

White spot syndrome virus VP24 interacts with VP28 and is involved in virus infection

Xixian Xie¹ and Feng Yang²

Correspondence

Feng Yang

mbiotech@public.xm.fj.cn

¹School of Life Science, Xiamen University, 178 Daxue Road, Xiamen 361005, People's Republic of China

²Key Laboratory of Marine Biogenetic Resources, Third Institute of Oceanography, Xiamen, People's Republic of China

Received 30 September 2005

Accepted 22 February 2006

White spot syndrome virus (WSSV) is one of the most virulent pathogens causing high mortality in shrimp. Herein, the characterization of VP24, a major structural protein of WSSV, is described. When purified virions were subjected to Nonidet P-40 treatment to separate the envelopes from the nucleocapsids, VP24 was found to be present exclusively in the envelope fraction. Triton X-114 extraction also indicated that VP24 behaves as an envelope protein. Immunoelectron microscopy further confirmed that VP24 is located in the virion envelope. Far-Western experiments showed that VP24 interacts with VP28, another major envelope protein of the WSSV virion. To investigate the function of VP24, WSSV was neutralized with various amounts of anti-VP24 IgG and injected into crayfish. The results showed that anti-VP24 IgG could partially attenuate infection with WSSV. It is concluded that VP24 is an envelope protein and functions at an early stage in virus infection.

INTRODUCTION

White spot syndrome virus (WSSV) represents a novel genus of DNA viruses, *Whispovirus*, belonging to the family *Nimaviridae* (Mayo, 2002). It is a major pathogen in shrimp aquaculture and can also infect most species of crustacean (Chou *et al.*, 1995; Lo *et al.*, 1996; Chang *et al.*, 1998; Wang *et al.*, 1998; Chen *et al.*, 2000; Corbel *et al.*, 2001; Sahul Hameed *et al.*, 2001). First appearing in the 1990s in Taiwan, WSSV spread quickly to South-East Asia, the Indian sub-continent and Central and Latin America, causing catastrophic economic losses. Electron-microscopy studies reveal that WSSV is an enveloped, non-occluded and bacilliform virus (Wang *et al.*, 1995; Wongteerasupaya *et al.*, 1995). The virus contains a double-stranded, circular DNA of about 300 kb, which has been sequenced completely in three WSSV isolates (van Hulten *et al.*, 2001a; Yang *et al.*, 2001; Chen *et al.*, 2002). Approximately 180 open reading frames (ORFs) are revealed by the analysis of the WSSV genomic DNA sequence (GenBank accession no. AF332093).

In previous studies, major structural proteins of WSSV virions were separated by gel electrophoresis and analysed by mass spectrometry, and more than 30 polypeptides matching WSSV ORFs were identified with a molecular mass range of 7–660 kDa (Huang *et al.*, 2002a; Tsai *et al.*, 2004; Zhang *et al.*, 2004). At present, more attention has been paid to the viral envelope proteins, as these commonly play key roles in the primary-infection phase. So far, about 10 genes of WSSV have been identified to encode envelope proteins by using Western blotting or immunoelectron

microscopy (van Hulten *et al.*, 2000b, 2002; Huang *et al.*, 2002b; Zhang *et al.*, 2002a, b, 2004; Li *et al.*, 2005, 2006; Zhu *et al.*, 2005, 2006). Some of these envelope proteins, such as VP28, VP68, VP281 and VP31, were believed to be involved in WSSV infection by *in vivo* neutralization experiments (van Hulten *et al.*, 2001b; Li *et al.*, 2005; Wu *et al.*, 2005) and VP26 was supposed to help the viral nucleocapsid to move toward the nucleus by interacting with actin or cellular actin-binding proteins (Xie & Yang, 2005). However, due to the lack of an established shrimp-cell line, little is known about the molecular events underlying the WSSV life cycle and mode of infection.

VP24, the product of the *wsv002* gene of WSSV (Yang *et al.*, 2001), is a major structural protein of WSSV. Our initial inspection of the VP24 gene sequence suggested that it might be a component of the viral envelope. However, VP24 was thought to be a nucleocapsid protein (van Hulten *et al.*, 2000a). In this report, a more precise localization of VP24 within WSSV virions was determined. In addition, far-Western and neutralization experiments were conducted to further characterize the possible role of VP24 in WSSV infection.

