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ORIGINAL ARTICLE

Uncovering the Mechanisms of Shrimp Innate Immune Response by RNA Interference

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Abstract Because of the importance of shrimp in world aquaculture, there is much interest in understanding their immune system in order to improve their resistance to pathogenic microorganisms. An effective tool in studying genes involved in the immune response in shrimp is RNA interference (RNAi). RNAi, first recognized as an antiviral response against RNA viruses, is a cellular mechanism that is triggered by double-stranded RNAs and results in the degradation of homologous genes. In this review, we describe the current studies of genes in shrimp that employed RNAi technology to elucidate or confirm their functions. We also review the potential of RNAi to elicit antiviral response in shrimp.

Keywords Shrimp · RNAi · Innate immunity

Introduction

Farmed shrimp are a popular food that fetch a high price in both local and international markets. Like other farmed organisms that are cultured at high density, shrimp are vulnerable to a wide array of bacterial and viral pathogens. One approach to controlling microbial pathogens is to better understand the shrimp immune system. While shrimp do not possess an adaptive immune system, they possess an innate immune system that is capable of protecting themselves against pathogens (Lee and Söderhäll 2002). It is a complex system that is poorly understood.

A recent technology for studying immune function is RNA interference or simply RNAi. RNAi is a process whereby small double-stranded RNAs (dsRNAs) trigger the post-transcriptional suppression or silencing of homologous genes in a sequence-specific manner (Fire et al. 1998; Hannon 2002; Tuschl et al. 1999). It has been recognized to be a natural defense mechanism in plants, nematode, insect, and mammals against foreign RNAs such as virus and transposons (Hannon 2002; Waterhouse et al. 2001). First observed in plants where it was referred to as post-transcriptional gene silencing (Vaucheret et al. 2001) or co-suppression (Jorgensen 1990), RNAi, gained considerable attention through the work of Fire et al.(1998) in *Caenorhabditis elegans*. Early studies showed that both sense and anti-sense RNA can effectively inhibit gene expression (Fire et al. 1991; Guo and Kemphues 1995). Fire and his colleagues found that injection of dsRNAs or a mixture of both RNA strands can silence genes with sequences complementary to the introduced dsRNAs and that injected dsRNAs are more potent as a silencing trigger than either sense or antisense RNA strands (Fire et al. 1998).

The RNAi silencing pathway involves the cleavage of long dsRNA by the RNAs-III-like enzyme Dicer into short RNA duplexes, around 21–28 bp in length, called short interfering RNAs (siRNAs) or microRNAs (miRNAs) in the case of miRNA precursors (Hammond et al. 2000; Elbashir et al. 2001). These short RNA duplexes will subsequently unwind and assemble into the RNA-induced silencing complex (RISC) (Hammond et al. 2000). The single-stranded siRNA in the RISC eventually guides the degradation of complimentary target mRNAs leading to translational repression of the target gene (Martinez et al. 2002).

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Although relatively new, RNAi has become a staple in recent molecular studies as a functional genomics tool. RNAi has been used to elicit gene function in worms (Mori et al. 2008; Palakodeti et al. 2008), insects (Cruz et al. 2008; McGregor et al. 2008), to plants (Sappl et al. 2008; Bhuiyan et al. 2008) and livestock (Golding et al. 2006), as well as in cancerous tissues (Lam et al. 2008; Sun et al. 2008). Here we review the recent work in shrimp that has employed RNAi technology and discuss the potential of RNAi to further the understanding of the immune mechanisms in shrimp.

RNAi in Shrimp

The innate immune response in shrimp is mediated by cellular and humoral mechanisms (Tincu and Taylor 2004). Cellular responses include phagocytosis, nodule formation, and encapsulation while humoral responses are effected by three major mechanisms: melanization through the prophenoloxidase (proPO) cascade, clotting mechanism, and release of anti-microbial peptides present in the hemolymph. Activation of the humoral response in shrimp begins with the recognition of microbial cell wall components by pattern recognition proteins in the hemolymph which will trigger the release of antimicrobial peptides and activate the proPO cascade.

Clotting System

Hemolymph coagulation or clotting is an integral part of the invertebrate immune system and the hemolymph-clotting phenomenon was first identified as a prominent defense system in horseshoe crab (*Limulus polyphemus*) by Bang in 1956 (Iwanaga and Lee 2005). Clotting serves to prevent blood loss due to injury and prevent microbes from entering the hemocoel and may also be linked with the release of antimicrobial substances (Iwanaga 2002). The key components in hemocyte coagulation are the clotting protein (CP) and transglutaminase (TGase), which mediates the polymerization or crosslinking of CP to form stable clots (Kopacek et al. 1993; Hall et al. 1999). Clotting is essentially achieved through the activation of TGase, which is released in the hemocytes or tissues, by Ca2+ ions in the plasma leading to the crosslinking of plasma CP molecules into large aggregates (Hall et al. 1999).

