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Involvement of *Lv*SID-1 in dsRNA uptake in *Litopenaeus vannamei*

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ABSTRACT

In the past decade, RNA interference (RNAi) has emerged as a successful tool for functional genomics and anti-viral applications in shrimp. However, the mechanism of extracellular dsRNA uptake into shrimp cells has not been determined. Systemic RNA interference defective-1 protein (SID-1) is a transmembrane protein which is required for dsRNA transport between cells in several organisms including *Caenorhabditis elegans* but not in Drosophila and some insects. Recently, a SID-1 homolog (*Lv*SID-1) was identified in whiteleg shrimp (*Litopenaeus vannamei*). Therefore, this study aimed to evaluate whether *Lv*SID-1 is involved in the uptake of dsRNA into shrimp cells. *Lv*SID-1 mRNA expression was up-regulated in gills and muscles but not in hepatopancreas of the shrimp that received long dsRNA introduced by injection. To elucidate the role of *Lv*SID-1 in dsRNA uptake, a strategy of sequential introduction of dsRNAs was employed. Shrimp were initially injected with a long dsRNA to induce *Lv*SID-1 mRNA expression. In a first experiment hemocytes of the *Lv*SID-1 induced shrimp were then collected and seeded in a chamber slide before incubation with Cy3-labeled dsRNA to monitor cellular uptake. Under a confocal microscope, the Cy3 signal in the *Lv*SID-1 induced hemocytes was significantly higher than the signal in naïve hemocytes. In a second experiment, *Lv*SID-1 induced shrimp were separately injected with a second dsRNAs specific to a shrimp endogenous gene (signal transduction and transcription protein (STAT) or clathrin heavy chain (CHC)). Levels of STAT or CHC suppression in the *Lv*SID-1 induced-shrimp compared with the control shrimp reflected the efficiency of uptake of the second dsRNA. Significantly, improved suppression of STAT and CHC was found in gills of the *Lv*SID-1 induced shrimp. These results suggest that *Lv*SID-1 participates in the uptake of injected dsRNA into shrimp cells. This study reports for the first time the involvement of the *Lv*SID-1 in dsRNA uptake in shrimp, which could help to improve the potency of RNAi mediated anti-viral approaches in shrimp in the future.

1. Introduction

RNA interference (RNAi) is a highly conserved post-transcriptional gene regulatory mechanism in eukaryotes (Campbell and Choy, 2005). Over the past decade, it has become a successful tool for functional genomics (Phetrungnapha et al., 2015; Sagi et al., 2013) with potential anti-viral applications (Assavalapsakul et al., 2014; Escobedo-Bonilla, 2011; Sagi et al., 2013; Shekhar and Lu, 2009) in shrimp. Moreover, much progress has been made in recent years with regard to diet-delivered RNAi due to its potential for practical usage in the farm, although its efficiency is currently highly variable (Chimwai et al., 2016; Sanitt et al., 2016; Sanitt et al., 2014; Sarathi et al., 2008; Sellars et al., 2011; Treerattrakool et al., 2013). Uptake of dsRNA by cells and its subsequent processing by the cellular core RNAi machinery prior to the target gene silencing are two main steps in the RNAi process. Although the core components and their roles are intensively studied nowadays (Agrawal et al., 2003; Chen et al., 2012; Dechklar et al., 2008), the mechanism of the cellular uptake of dsRNA by cells remains to be fully characterized.

In invertebrates, uptake of an extracellular dsRNA can occur through two different mechanisms. The first transmembrane channel-mediated mechanism, requires a multispan transmembrane protein called systemic RNA interference defective (SID), which was originally identified in *Caenorhabditis elegans* (*C. elegans*) (Winston et al., 2002; Winston et al., 2007). The SID is necessary for systemic RNAi in *C. elegans* (Rocheleau, 2012) and some insects (Aronstein et al., 2006; Cappelle et al., 2016; Miyata et al., 2014). A second, endocytosis-mediated mechanism, which requires receptor recognition and an endocytosis pathway, has been described in some insects particularly in *Drosophila melanogaster* (*D. melanogaster*) (Saleh et al., 2006; Ulvila et al., 2006).