METHODS

Expression and purification of recombinant VP24 (rVP24) in *Escherichia coli*. The WSSV VP24 gene (*wsv002*) was amplified from the genomic DNA of WSSV with the forward primer 5'-GAGAGGATCCACCAACATAGAACTTAAC-3' and the reverse primer 5'-GAGAGAATTCTTTTTCCCAACCTTAAAC-3', carrying

recognition sequences for the *Bam*HI and *Eco*RI restriction enzymes (underlined). The amplified DNA fragment contained sequences corresponding to VP24 aa 26–208. The PCR product was digested with *Bam*HI and *Eco*RI and cloned into pET-His (Shenzhen Gene Power). The recombinant plasmid was transformed into *E. coli* BL21 (DE3) and cultures were induced with 0.1 mM IPTG for 6 h at 30 °C and harvested. The bacterial pellets were resuspended and sonicated and the supernatant recovered after centrifugation was loaded onto a column of Ni-NTA resin (Qiagen). The recombinant protein was purified under native conditions according to the instructions of the QIAexpressionist system (Qiagen).

Preparation of antibody. The purified proteins were used as antigen to immunize mice by intradermal injection once every 10 days. Antigen (20 µg) was mixed with an equal volume of Freund's complete adjuvant (Sigma) for the first injection. Subsequent injections were conducted by using 20 µg antigen mixed with an equal volume of Freund's incomplete adjuvant (Sigma). Four days after the last injection, mice were exsanguinated and antisera were collected. The immunoglobulin (IgG) fractions were purified by protein A-Sepharose (Amersham Biosciences) and stored at –70 °C.

Virus purification and detergent extraction of intact virus. WSSV inoculum was prepared from *Penaeus japonicus* shrimp with pathologically confirmed infection. The infection of healthy crayfish, *Procambarus clarkii*, and the purification of virus were performed as described previously (Xie *et al.*, 2005). Briefly, the tissues of infected crayfish, excluding the hepatopancreas, were homogenized in TNE buffer (50 mM Tris/HCl, 400 mM NaCl, 5 mM EDTA, pH 8.5) and then centrifuged at 3500 g for 5 min at 4 °C. After filtering by nylon net (400 mesh), the supernatant was centrifuged at 30 000 g for 30 min at 4 °C. Then, the upper loose pellet was rinsed out carefully and the lower white pellet was suspended in 10 ml TN buffer (20 mM Tris/HCl, 400 mM NaCl, pH 7.4). After centrifugation at 3500 g for 5 min, the virus particles were sedimented by centrifugation at 30 000 g for 20 min at 4 °C and then resuspended and kept in 1 ml TN buffer.

Viral envelope was separated from the nucleocapsids as described by van Hulst *et al.* (2000b). Briefly, intact virions were mixed with an equal volume of 2 % Nonidet P-40 (NP-40) and incubated for 30 min at room temperature with gentle shaking. The extract was separated into soluble (envelope) and insoluble pellet (nucleocapsid) fractions by centrifugation at 30 000 g for 20 min at 4 °C.

For the Triton X-114 extraction (Bordier, 1981), intact virions were mixed with an equal volume of 2 % Triton X-114 and incubated at 4 °C for 30 min. The mixture was sedimented at 15 000 g for 5 min to separate the phases. Both phases were subjected to a second round of Triton X-114 extraction. All samples were concentrated by acetone precipitation at –20 °C and analysed by SDS-PAGE, transferred to a membrane and detected with anti-VP24 serum.

Immunoelectron microscopy. Purified WSSV virion and nucleocapsid suspensions were mounted on Formvar- or carbon-coated nickel grids (300 mesh), respectively, and incubated for 15 min at room temperature. Then, the grids were blocked with 2 % BSA in PBS for 1 h, followed by incubation with anti-VP24 serum (1:1000 dilution in 2 % BSA) for 1 h. After washing three times with PBS, the grids were incubated with goat anti-mouse IgG conjugated to 10 nm colloid gold (Sigma) for 1 h. Subsequently, the grids were washed three times with PBS and briefly negatively stained with 2 % phosphotungstic acid (PTA; pH 7.0) for 1 min. Specimens were examined under a transmission electron microscope (JEM 100 cxII). For control experiments, pre-immune mouse serum was used to replace the primary antibody indicated above.

Gel electrophoresis and Western blot. Proteins dissolved in loading buffer were separated by standard SDS-PAGE (Laemmli,

1970) with a 12 % resolution gel and a 4 % stacking gel and then transferred onto a PVDF transfer membrane (Amersham Biosciences). The blot was immersed in incubation buffer (20 mM Tris/HCl, 150 mM NaCl, 0.05 % Tween 20, 3 % non-fat milk, pH 7.5) at 4 °C overnight followed by incubating with anti-VP24 serum (1:5000 dilution) for 2 h. Subsequently, alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Promega) (1:7500 dilution) was used as the secondary antibody and detection was performed with a substrate solution containing 4-chloro-1-naphthol and X-phosphate (Roche).