CP in crustaceans, first cloned and characterized in crayfish, is homologous to vitellogenins, proteins present in females of egg-laying animals, but differs from vitellogenins in function and are expressed in both males and females (Hall et al. 1999). It was later cloned in a number of shrimp species (Perazzolo et al. 2005; Reyes-Izquierdo and Vargas-Albores 2001; Yeh et al. 1999). On the other hand, tissue localization and cloning of crustacean TGase was done in crayfish (Wang et al. 2001) and also identified in shrimp (Chen et al. 2005; Huang et al. 2004).

RNAi was used to show that the transcription and translation of TGase and CP were effectively silenced in shrimp. Injection of dsRNAs homologous to TGase and CP inhibit blood coagulation in vivo. The effects of the knockdown of both genes showed that TGase- and CP-depleted shrimp hemolymph failed to coagulate at room temperature in contrast to control samples where coagulation was observed moments after their hemolymph were withdrawn. TGase- and CP-depleted shrimp were also more susceptible to white spot virus and *Vibrio penaeicida* infection (Maningas et al. 2008). These results provided further evidence that both genes are involved in the shrimp immune response.

Antimicrobial Peptides

Antimicrobial peptides (AMPs) constitute another major component of the innate immune defense system in marine invertebrates. AMPs, which have masses less than 10 kDa, have a role in the first line of host defense in many animal species (Boman 1995). Among AMPs' advantages are their small size, which allows them to be easily synthesized and be rapidly diffused to the point of infection and their ability to function without either high specificity or memory (Relf et al. 1999). Many AMPs also show remarkable specificity for prokaryotes with low toxicity to eukaryotic cells, making them good candidates for development as potential new antibiotics (Zasloff 1992). Shrimp have a number of AMPs including penaedins (Destoumieux et al. 1997), histones (Patat et al. 2004), crustin (Bartlett et al. 2002; Supungul et al. 2004), anti-lipopolysaccharide factor (ALF) (Somboonwiwat et al. 2005; Liu et al. 2005), and hemocyanin fragments (Destoumieux-Garzon et al. 2001).

To date, RNAi was used to determine or confirm the functions of only two AMPs: ALF and crustin. ALF is a potent anticoagulant that acts by inhibiting the endotoxin- or lipopolysaccharide-mediated activation of the coagulation cascade (Tanaka et al. 1982). ALF gene expression was also found to increase in response to *V. harveyi* infection in black tiger shrimp, *Penaeus monodon* (Tharntada et al. 2008) and was proposed to be a viable prophylactic marker in shrimp (de Lorgeril et al. 2008). Silencing ALF in *L. vannamei* significantly increased the mortality of *V. penaeicida*- and *Fusarium oxysporum*-challenged shrimp, indicating the importance of ALF in bacterial clearance and defense against filamentous fungus infection (de la Vega et al. 2008). Furthermore, this work on ALF was also the first to demonstrate in vivo that a single antimicrobial peptide is necessary for shrimp resistance against diverse pathogens.

Crustin is a cysteine-rich antimicrobial peptide that acts against Gram-positive bacteria (Relf et al. 1999) including *Vibrio harveyi*, a major pathogenic bacterium in shrimp (Amparyup et al. 2008). Injection of dsRNA specific for crustin resulted in a significant increase in mortality of crustin-depleted shrimp after *V. penaeicida* challenge (Shockey et al. 2008). Interestingly, silencing crustin caused no significant mortality after *F. oxysporum* challenge (Shockey et al. 2008). Although both studies confirmed the importance of AMPs in the shrimp immune response, they also demonstrated that AMPs may vary in activity against various pathogenic organisms.

ProPO System

Melanization, through the proPO cascade, of pathogens and of damage tissues is regarded as a major innate response mechanism regulated by the enzyme phenoloxidase (PO). The proPO system is also considered a non-self recognition system since it is activated by small amounts of microbial components such as lipopolysaccharides, peptidoglycans, or β-1,3-glucans (Soderhall and Cerenius 1998). These microbial components or pathogen associated molecular patterns are recognized by pattern recognition receptors which will initiate a serine protease cascade leading to the eventual conversion of the inactive enzyme precursor, proPO, into phenoloxidase (PO). PO will in turn catalyze the oxidation of tyrosine to produce toxic quinones and finally leading to the formation of melanin (Cerenius et al. 2008; Soderhall and Cerenius 1998). Melanin could then bind to the surface of bacteria and increase the adhesion of hemocytes to bacteria, thus accelerating their removal by nodule formation (da Silva 2002). Invertebrate proPO was initially cloned and characterized in freshwater crayfish *Pacifastacus leniusculus* (Aspan et al. 1995), and to date, this gene has already been identified in shrimp and in several invertebrate species (Adachi et al. 1999; Ai et al. 2008, 2009; Aladaileh et al. 2007; Amparyup et al. 2009, reviewed in Cerenius and Soderhall 2004).

