Viral ubiquitin ligase WSSV222 is required for efficient white spot syndrome virus replication in shrimp

Fang He,1 Syed Musthaq Syed,1 A. S. Sahul Hameed2 and Jimmy Kwang1,3

1Animal Health Biotechnology, Temasek Life Sciences Laboratory, National University of Singapore, 1 Research Link, Singapore 117604, Singapore
2OIE Reference Laboratory for WTD, Department of Zoology, C. Abdul Hakeem College, Melvisharam 632 509, Vellore Dist., Tamil Nadu, India
3Department of Microbiology, Faculty of Medicine, National University of Singapore, Block MD4, 5 Science Drive 2, Singapore 117597, Singapore

The E3 ligase WSSV222 of white spot syndrome virus (WSSV) is involved in anti-apoptosis regulation by ubiquitin-mediated degradation of tumour suppressor-like protein (TSL), a shrimp tumour suppressor. In the present study, WSSV222 gene expression was silenced by using specific small interfering RNA (siRNA) in Sf9 and BHK cells. Based on the results of the in vitro silencing, WSSV-challenged shrimp were treated with anti-WSSV222 siRNA to knock down WSSV222 protein expression. The survival rate of shrimp and the efficiency of WSSV replication were assessed to evaluate the efficacy of anti-WSSV222 siRNA in regulating WSSV infection in shrimp. The anti-WSSV222 siRNA reduced the cumulative mortality in shrimp challenged with 10^3 copies of WSSV and delayed the mean time to death in shrimp challenged with the higher dose of 10^6 copies. The results of real-time quantitative PCR showed that virus replication was delayed and reduced in WSSV-challenged shrimp treated with anti-WSSV222 siRNA in comparison with challenged shrimp treated with random-control siRNA. Co-immunoprecipitation assays revealed that WSSV222 silencing inhibited the degradation of TSL in WSSV-challenged shrimp, indicating the requirement for WSSV222 for efficient replication of WSSV in shrimp.

INTRODUCTION

White spot syndrome virus (WSSV) is a virulent shrimp pathogen responsible for high mortality in cultured shrimp, raising major concerns in the aquaculture industry. Disease outbreaks can reach a cumulative mortality of up to 100% within 3–7 days of infection (Escobedo-Bonilla et al., 2008). Histopathological and molecular features of cell apoptosis such as nuclear hypertrophy and DNA fragmentation have been detected in shrimp tissue in the early stages of WSSV infection (Sahtout et al., 2001; Wang et al., 1999). Apoptosis plays a critical role in vertebrate defence against viral pathogens (McLean et al., 2008; O’Brien, 1998). Upon virus infection, host-induced apoptosis in individual infected cells limits viral replication, infectivity and spread. Similarly, in invertebrates, apoptosis is an extremely powerful response to virus infection, which has been mainly characterized in insects with baculovirus infection (Clem, 2005). Several studies have been carried out to investigate changes in the level of apoptosis-related gene expression in WSSV-infected shrimp, such as shrimp caspase and tumour proteins (Bangrak et al., 2004; Leu et al., 2008; Wang et al., 2008b). Some viral proteins function as anti-apoptosis proteins against the host defence system for successful viral replication in the host. In WSSV, a few viral proteins have been found to inhibit apoptosis using different mechanisms (Wang et al., 2004). WSSV449 was identified as a caspase inhibitor (Leu et al., 2008) and anti-apoptosis protein WSSV222 has been found to target tumour suppressor-like protein (TSL), a shrimp tumour suppressor, by ubiquitination (He et al., 2006).