Amongst the identified SID proteins (SID-1, SID-2, SID-3 and SID-5), the SID-1 protein is the best characterized in *C. elegans* and other animals including mammals (Winston et al., 2002; Duxbury et al., 2005;

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Feinberg and Hunter, 2003; Tsang et al., 2007). Although SID-1 orthologs sharing 11 transmembrane domains (like *C. elegans* SID-1) have been found in many organisms, participation of these SID-1s in dsRNA uptake remains obscure, particularly in insects. The SID-1 homologs (single or multiple) have been identified in most insects such as red flour beetle, *Tribolium castaneum* (Tomoyasu et al., 2008), locust, *Locusta migratoria* (Luo et al., 2012), and *Schistocerca gregaria* (Wynant et al., 2014). Knockdown of their SID-1 homologs did not impair RNAi indicating that their SID-1s are not required for cellular dsRNA uptake. However, SID-1 homologs seem to facilitate systemic RNAi in some insects such as the honey bee (*Apis mellifera*) (Aronstein et al., 2006), the western corn rootworm (*Diabrotica virgifera*) (Miyata et al., 2014), and the Colorado potato beetle (*Leptinotarsa decemlineata*) (Cappelle et al., 2016).

In shrimp, the sequence of a SID-1 homolog was first identified in *Litopenaeus vannamei* (*L. vannamei*) (Labreuche et al., 2010). It encodes a putative protein with an extracellular N-terminal domain and contains 11 predicted transmembrane domains similar to the SID-1 protein of *C. elegans* (*Ce*SID-1). Moreover, up-regulated expression of the SID-1 upon dsRNA injection has been observed in the gills of *L. vannamei* (Labreuche et al., 2010) and in hemocytes of Kuruma shrimp, *Marsupenaeus japonicas* (Maralit et al., 2015). With these characteristics, the shrimp SID-1 (*Lv*SID-1) may function as a transmembrane channel and participate in extracellular dsRNA uptake in shrimp cells like the *Ce*SID-1. Therefore, this study aimed to evaluate whether *Lv*SID-1 is involved in the uptake of dsRNA into shrimp cells. Using sequential dsRNAs administration, shrimp were initially injected with a long dsRNA to induce *Lv*SID-1 mRNA expression. In a first experiment hemocytes of the *Lv*SID-1 induced shrimp were used to monitor cellular uptake of Cy3-labeled dsRNA. In a separated experiment, the *Lv*SID-1 induced shrimp were injected with a second dsRNAs specific to shrimp endogenous mRNAs. Levels of gene suppression in the *Lv*SID-1 induced shrimp as compared to control shrimp reflect the capability of cells to uptake the second dsRNA.

2. Materials and methods

2.1. Shrimp specimens

Post larval (Pl_{15}-Pl_{20}) white leg shrimp (*L. vannamei*) were purchased from Chuchai farm in Chonburi province, Thailand. They were maintained with aeration in a 500 L tank with seawater at 10 ppt salinity. They were fed daily with shrimp commercial diet (CP) until they reached a juvenile stage of roughly 300 mg or 1–2 g (only for the Cy3-labeled dsRNA uptake experiment) of body weight. During experiments shrimp were kept in individual cages in the same tank to avoid cannibalism.