Biotinylation of rVP24 protein and far-Western experiment. Purified rVP24 protein was dialysed against PBS and adjusted to a concentration of 1 mg ml⁻¹. Two hundred micrograms of EZ-Link sulfo-NHS-LC-biotin (Pierce) was added directly to 1 ml rVP24 protein solution. The mixture was incubated at room temperature for 30 min and dialysed against PBS to remove the unreacted biotin reagent.

The viral structural proteins were separated by SDS-PAGE, transferred to a PVDF membrane and renatured gradually at 4 °C overnight in HEPES buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1 % Tween 20, 10 % glycerol, pH 7.5) containing 5 % non-fat milk. The blot was washed and incubated with 0.5 µg biotinylated rVP24 in 5 ml incubation buffer for 1 h at room temperature. The blot was subsequently washed three times and incubated with AP-conjugated streptavidin (Promega) for 1 h at room temperature. After washing three times, the detection was performed with a substrate solution containing 4-chloro-1-naphthol and X-phosphate. Biotinylated glutathione S-transferase (GST) was incubated with viral structural proteins as a control.

Coimmunoprecipitation experiments. The viral envelope proteins were dialysed against PBS and labelled with EZ-Link sulfo-NHS-LC-biotin as described above. The biotinylated envelope proteins were incubated with anti-VP24 serum (1:250 dilution) overnight at 4 °C. Subsequently, 10 µl protein A-Sepharose beads was added to the mixture and incubated for 1 h at 4 °C. The Sepharose beads were collected by centrifugation and washed five times with 500 µl PBS. The bound proteins were dissociated from the antibody by boiling in loading buffer for 5 min and separated on 12 % SDS-PAGE gels. The separated proteins were transferred onto a PVDF membrane and the biotinylated proteins were detected as described above.

In vivo neutralization assay. Virus concentration was quantified by competitive PCR as described by Xu *et al.* (2001). The *in vivo* neutralization experiment was performed as described by van Hulst *et al.* (2001b). Prior to injection, 100 µl virions (equivalent to 10⁶ copies) was incubated with anti-GST IgG (group 2, 20 µg) or with various amounts of anti-VP24 IgG (group 3, 5 µg; group 4, 10 µg; group 5, 20 µg). Then, the mixture of antibody and WSSV was injected intramuscularly into crayfish. At the same time, a negative control (0.9 % NaCl, group 6) and a positive control (WSSV only, group 1) were included in the injection. For each treatment, 20 crayfish were used. Crayfish mortality caused by WSSV was monitored daily.

RESULTS

Expression and purification of rVP24

Computer analysis (ANTHEPROT software) of the 208 aa of VP24 showed that it has a strong hydrophobic region typical of membrane-spanning proteins, i.e. a stretch of 10–23 hydrophobic amino acids (Fig. 1), consistent with the hydrophilic analysis (van Hulst *et al.*, 2000a). For the convenience of purification of the soluble VP24, a truncated

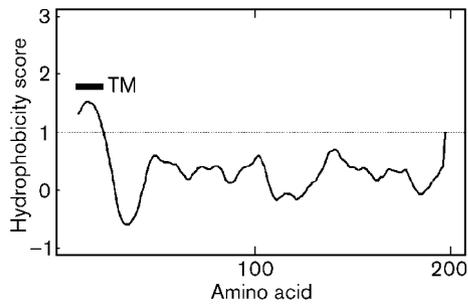


Fig. 1. Transmembrane analysis of VP24 amino acids translated from the DNA sequence. The potential transmembrane (TM) domain is indicated.

form of VP24 without the N-terminal transmembrane domain from aa 1 to 25 was cloned into a bacterial expression vector upstream of a (His)₆ tag. rVP24 was expressed as a soluble protein and purified through an Ni-NTA affinity column.

VP24 associates with viral envelopes

The location of VP24 in the virion is controversial (see Introduction). In this study, we used some different criteria that are used widely to categorize the physical properties of proteins to define the localization of VP24. First, purified WSSV virions were subjected to treatment with 1 % NP-40. Typically, envelope-associated proteins can be released from virions with a non-ionic detergent. As shown in Fig. 2, following NP-40 treatment, the typical envelope proteins (VP28 and VP19) were present exclusively in the envelope

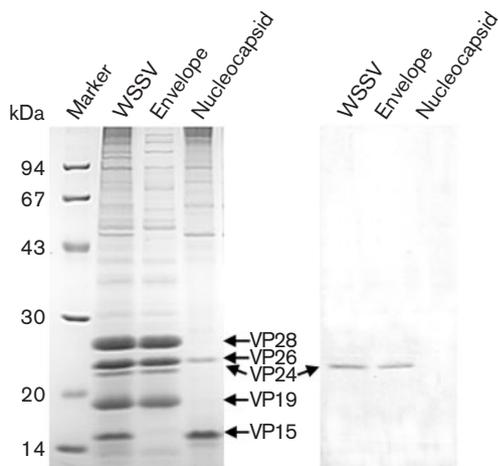


Fig. 2. Purified WSSV virions are subjected to treatment with 1 % NP-40. The left panel shows the gel samples, whilst the right panel shows a Western blot of an equivalent gel probed with anti-VP24 serum. The major structural proteins, VP28, VP26, VP24, VP19 and VP15, are indicated by arrows.