WSSV222, containing a RING domain, has been characterized as a viral E3 ubiquitin ligase (He et al., 2006). Its specific interaction with TSL in shrimp results in ubiquitination and degradation of TSL, which has been studied in both mammalian and shrimp cells. WSSV222 functions as an anti-apoptosis protein, as it can rescue TSL-induced apoptosis in baby hamster kidney (BHK) cells based on ubiquitination-mediated degradation of TSL. However, in WSSV-infected shrimp, the function of WSSV222 and its requirement for efficient WSSV replication is not clear. Small interfering RNA (siRNA) can
induce specific gene silencing in shrimp as a classical RNA interference (RNAi) effect (Elbashir et al., 2002). This technique has been applied in several studies to silence the genes of WSSV in order to protect shrimp from virus infection (Wang et al., 2008b; Westenberg et al., 2005; Xu et al., 2007). In this context, the present study aimed to knock down expression of WSSV222 using specific siRNA and to evaluate the effects of WSSV222 silencing by determining the survival rate of WSSV-challenged shrimp and the efficiency of WSSV replication.

METHODS

Synthesis of siRNAs. The sequence of siRNA used to silence expression of WSSV222 was designed according to the design rule for RNAi using the WSSV222 gene sequence (Elbashir et al., 2002). The sequence of the anti-WSSV222 siRNA was 5'-GTGGAGTGTG-TGCAACATC-3'. For a random-control siRNA, the sequence of the anti-WSSV222 siRNA was rearranged at random and the sequence was designed as 5'-GACGTAGTCTGATGTAGG-3'. The siRNA oligonucleotides were synthesized in vitro by Sigma and annealed according to a protocol reported previously (Elbashir et al., 2002). Briefly, both the sense and antisense RNA oligonucleotides were mixed with annealing buffer (Promega) at a final concentration of 40 μg ml⁻¹. The annealing reaction was heated at 90 °C for 3 min and at 37 °C for 15 min. The annealed siRNA complex was used immediately or stored at −20 °C for up to 1 month.

Shrimp culture, WSSV infection and siRNA injection. Shrimp (Penaeus monodon) of approximately 12 g body weight were collected and screened for WSSV by PCR using WSSV-specific primers (5'-TCGGCATCTGTGATGTAGG-3' and 5'-CTTGGCCACATC-TGCAACATC-3'). WSSV-negative shrimp (five per group for the survival test) were stocked in individual 10 litre containers and acclimatized for 2–3 days prior to the experiment. The experimental shrimp were injected intramuscularly at the second abdominal segment with anti-WSSV222 siRNA or random-control siRNA at a concentration of 20 μM in 100 μl PBS per shrimp 1 day prior to WSSV challenge (Xu et al., 2007). For negative controls, shrimp were injected with 0.9 % NaCl. A dose of 10⁵ or 10⁶ virus copies per shrimp was used in the challenge experiments.

In vitro silencing of WSSV222. Spodoptera frugiperda pupal ovarian (S9) cells (Invitrogen) were grown at 28 °C in serum-free medium (SF-900 II SFM; Gibco-BRL) supplemented with 100 μg gentamicin ml⁻¹ and transfected with siRNA by using Effectene transfection reagent (Qiagen). The full-length WSSV222 gene was inserted into pFAST-HTa (Invitrogen) in the SalI restriction site and the enhanced green fluorescent protein (EGFP) gene was inserted in the SalI and NorI sites. The recombinant WSSV222–EGFP baculovirus was produced according to the manufacturer’s instructions (Invitrogen).

BHCl cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C. The full-length WSSV222 gene was inserted into pEGFP-N1 (Clontech) by using the BamHI and HindIII restriction sites. The cells were transfected with pWSSV222-EGFP (He et al., 2006) and anti-WSSV222 siRNA or nonspecific siRNA (negative control) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The cells were observed under a fluorescence microscope at 24 h post-transfection for gene expression or silencing.

