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Short communication

Antiviral effect of PmRab7 knock-down on inhibition of Laem-Singh virus replication in black tiger shrimp

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ABSTRACT

PmRab7 is a *Penaeus monodon* small GTPase protein possibly involved in replication of several shrimp viruses. In this study RNA interference (RNAi) using double-stranded RNA (dsRNA) targeting PmRab7 gene (dsRNA-PmRab7) was employed to silence the expression of PmRab7 to investigate the inhibitory effect on Laem-Singh virus (LSNV) replication. Injection of dsRNA-PmRab7 24 h before challenge with the virus resulted in a drastic decrease of PmRab7 mRNA and complete inhibition of LSNV replication at 3 days post-challenge. In a therapeutic mode, shrimp injected with dsRNA-PmRab7 1 day but not at 3 or 5 days post-LSNV challenge resulted in inhibition of LSNV replication. These results pave the way to use dsRNA-PmRab7 to prevent or cure LSNV infection in shrimp.

Diseases, especially caused by viruses have been the most serious cause of shrimp production loss worldwide. Since 2002, Thailand has encountered a serious problem called monodon slow growth syndrome (MSGS) of black tiger shrimp. The cause of MSGS is still unknown however, a new RNA virus called Laem-Singh virus (LSNV) was identified from slow growth black tiger shrimp (Sritunyalucksana et al., 2006a). A partial genome sequence of the putative RNA dependent RNA polymerase of LSNV was identified (GenBank Accession No. DQ127905). Interestingly, the LSNV positive in the fasciculated zone of the eye was found only in the slow growth but not in the normal growth shrimp from an MSGS pond (Prathoomthai et al., 2008) suggesting the association of retinopathy and slow growth is caused by LSNV. To date, an effective control to prevent or cure LSNV infection has not been established in farmed shrimp.

Several approaches have been used to inhibit viral replication in shrimp. *In vivo* neutralization using antibody against the viral proteins has been employed extensively to prevent viral replication. Anti-VP28 antibody or antibody against other white spot syndrome virus (WSSV) envelop proteins can cause a reduction in the mortality of WSSV-infected shrimps (van Hulten et al., 2001; Wu et al., 2005). Recently, RNA interference (RNAi) using double-stranded RNA (dsRNA) corresponding to viral genes showed promise in combating viral replication in shrimp. DsRNAs corresponding to the viral genes of white spot syndrome virus (WSSV) or yellow head virus (YHV) have been used to prevent or cure viral infection (Robalino et al., 2005, 2004; Tirasophon et al., 2007; Westenberg et al., 2005; Yodmuang et al., 2006). Moreover, dsRNA targeting a specific viral gene can inhibit only the specific corresponding virus (Yodmuang et al., 2006). Therefore, specific dsRNA targeting of a host gene important for viral entry and viral life cycle could be an alternative approach for inhibition of viral replication. Inhibition of viral entry by dsRNAs targeting the viral binding proteins such as the putative YHV receptor (Assavalapsakul et al., 2006) or WSSV-VP28 binding protein now known to be PmRab7 (Ongvarrasopone et al., 2008; Sritunyalucksana et al., 2006b), demonstrated inhibition of YHV or WSSV replication, respectively (Ongvarrasopone et al., 2008; Sritunyalucksana et al., 2006b).