2.2. Production and verification of dsRNA

Each plasmid containing an appropriate dsRNA expression cassette was transformed into *E. coli* HT115 (a RNase III defective strain) (Ongvarrasopone et al., 2007). Six different dsRNAs namely dsSTAT (406 bp) (Attasart et al., 2013), dsGFP (380 bp) (Yodmuang et al., 2006), dsNS1 (425 bp) (Attasart et al., 2010), dsRab7 (393 bp) (Ongvarrasopone et al., 2008), dsCHC (402 bp) (Posiri et al., 2015) and dsRR2 (398 bp) (Attasart et al., 2009), targeting the shrimp signal transduction and transcription protein (STAT), jelly fish GFP, *Penaeus monodon* densovirus ns1, shrimp Rab7, shrimp chlathrin heavy chain and white spot syndrome virus rr2 mRNAs respectively, were used in this study. Bacterial cultures were grown overnight from a single colony in LB medium containing 100 μg/ml ampicillin and 12.5 μg/ml tetracycline. The next day 0.5 OD of starter culture was inoculated in 15 ml of new medium and cells were grown until OD_{600} reached 0.4. Expression of dsRNA was induced with 0.4 mM of isopropyl-β-d thiogalactopyranoside (IPTG). The culture was grown for a further 4 h after induction. Cells were collected and dsRNA was extracted according to the protocol of Posiri et al. (2013), with some minor modifications. Briefly, one OD of bacterial cells, which was collected by centrifugation at 8,000 × *g* at 4 °C for 5 min, was resuspended in 100 μl 75% ethanol in phosphate buffer saline (PBS) and incubated at room temperature for 5 min. The treated cells were collected by centrifugation at 8000 × *g* for 5 min at 4 °C. The cell pellet was resuspended in 100 μl of 150 mM NaCl and incubated at room temperature for 1 h. The cell suspension was centrifuged at 12000 × *g* at 4 °C for 5 min to generate a cell-free supernatant. The dsRNA in the supernatant was diluted before loading into agarose gels to estimate the concentration by comparing with a standard marker. To assess dsRNA structure, 2 μg of dsRNA were digested with RNase A or RNase III enzymes. For the RNase A digestion reaction, 2 μg of dsRNA was incubated with 0.01 μg of RNase A in a final 1 × RNase A buffer (500 mM sodium acetate, 10 mM Tris-Cl pH 7.5) in a total volume of 10 μl. For the RNase III digestion, 2 μg of dsRNA was incubated with 1 × $MnCl_2$ (20 mM), 0.5 U of RNase III in a final 1 × RNase III buffer (50 mM Tris-Cl, 1 mM DTT, 50 mM NaCl, pH 7.5 at 25 °C). The reactions were incubated at 37 °C for 5 min after which they were immediately loaded onto gels.

2.3. Evaluation of the LvSID-1 involvement in cellular dsRNA uptake in shrimp

Using sequential dsRNAs administration, shrimp were firstly injected with an unrelated dsRNA specific to the ns1 gene of *Penaeus monodon* densovirus (dsNS1) to induce *Lv*SID-1 mRNA expression, and then hemocytes of the *Lv*SID-1 induced shrimp were collected and seeded in a chamber slide before incubating with second Cy3-labeled dsRNA to monitor cellular uptake of dsRNA compared with naïve cells (2.4). In a separated experiment, the *Lv*SID-1 induced shrimp were injected with a second dsRNA specific to shrimp STAT (dsSTAT) or shrimp clathrin heavy chain (CHC) (dsCHC) mRNAs (2.5). Levels of STAT or CHC suppression in the *Lv*SID-1 induced shrimp as compared to control shrimp reflect the capability of cells to uptake the second dsRNA.

2.4. Examination of cellular uptake of Cy3-labeled dsRNA in shrimp hemocytes

A dsRNA (393 bp) targeted to the shrimp Rab7 transcript (dsRab7) was labeled with Cy3 using the Silencer® siRNA labeling kit (Ambion). The Cy3-labeled dsRab7 was verified by observation of its mobility shift in gel electrophoresis. The Cy3 dye solution, diluted to the same absorbance as the Cy3-labeled dsRab7 was used as a negative (background) control.

Hemolymph of the *Lv*SID-1 induced shrimp (in parallel with the control shrimp) were withdrawn and mixed with AC-1 solution (27 mM Sodium citrate, 34.33 mM NaCl, 104.5 mM Glucose, 198.17 mM EDTA, pH 7.0) at a 1:1 ratio before seeding in a chamber slide (Lab-Tek® Chamber Slide™). After 20–30 min, the supernatant was replaced with sterile 1 × PBS. The attached hemocytes were incubated at 4 °C for 15 min to inactivate endocytosis activity. Then PBS was replaced with cold Cy3-dsRab7 (1.5 μg/200 μl/well) or Cy3 Dye solution and hemocytes were incubated at 4 °C for 10 min before synchronization of endocytosis by incubating at 28 °C for 1 h. Cells were washed with PBS twice before fixing with 4% paraformaldehyde for 20 min. Then the fixed cells were washed twice with PBS (3 min each) followed by incubation in 0.1% Triton X-100 in PBS for 15 min before washing with PBS. After washing three times, cell nuclei were stained with DAPI (Invitrogen) for 10 min and cells were washed three times with PBS (3 min each). Thereafter, the chamber and gasket were removed, cells were coated with Prolong® Gold antifade reagent (Molecular Probes®) and covered with a coverslip. The fluorescence signal was observed under a Zeiss LSM800 confocal microscope using laser wavelength at 548 nm (Cy3) and 561 nm (DAPI) for excitation and 561 nm (Cy3) and