RT-PCR and real-time quantitative PCR. Total RNA from the head tissue of shrimp was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. After treatment with DNase I, the RNA samples were stored in aliquots at −80 °C until further use. Genomic DNA was extracted by using a QIAamp Miniprep kit (Qiagen). Subsequently, RT-PCR amplification of the genes for WSSV222 and the envelope protein VP28 was performed with reverse transcriptase (Stratagene) according to the manufacturer’s protocol as described previously (Khadijah et al., 2003). Briefly, a gene-specific reverse primer was used for reverse transcription, and nested PCR was employed for gene amplification. Actin-specific primers (5’-GAGGTATCTGACTCTCAAG-3’ and 5’-GTCACCAGCTACAGTATG-3’) were also used as an internal control for RNA quality and amplification efficiency. Shrimp haemolymph was collected from the ventral sinus by inserting a 22-gauge needle containing heparin sodium (500 U ml⁻¹, pH 7.2) (Wang et al., 2008b) at 0, 3, 6, 12, 24, 36, 48, 72 and 96 h post-injection. Real-time quantitative PCR was performed using a Master SYBR Green I system and a LightCycler (Roche) as recommended by the supplier with cDNA or genomic DNA from shrimp haemolymph. Primers 5’-CCTACTACTAGCACAAGC-3’ and 5’-TGCAATTCA-TCTGGATCACAGA-3’ were used to amplify WSSV222, and primers 5’-TGGCCATCAGCAGTGGTTG-3’ and 5’-CTTTGGCACCAC-TCTGACATCC-3’ to amplify VP28. A plasmid containing either WSSV222 or VP28 was used as template in each real-time quantitative PCR to standardize the gene copy number.

Co-immunoprecipitation and Western blot analysis. Co-immunoprecipitation assays were performed with total protein extracted from shrimp haernocytes of different experimental shrimp groups. Protein samples were prepared by using a commercial protein extraction kit (Pierce). Briefly, anti-TSL antibody from mouse was incubated with protein A beads (Roche) at room temperature for 1 h. The beads were washed and further incubated with protein samples at 4 °C overnight. Washed beads were then analysed by Western blotting with anti-TSL antibody from guinea pig (He et al., 2006).

Western blot analysis was performed to determine protein expression in haemocytes with anti-TSL (He et al., 2006) or anti-actin monoclonal antibody (Santa Cruz). After separation by SDS-PAGE, the samples were transferred to PVDF membrane. The membrane was blocked for 1 h with 5 % BSA. Blots were probed with a 1:1000 dilution of primary antibody followed by a 1:2000 dilution of horseradish peroxidase-conjugated rabbit anti-mouse or anti-guinea pig IgG (Dako) prior to development with ECL Western blot substrate (GE Healthcare).

Mice and guinea pigs were boosted three times with the same quantities of antigen emulsion of purified glutathione S-transferase–TSL recombinant protein from Escherichia coli every other day for 14 days. Ten days after the final booster injection, the animals were sacrificed by exsanguination and sera were collected.

Fluorimetric assay of caspase activity. A colorimetric assay of caspase-3-like (DEVDase) proteolytic activity was performed using an ApoAlert Caspase-3 Colorimetric Assay kit (Clontech). Shrimp haemocytes were harvested from 1 ml haemolymph containing heparin sodium by centrifugation at 800 g for 5 min. Shrimp haemocytes were lysed in 50 μl lysis buffer on ice for 10 min and centrifuged at 16000 g for 10 min and the supernatant collected. A 50 μl volume of supernatant was added to an equal volume of 2 x reaction/dithiothreitol buffer supplemented with the caspase-3 substrate DEVD-AFC (50 μM) and incubated at 37 °C for 2 h. The absorbance values at 400 nm for caspase-3 were determined. The p-nitroanilide released (nmol h⁻¹) was calculated from the standard curve.

Statistical analysis. Numerical data from three independent experiments were analysed by one-way analysis of variance or Student’s t-test (GraphPad software) based on the mean ± S.D. of triplicate assays. The mean time to death was determined as
RESULTS

