline, and 48 h later injected again with either saline (negative controls) or a WSSV inoculum used at a 5×10^{-8} dilution (weight of infected tissue:volume of saline), to typically yield mortalities close to 80% of injected but otherwise untreated shrimp. Shrimp were kept in culture flasks for 10 days following infection. Cumulative mortality was recorded daily.

2.6. Quantitative real-time PCR

Quantitative real-time RT-PCR (qPCR) was performed on an ABI 7500 system as previously described (Labreuche et al., 2009). Amplification efficiencies for all qPCR primers were determined according to Pfaffl et al. (2002) and the specificity of the PCR amplification verified from the melting curve. Each run included the cDNA control, negative controls (total RNA treated with DNase I), and blank controls (water). The relative mRNA expression levels were determined using the two standard curve methodology (QuantiTect® SYBR Green PCR Handbook) and S3A ribosomal protein (BF023924) was used as the internal reference (normaliser) mRNA. Primer sequences are indicated in Table 1.