Silencing of proPO homologs in shrimp resulted in a significant decrease in endogenous proPO mRNA levels in the hemocytes and a substantial reduction in total PO activity (Amparyup et al. 2009; Fagutao et al. 2009). *P. monodon* proPO-silenced shrimp were also found to be more susceptible to *V. harveyi* infection (Amparyup et al. 2009). On the other hand, injection of dsRNA homologous to proPO in kuruma shrimp resulted in an abrupt increase in mortality of proPO-depleted samples even in the absence of a microbial challenge and a significant increase in endemic bacteria in the shrimp hemolymph (Fagutao et al. 2009). These reports confirm the importance of proPO in shrimp immune response.

Silencing of other Genes in Shrimp

RNAi technology also proved effective in analyzing the function of other genes in shrimp. Silencing of the lymphoid cell-expressed receptor for yellow head virus (YHV) in *P. monodon* (pmYRP65) conclusively proved its role as a receptor for YHV, and it resulted in the complete inhibition of virus entry (Assavalapsakul et al. 2006). Gene silencing of β-integrin, another prominent cell-surface receptor, effectively inhibited white spot syndrome virus (WSSV) infection, which suggests that β-integrin acts as a cellular receptor for WSSV (Li et al. 2007). RNAi has also been used to elucidate the function of genes involved in growth, molting, and reproduction in shrimp. Injection of *P. monodon* with dsRNA specific for the gonad-inhibiting hormone, an important peptide hormone that controls reproduction in crustaceans, increased the level of vitellogenin, the precursor of egg yolk protein levels in the ovary (Treerattrakool et al. 2008). In contrast, silencing of the molt-inhibiting hormone in *Metapenaeus ensis* resulted in decreased vitellogenin expression (Tiu and Chan 2007). RNAi studies also demonstrated the functions of the crustacean vitellogenin receptor (Tiu et al. 2008) and two enzymes that regulate molting and reproduction, hyperglycemic hormone (Lugo et al. 2006; Tiu et al. 2007), and farnesoic acid *O*-methyltransferase. Knockdown of caspase, a main effector for apoptosis in kuruma shrimp, resulted in the increase of WSSV copies, indicating that apoptosis plays a key role in the antiviral response in shrimp (Wang et al. 2008). A separate RNAi assay on a caspase homolog, caspase 3, in *L. vannamei*, however, showed that dsRNA for caspase 3 provided significant protection from low-dose WSSV challenge, which suggests that there could be factors other than apoptosis that contribute to the pathogenicity of the virus (Rijiravanich et al. 2008). Both studies provided evidence that a number of caspases are involved in inducing apoptosis in shrimp. Silencing of Rab GTPases, proteins involved in phagosome formation and maturation, resulted in the increase of WSSV copies and a decrease in phagocytic activity against *Vibrio parahaemolyticus* (Wu et al. 2008; Zong et al. 2008). These findings confirm the role of phagocytosis in the host defense against pathogens. In contrast, silencing of Rab7 effectively inhibited WSSV and YHC infection in *P. monodon*, suggesting that Rab7 has a role in viral replication (Ongvarrasopone et al. 2008). QM protein, a tumor suppressor in shrimp, was also effectively silenced and shown to regulate proPO activation (Xu et al. 2008). Genes that are key components of the RNAi pathway were also assayed using dsRNAs. Silencing of Dicer-1, which is responsible for the cleavage of dsRNAs, increased the viral loads in shrimp, resulting in sharp increase in mortality demonstrating that Dicer-1 is involved in the antiviral response (Su et al. 2008). Silencing of

argonaute, another gene involved in RNAi, resulted in impaired RNAi (Dechklar et al. 2008). Meanwhile, silencing of a novel relish homolog, a gene associated with Rel/NF-κB family that is involved in the Imd pathway, resulted in the suppression of the expression of the AMP penaeidin (Li et al. 2009). This study further implied that an Imd pathway may also be present in shrimp. Table 1 shows the list of genes in shrimp that were analyzed using RNAi.