WSSV222 silencing in cultured cells and WSSV-infected shrimps

The siRNA-based WSSV222 silencing was primarily studied in BHK cells, which can support efficient WSSV222 expression, as observed in a previous study (He et al., 2006). After co-transfection of BHK cells with the siRNA and pWSSV222-EGFP vector, the expression of WSSV222-EGFP protein was determined and a marked reduction in EGFP fusion protein expression was noted in cells transfected with the specific anti-WSSV222 siRNA (Fig. 1a), compared with the random-control siRNA or no-siRNA control. The level of WSSV222 mRNA was quantified in the transfected cells by a WSSV222-specific real-time RT-PCR assay, normalized to actin mRNA levels. A molar ratio of 10:1 for anti-WSSV222 siRNA to WSSV222 target specifically silenced WSSV222 gene expression by 88%, whereas a 1:1 ratio resulted in only 37% silencing compared with the no-siRNA control. In the presence of the random-control siRNA, however, even at a 10:1 ratio, a silencing of only 17% was found, whilst at ratios of 5:1 and 1:1, there was no significant decrease in WSSV222 mRNA levels compared with the no-siRNA control. These results indicated that the siRNA designed in the present work to target the WSSV222 gene efficiently and specifically silenced WSSV222 expression in vitro.

To test further the effectiveness of anti-WSSV222 siRNA in insect cells, Sf9 cells were transfected with the specific anti-WSSV222 siRNA or with random-control siRNA. At 24 h post-transfection, the cells were infected with recombinant baculovirus expressing the WSSV222–EGFP fusion protein. A reduction in fluorescence intensity from WSSV222–EGFP was observed in the group treated with anti-WSSV222 siRNA (Fig. 1b), indicating a decrease in WSSV222–EGFP expression. In addition, a decrease in WSSV222–EGFP transcription in the anti-WSSV222 siRNA group was detected in a WSSV222-specific real-time RT-PCR. These results confirmed the efficacy of WSSV222-specific siRNA in invertebrate cells and suggest its potential for the inhibition of virus infection.

Based on the results of in vitro studies, an attempt was made to utilize anti-WSSV222 siRNA to protect the shrimp from WSSV. The efficacy of anti-WSSV222 siRNA was evaluated in shrimp injected with 10^7 or 10^8 WSSV copies. Three shrimp were tested at each time point. As indicated in the real-time RT-PCR results, WSSV222 transcription increased over the course of WSSV infection with either dose of virus (Fig. 2a). The dose response of siRNA in shrimp was also analysed by injection of 10^5 or 10^6 virus copies together with different concentrations of anti-WSSV222 siRNA in 100 μl PBS. In real-time RT-PCR for WSSV222 at 48 h post-infection (p.i.), the effects of siRNA were shown to be equivalent at 20 and 40 μM anti-WSSV222 siRNA (Fig. 2b). The inhibitory effect declined rapidly at siRNA concentrations lower than 20 μM, suggesting that the optimum concentration of anti-WSSV222 siRNA was 20 μM in each shrimp. Thus, a concentration of 20 μM anti-WSSV222 siRNA was used in further experiments. In RT-PCR, no WSSV222 mRNA expression could be detected in any of the shrimp with anti-WSSV222 siRNA until 3 days after WSSV infection, whilst WSSV222 transcription was clearly observed in the infected shrimp with random-control siRNA as early as 3 h.p.i. (see Fig. 4a). This result confirmed the silencing of the WSSV222 gene by anti-WSSV222 siRNA in WSSV-infected shrimp.

WSSV222 silencing delays time of death in WSSV-infected shrimp

In order to study WSSV222 function during WSSV infection, the mortality rate was compared among different experimental groups of shrimp. In shrimp challenged with 10^8 copies of WSSV, 100% mortality was reached by 4–6 days p.i. (Fig. 3a). However, the mean time to death in the group with WSSV222 silencing was 4.20 ± 0.82 days,
which was longer than either the no-siRNA WSSV control group (2.13 ± 0.55 days) or the random-control siRNA group (3.46 ± 0.60 days) (P < 0.01). These data indicated that the knockdown of WSSV222 using anti-WSSV222 siRNA resulted in a delay in time of death of WSSV-infected shrimp, although it could not rescue shrimp from death in the high-dose WSSV challenge.