PmRab7 cDNA contains 1357 bp encoding 205 amino acids. PmRab7 contains conserved motifs involved in guanosine-5′-triphosphate (GTP) binding, GTPase activity, an effector binding domain and an isoprenylation site similar to other mammalian Rab proteins. Knock-down of PmRab7 expression by RNAi using dsRNA-PmRab7 not only inhibited WSSV but also inhibited RNA viruses including YHV (Ongvarrasopone et al., 2008) and TSV (unpublished data). The mechanism underlying this phenomenon in shrimp is unclear. It is noted that mammalian Rab7 plays important roles in endosomal trafficking processes in transportation of the cargo from an early to a late endosome or from a late endosome to a lysosome and is involved in lysosome biogenesis and phagocytosis (Bucci et al., 2000; Dale et al., 2004; Feng et al., 1995; Meresse et al., 1995; Vitelli et al., 1997; Zhang et al., 2009). In addition, Rab7 is involved in sorting of virus and in the formation of transport vesicles (Vidricaire and Tremblay, 2005; Vonderheit and Helenius, 2005). This evidence suggests that PmRab7 may function in the viral trafficking pathway and play an essential role during viral repli-

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cation inside the cells. Several viruses enter into host cells via an endocytosis pathway (Sieczkarski and Whittaker, 2002) and may rely on cellular machinery such as Rab proteins for viral infection and replication in the host cells. The cellular target that is required for LSNV entry and replication in host cells is unknown. Therefore, the purpose of this study is to investigate whether knock-down of PmRab7 by dsRNA-PmRab7 exhibits antiviral activity for LSNV in either a preventative or curative manner.

Double-stranded RNA of PmRab7 was produced by *in vivo* bacterial expression (Ongvarrasopone et al., 2007). *E. coli* HT115 containing pET-17b-StRab7 (at nucleotide position 246–639 of GenBank Accession No. DQ321062) was used to express dsRNA-PmRab7. The bacterial culture was grown until OD_{600} reached 0.4 and induced with isopropyl-β-D-thiogalactoside to a final concentration of 0.1 mM for 4 h at 37 °C. The bacterial pellet was collected and the dsRNA-PmRab7 was purified according to the method described earlier. Similarly, an unrelated dsRNA-GFP was also produced using an *in vivo* bacterial expression system (Ongvarrasopone et al., 2007). LSNV inoculum was prepared from lymphoid tissues of the naturally LSNV-infected brood stock shrimp (size 100–120 g) according to the method described previously (Xu et al., 2007) and was stored at −80 °C. The viral nucleic acid was purified from the crude LSNV stock lysate using high pure viral nucleic acid kit (Roche Diagnostics, Germany) and subjected to reverse transcription (RT) and PCR to determine the viral titer using primers LSNVF: 5′-GCT CTT TGC GCC TAT GAA TG-3′ and LSNVR: 5′-GCC CCA GAA ACG TAT TGG CAC-3′. To confirm that the prepared LSNV inoculum was infectious, shrimps were injected with 30 μl LSNV inoculum intramuscularly into the second abdominal segment using 0.5 ml insulin syringe with 29G × 1/2″ needle. The LSNV infectivity was determined by RT-PCR analysis from gill tissues of LSNV-injected shrimp. The viral titer (~10^7 copies) that gave 100% infectivity within 3–5 days was chosen in this study. Prior performing each set of experiment, shrimps (size 1 g) were acclimatized for 3 days to observe signs of lethal infection from viruses such as YHV or WSSV. In addition, screening of the LSNV expression by RT-PCR was performed. Shrimps that were free from LSNV were used in this study. The health status of the experimental shrimps relative to their LSNV infection status are not different.