RNAi as an Antiviral Tool in Shrimp

A number of studies have also shown that injection of dsRNAs or siRNAs specific for viral genes can effectively inhibit the corresponding viral infections (Kim et al. 2007; Tirasophon et al. 2005; Tirasophon et al. 2007; Westenberg et al. 2005; Wu et al. 2007; Xu et al. 2007; Yodmuang et al. 2006). In addition, administration of bacterially synthesized dsRNA specific for WSSV vp28 gene also conferred protective effects against WSSV (Sarathi et al. 2008a, b). These studies suggest that sequence-specific silencing of viral genes may provide a potential therapeutic strategy against viral infections.

Furthermore, recent studies have demonstrated that non-specific RNAi, RNAi constructs that are not homologous to any gene in shrimp, can protect against viral infection. Introduction of non-specific dsRNA to shrimp resulted in the induction of an antiviral immune response even against unrelated viruses, WSSV, and Taura syndrome virus, suggesting that dsRNA can effectively induce a general antiviral immune response (Robalino et al. 2004). Similar results were observed when shrimp was injected with dsRNA and siRNA specific for green fluorescent protein (GFP), a gene absent in shrimp (Maningas et al. 2008; Westenberg et al. 2005; Yodmuang et al. 2006), indicating that innate antiviral defense in shrimp can be effectively triggered by sequence-independent dsRNA or siRNA. These protective effects, however, were achieved when dsRNAs/siRNAs were given prior to viral challenge. A separate study showed that unrelated GFP-dsRNA failed to elicit protective effects in shrimp with active YHV replication, whereas injection with YHV specific protease dsRNA was effective, suggesting that innate antiviral and sequence-specific mechanisms play distinct roles (Tirasophon et al. 2007).

Conclusions

RNAi have undoubtedly revolutionized the field of biology and emerged as a powerful tool for studying gene function in various organisms. In shrimp, the efficiency of RNAi technology in elucidating gene function in vivo has made it

Table 1 Summary of genes in shrimp that were analyzed for their function using RNAi

Shrimp	Gene	RNAi construct	Authors	Year
Litopenaeus schmitti	Crustacean hyperglycemic hormone	dsRNA	Lugo et al.	2006
Penaeus monodon	Yellowhead virus receptor protein 65 (pmYRP65)	dsRNA	Assavalapsakul et al.	2006
Marsupenaeus japonicus	β-integrin	dsRNA	Li et al.	2007
Metapenaeus ensis	Molt-inhibiting hormone	dsRNA	Tiu and Chan	2007
Litopenaeus vannamei	Ion transport peptide	dsRNA	Tiu et al.	2007
Marsupenaeus japonicus	Rab GTPase	siRNA	Zong et al.	2008
Marsupenaeus japonicus	QM protein	siRNA	Xu et al.	2008
Litopenaeus vannamei	Caspase-3	dsRNA	Rijiravanich et al.	2008
Litopenaeus vannamei	Farnesoic acid *O*-methyltransferase	dsRNA	Hui et al.	2008
Penaeus monodon	Argonaute	dsRNA	Dechklar et al.	2008
Penaeus monodon	Dicer-1	dsRNA	Su et al.	2008
Marsupenaeus japonicus	Rab protein	siRNA	Wu et al.	2008
Marsupenaeus japonicus	Caspase	siRNA	Wang et al.	2008
Penaeus monodon	Gonad-inhibiting hormone (GIH)	dsRNA	Treerattrakool et al.	2008
Penaeus monodon	vitellogenin receptor (VgR)	dsRNA	Tiu et al.	2008
Penaeus monodon	Rab7	dsRNA	Ongvarrasopone et al.	2008
Litopenaeus vannamei	Anti-lipopolysaccharide factor (ALF)	dsRNA	de la Vega et al.	2008
Marsupenaeus japonicus	Transglutaminase	dsRNA	Maningas et al.	2008
Marsupenaeus japonicus	Clotting protein	dsRNA	Maningas et al.	2008
Litopenaeus vannamei	Crustin	dsRNA	Shockey et al.	2008
Penaeus monodon	Prophenoloxidase	dsRNA	Amparyup et al.	2009
Marsupenaeus japonicus	Prophenoloxidase	dsRNA	Fagutao et al.	2009

possible to evaluate the complexities of gene regulation in this group. Furthermore, the limitless potential of using RNAi in shrimp opens the door for the discovery of novel genes that might be involved in immune response. RNAi also has the potential of being an excellent therapeutic alternative to combat viral diseases in shrimp. Further studies are needed to explore the prophylactic activity of dsRNAs in order to develop a cheaper and safer substitute to antibiotics. It is equally important to develop means to make these dsRNA feasible for use on a commercial scale.

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