WSSV challenge was also performed with a lower dose of 10^3 virus copies to reveal WSSV222 function in more detail. As shown in Fig. 3(b), the mortality in WSSV-infected shrimp decreased significantly with anti-WSSV222 siRNA (53.33%), compared with either the no-siRNA (100%) and random-control siRNA (100%) groups (P < 0.01). In addition, among those dead shrimp subjected to anti-WSSV222 siRNA, the death of shrimp started at 5 days p.i., whilst the earliest death occurred at 4 days p.i. in the random-control siRNA group and at 3 days p.i. in the no-siRNA group. Taken together, these studies indicated that WSSV222 contributes to the severity of WSSV infection and suggest that WSSV222 silencing could provide mild protection in shrimp from WSSV infection.

Delayed and reduced WSSV replication in shrimp with WSSV222 silencing

After the knockdown of WSSV222 expression in infected shrimp, the effects of anti-WSSV222 siRNA on WSSV replication were investigated by RT-PCR using primers for the WSSV VP28 gene, which encodes the envelope protein. In the random-control siRNA control group, VP28 mRNA was observed in WSSV-infected shrimp as early as 12 h p.i. and gradually increased at subsequent time points (Fig. 4a). However, following silencing of WSSV222 expression, VP28 mRNA expression was delayed and detected only after 24 h p.i., suggesting that WSSV replication was delayed due to WSSV222 silencing. To confirm this finding, the level of VP28 or WSSV222 mRNA was quantified in different groups by using gene-specific real-time RT-PCR (Fig. 4b). During WSSV infection, in addition to delayed virus replication, a reduction in WSSV expression was also observed in the anti-WSSV222 siRNA group. As it is a DNA virus, WSSV copies in infected shrimp were also determined by a real-time PCR based on shrimp DNA templates (Fig. 4c). At 72 h p.i., in WSSV-infected shrimp, WSSV was quantified as 2.56 ± 0.46 × 10^7 virions (μl haemolymph)^{-1}. A similar result was found in the random-control siRNA control.
WSSV222 is required for TSL degradation in WSSV-infected shrimp

TSL is degraded by WSSV222 via the 26S proteasome pathway in mammalian cells. In this study, co-immunoprecipitation with anti-TSL antibody was used to detect the expression of TSL in shrimp during WSSV infection. A reduction in TSL expression was observed in samples from WSSV-infected shrimp when compared with uninfected shrimp. MG132 is an inhibitor of the 26S proteasome. Each shrimp was injected with 20 μM MG132 1 day before infection. There was no reduction in TSL expression in MG132-treated shrimp after WSSV infection (Fig. 5a), confirming TSL degradation in WSSV-infected shrimp. Based on this result, the effects of WSSV222 silencing on TSL expression in WSSV-infected shrimp were studied further. As observed with anti-TSL antibody, reduced TSL degradation was detected in shrimp with knockdown of WSSV222 during WSSV infection, whilst a decrease in TSL was observed in the random-control siRNA group. These results indicated that TSL degradation relies on WSSV222 expression (Fig. 5b). Taken together, we concluded that WSSV222 is essential for efficient WSSV replication in shrimp. 

WSSV222 is required for WSSV replication

WSSV222 is required for WSSV replication in shrimp treated with anti-WSSV222 siRNA, whilst the copy number of virions decreased to 0.13 ± 0.07 × 10^7 (μl haemolymph)^{-1} in the group with anti-WSSV222 siRNA. These results thus demonstrated delayed and reduced WSSV replication in shrimp treated with anti-WSSV222 siRNA, indicating that WSSV222 is essential for efficient WSSV replication in shrimp.

Fig. 4. WSSV222 silencing results in a delay and reduction in WSSV gene expression in shrimp challenged with WSSV (10^6 copies). (a) RT-PCR detection of the VP28 and WSSV222 genes in WSSV-challenged shrimp treated with anti-RSSV222 siRNA or random-control siRNA at different time intervals. Shrimp actin was used as an internal control. Total RNA was extracted from shrimp head tissues. (b, d) WSSV gene expression was quantified by a VP28-specific (left graph) or WSSV222-specific (right graph) real-time RT-PCR during the infection time course. Total RNA was extracted from shrimp haemolymph. The data were normalized based on the expression level of shrimp actin. WSSV expression is indicated in gene copies (μl haemolymph)^{-1} at the various time points shown. Three independent experiments were conducted (P<0.05). (c) WSSV copies were quantified by a VP28-specific real-time PCR in WSSV-challenged shrimp treated with anti-WSSV222 siRNA or random-control siRNA. DNA was extracted from shrimp haemolymph at 72 h p.i. WSSV replication is indicated as WSSV copies×10^7 (μl haemolymph)^{-1}. Three independent experiments were conducted (P<0.01).
required for TSL degradation in shrimp during WSSV infection.