For prevention study (Fig. 1), shrimps (1 g) were divided into 4 groups. Shrimps were injected intramuscularly with 150 mM NaCl, dsRNAs such as dsRNA-PmRab7, or dsRNA-GFP (0.63 μg g^{-1} shrimp) 1 day before LSNV challenge. Gills were collected 3 days after LSNV challenge for total RNA extraction and RT-PCR analysis (Supplemented Fig. 1). Shrimps injected with 150 mM NaCl only were used as a control group. Injection of dsRNA-PmRab7 1 day prior to challenging with LSNV (10^7 copies) caused significant reduction of endogenous PmRab7 expression approximately 75% and resulted in 100% inhibition of LSNV-infected shrimp after 3 days LSNV challenge (Fig. 1). In contrast, 2 of 5 shrimps injected with dsRNA-GFP showed high levels of LSNV expression and the rest 3 shrimps showed very low levels. Shrimp injected with LSNV alone showed 100% LSNV infection by 3 days whereas LSNV replication was not detected in shrimp injected with 150 mM NaCl (Fig. 1A). The relative expression of PmRab7 in dsRNA-PmRab7 → LSNV group (0.14 ± 0.03) was decreased approximately 73% when compared to NaCl → LSNV group (0.52 ± 0.05) and 75% when compared to dsRNA-GFP → LSNV group (0.56 ± 0.10) (Fig. 1A and B). Expression of PmRab7 was similar in shrimp injected with NaCl, LSNV alone and dsRNA-GFP → LSNV. The results clearly demonstrated that dsRNA-PmRab7, not dsRNA-GFP could significantly knock-down PmRab7 gene and the silencing of PmRab7 resulted in prevention of LSNV replication. The mechanism of LSNV entry into shrimp cells has not been elucidated due to the lack of knowledge of the complete genome sequence of LSNV and its binding proteins. The effect of knocking down PmRab7 on the inhibition

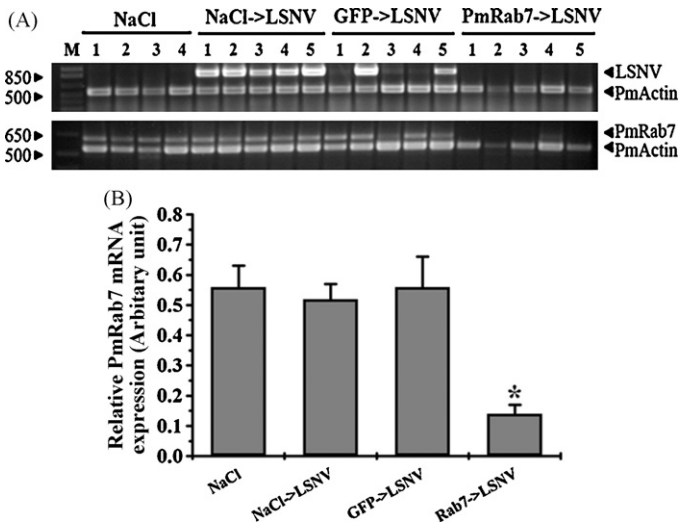

Fig. 1. Inhibition of PmRab7 by dsRNA-PmRab7 for 1 day before LSNV challenge prevents replication. Representative gel of RT-PCR products of LSNV, PmRab7 and PmActin from gills after 3 days challenge (A). M is 1 kb plus DNA marker. Injection of 150 mM NaCl alone ($n = 4$), NaCl → LSNV ($n = 5$), 0.63 μg g^{-1} shrimp of dsRNA-GFP ($n = 5$) and 0.63 μg g^{-1} shrimp of dsRNA-PmRab7 ($n = 5$). The relative mRNA expression of PmRab7 (B) was normalized with PmActin and expressed as mean ± SEM. (*) Statistically significant difference in group injected with NaCl → LSNV and dsRNA-PmRab7 → LSNV ($P < 0.05$).

of LSNV replication suggests a possibility that LSNV may enter and replicate in shrimp cells via the endocytosis pathway and may rely on the host cell endocytic machinery, including PmRab7, for virus trafficking and replication.