**WSSV222 contributes to the regulation of WSSV-associated apoptosis in shrimp**

To study further the effects of WSSV222 knockdown on cell apoptosis in shrimp, caspase-3 expression in shrimp haemocytes was quantified during WSSV infection (Fig. 6). At 24 h after infection with $10^3$ WSSV copies, increased caspase-3 expression was detected in all WSSV-infected groups compared with the mock group. With WSSV222 silencing, a higher increase in caspase-3 expression was observed, in comparison with the no-siRNA and the random-control siRNA group, suggesting that WSSV222 might contribute to the inhibition of host-induced apoptosis at an early stage of infection. However, at 72 h p.i., the caspase-3 level in shrimp with WSSV222 silencing was significantly lower than the random-control siRNA group, as well as the no-siRNA group, although it was higher than the level detected in the anti-WSSV222 siRNA group at 24 h p.i. These results confirmed that WSSV222 silencing reduces the severity of virus infection in shrimp; they also indicated that the knockdown of WSSV222 has opposing effects on shrimp apoptosis at different stages of WSSV infection and verified the anti-apoptosis function of WSSV222 in shrimp.

**DISCUSSION**

In this study, we have presented direct evidence for the requirement for WSSV222 for efficient WSSV replication in shrimp. As described previously, WSSV222 was able to mediate degradation of a shrimp tumour suppressor and to rescue TSL-induced apoptosis in mammalian cells, suggesting that its function is to antagonize host-induced apoptosis in shrimp during WSSV infection. The results of the present study showed that the knockdown of WSSV222 expression in WSSV-infected shrimps reduced WSSV infection severity and delayed WSSV replication, allowing recovery of TSL expression. Our study further characterized the important role of WSSV222 in WSSV replication in shrimp tissue.

During virus infection, apoptosis in host tissues plays two opposing roles in viral pathogenesis (Tschopp *et al.*, 1998). At the early stage of infection, especially in the initiation of infection, the virus counteracts host-induced apoptosis to allow efficient virus spread and replication (Everett & McFadden, 1999). For example, human papillomavirus uses ubiquitination in the proteolytic removal of the tumour suppressor protein p53 (McLean *et al.*, 2008; Stewart *et al.*, 2005). At the late stage of infection, apoptosis enhances viral pathogenicity by successful virus budding and viral signal transduction (McLean *et al.*, 2008; Rijiravanich *et al.*, 2008). The degradation of TSL after WSSV infection suggests that the shrimp tumour suppressor TSL is involved in host-induced apoptosis for host protection against virus in the early stages of infection. This degradation was inhibited by WSSV222 silencing, together with the early transcription of the WSSV222 gene at 3 h...
WSSV222 is required for WSSV replication

p.i., suggesting the possibility that WSSV222 is an early protein in WSSV. Silencing of WSSV222 delayed death in the shrimp and reduced WSSV replication, strongly implying that WSSV222 functions in the early stages of WSSV infection to inhibit host-induced apoptosis. Hence, in WSSV222-silenced shrimp, host-induced apoptosis could successfully proceed and allow protection of shrimp due to the absence of WSSV222-mediated degradation of TSL. This process could further prevent virus spread to neighbouring tissues and suspend virus replication, which would lead to the delay in mortality and the reduction in WSSV replication observed in the present study.

A newly found shrimp caspase gene is believed to function like human caspase-3, which is one of the key executors of the apoptotic process (Rijiravanich et al., 2008; Wongprasert et al., 2007). Elevated caspase-3 expression has been associated with shrimp mortality from WSSV infection, supporting a link between caspase-induced apoptosis and death (Wongprasert et al., 2007). Therefore, shrimp caspase-3 activity in shrimp haemocytes was determined to evaluate the intensity of apoptosis in WSSV-infected shrimp, to elucidate the anti-apoptosis role of WSSV222.

WSSV222 silencing reduced the mortality in shrimp challenged with a low dose of WSSV and delayed the mean time to death after a high-dose challenge, suggesting that the protection by anti-WSSV222 siRNA against WSSV infection is mild. One possible reason is that the siRNA-based gene silencing did not support permanent protection in shrimp, as suggested in previous results (Wang et al., 2008b; Xu et al., 2007). Another possible explanation is that anti-WSSV222 siRNA is limited and is able to protect shrimp at a low level but not at a high level of WSSV infection. With an excess of virus in the challenge dose, anti-WSSV222 siRNA may block some WSSV222 expression, but leaky expression of WSSV222 may eventually promote virus replication and cause shrimp mortality.

A few viral proteins have been identified as promoting ubiquitination in the host for pathogenesis, such as auxiliary regulatory protein Vpr in human immunodeficiency virus type 1 and the V protein in simian virus 5 and human parainfluenza virus type 2 (Precious et al., 2005a, b; Zhao et al., 2004). The baculovirus inhibitor of apoptosis protein Op-IAP3 ubiquitinates pro-apoptotic cellular proteins as a viral E3 ligase (Green et al., 2004). In WSSV, the RING protein WSSV249 sequesters ubiquitin-conjugating E2s in shrimp and induces an increase in E2 expression after infection (Wang et al., 2005), suggesting enhanced ubiquitination in WSSV-infected shrimp. Here, by using the proteasome inhibitor MG132, TSL degradation was inhibited in WSSV-infected shrimps, indicating that TSL is regulated via the ubiquitin–26S proteasome pathway. Similarly, WSSV222 silencing prevented TSL degradation in shrimp during infection, revealing that WSSV222 is required in the ubiquitin-mediated regulation of TSL. These results indicate that WSSV222 plays an important role in WSSV-induced ubiquitination in shrimp, which contributes to efficient WSSV replication and pathogenesis.

It has been reported in several studies that significant WSSV gene expression can be detected in shrimp haemocytes by immunostaining (Wang et al., 2002, 2008a) or RT-PCR (Wang et al., 2008b). Shrimp haemocytes were shown to be the major target for WSSV attachment (Liang et al., 2005; Sritunyalucksana et al., 2006) and the main source of WSSV production (Syed Musthaq et al., 2006; Wang et al., 2002, 2008b). Therefore, WSSV copy numbers in haemocytes from shrimp indicate the intensity of WSSV infection (Wang et al., 2008b). In addition, shrimp haemocytes can easily be isolated from shrimp haemolymph as a pure, clean tissue sample without contamination from foreign proteins or tissues, compared with other shrimp tissues. Hence, shrimp haemocytes have been widely used in WSSV and shrimp studies to determine and quantify viral or cellular protein and gene expression accurately (Lin et al., 2002; Sritunyalucksana et al., 2006; Wang et al., 2008a). In this study, taking these advantages into consideration, haemocyte samples were exploited to study TSL protein expression and WSSV gene transcription. To date, no continuous shrimp cell line has been established supporting WSSV replication in vitro. Some researchers have reported that crayfish haemocytes also fail to support WSSV replication (Shi et al., 2005). This is possibly caused by differences in the haemocyte origins (Arts et al., 2007) and current technological limitations. Therefore, this does not hinder the use of shrimp haemocytes as a useful cellular platform in studies on the interaction and regulation of WSSV and its host.

The present study demonstrated a possible WSSV222 function in shrimp, a susceptible species for WSSV, during WSSV infection by using siRNA-induced WSSV222 silencing. These findings will help to provide a better understanding of viral anti-apoptosis mechanisms and alternative methods of inhibiting WSSV in cultured shrimp. Further studies should be carried out that focus on TSL function in the host defence against WSSV or other pathogens based on its correlation with WSSV222 in apoptosis regulation.

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