To study whether dsRNA-PmRab7 could be used as a therapeutic agent to inhibit LSNV replication in LSNV-infected shrimp, dsRNA-PmRab7 or dsRNA-GFP (0.63 μg g^{-1} shrimp) was injected at day 1, 3, or 5 post-LSNV (10^7 copies) challenge. The gill tissues were collected at day 7 post-LSNV challenge (Supplemented Fig. 1). The replication of LSNV (Fig. 2A) could rarely be detected in shrimp injected with dsRNA-PmRab7 at day 1 post-LSNV challenge. A previous report showed that dsRNA targeting the protease gene of YHV could cure YHV infected shrimp within 12 h post-YHV infection (Tirasophon et al., 2007). LSNV-infected shrimp injected with dsRNA-GFP demonstrated no effect in the reduction of LSNV

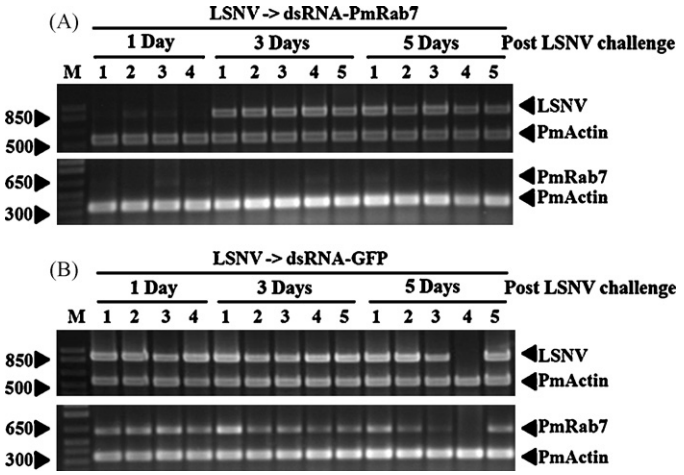

Fig. 2. Therapeutic effect of dsRNA-PmRab7 on LSNV replication. Representative gel of RT-PCR products of LSNV, PmRab7 and PmActin from gills at day 7 post-LSNV challenge. DsRNA-PmRab7 (A) or dsRNA-GFP (B) (0.63 μg g^{-1} shrimp) was injected at day 1 ($n = 4$), day 3 ($n = 5$), or day 5 ($n = 5$) post-LSNV (10^7 copies) challenge. M is 1 kb plus DNA marker.

and PmRab7 expression (Fig. 2B) whereas injection of dsRNA-GFP before LSNV challenge showed partial antiviral activity (Fig. 1). The discrepancy was due to the sequence independent dsRNA triggered the innate antiviral defense which resulted in partial protection in shrimps only if the dsRNA was given before LSNV was challenged (Fig. 1). This evident has been previously reported (Robalino et al., 2004; Tirasophon et al., 2007). Although the expression of PmRab7 was completely inhibited in all samples injected with dsRNA-PmRab7, inhibition of LSNV replication was not observed in LSNV challenged shrimp injected with dsRNA-PmRab7 at day 3 or 5. An explanation may be due to the possibility that the infection of target cells by LSNV had already occurred by day 3 or day 5 (Fig. 1). An increase in viral load before dsRNA injection can minimize the protective effect of dsRNA. The loss of RNAi potency may be due to the virus produced RNAi suppressor (de Vries and Berkhout, 2008). Whether some LSNV proteins act as a suppressor remained to be investigated.

Silencing of PmRab7 by dsRNA-PmRab7 has been shown to inhibit WSSV and YHV replication in *Penaeus monodon* (Ongvarrasopone et al., 2008) and TSV replication in *Litopenaeus vannamei* (unpublished data). DsRNA-PmRab7 could be a valuable tool to inhibit more than one viral infection in shrimp. Viruses that infect shrimp such as LSNV, TSV and YHV are RNA viruses. It is possible that they all enter into the cell via an endocytosis pathway and use endogenous Rab protein for viral trafficking and replication. An efficient approach to completely inhibit viral replication could be developed by using a combination of dsRNAs targeting the viral and the host genes.

In summary, our results demonstrated the potential of knockdown PmRab7 to provide antiviral activity for both preventive and therapeutic modes of LSNV infection. The antiviral effect of knockdown PmRab7 on diverse types of viruses including WSSV, YHV, TSV and LSNV suggests a promising use of RNAi in therapeutic intervention.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2010.06.013.

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