fraction and the nucleocapsid protein (VP15) was found completely in the nucleocapsid fraction, as expected. VP24 was only found in the envelope fraction, which was validated by Western blot. When the virions were subjected to treatment with other detergents, such as Triton X-100, VP24 also partitioned entirely in the envelope fraction, consistent with the literature data reported previously (Xie *et al.*, 2005).

Subsequently, to test whether the hydrophobicity of VP24 was sufficient for envelope association, we extracted purified WSSV virions with Triton X-114. Following phase separation, Triton X-114 separates proteins predominantly according to their hydrophobicity, with hydrophobic and hydrophilic proteins tending to partition into the detergent phase and the aqueous phase, respectively (Bordier, 1981). As envelope proteins contain hydrophobic sequences that anchor them in the envelope, this phase-separation technique can be used operationally to separate envelope proteins from soluble proteins. After this extraction technique, VP24 was found completely in the detergent phase, indicating that it behaves as a hydrophobic protein (Fig. 3).

In order to further confirm the envelope association of VP24, a more precise localization of VP24 in the virions of WSSV was determined by immunoelectron microscopy. The results showed that the gold particles could be seen on the viral envelopes when using anti-VP24 serum as the primary antibody (Fig. 4a), whereas no gold particles could be observed on the viral nucleocapsids when using the same antibody (Fig. 4b). The control experiments showed that no gold particles were found on the envelopes of WSSV virions (Fig. 4c) when using pre-immune mouse serum as the primary antibody. All of these studies demonstrated that VP24 is an envelope protein of WSSV.

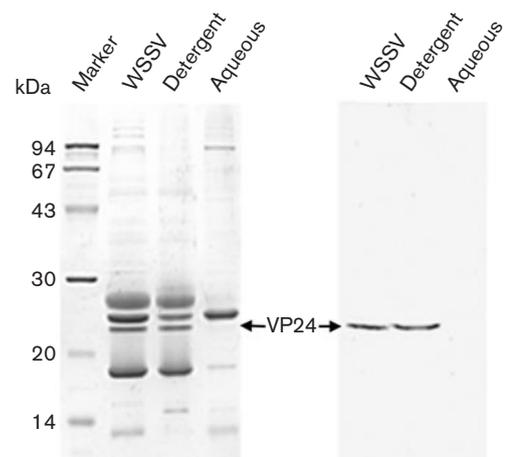


Fig. 3. Purified WSSV virions are subjected to treatment with 1 % Triton X-114. The left panel shows the gel samples, whilst the right panel shows a Western blot of an equivalent gel probed with anti-VP24 serum. VP24 is indicated by an arrow.

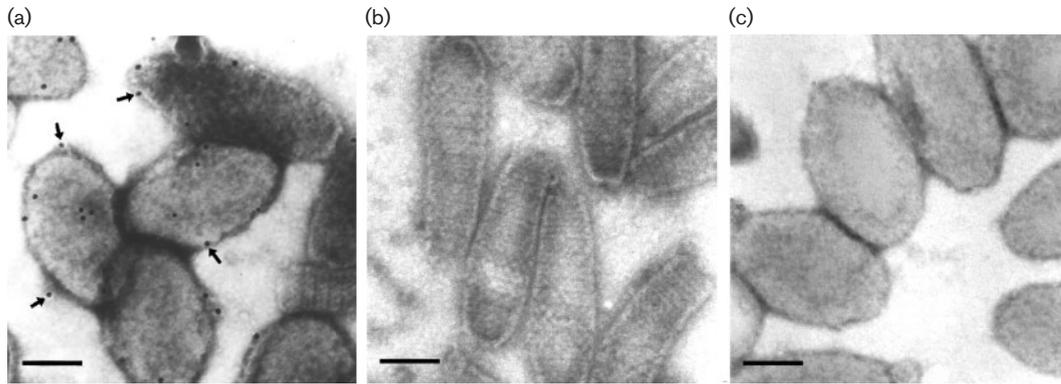


Fig. 4. Immunoelectron microscopy of intact virions and nucleocapsids with anti-VP24 serum followed by gold particle-conjugated secondary antibody. (a) Immunoelectron microscopy of purified WSSV virions using anti-VP24 serum. Gold particles are indicated by arrows. (b) Immunoelectron microscopy of WSSV nucleocapsids using anti-VP24 serum as the primary antibody. (c) Immunoelectron microscopy of WSSV virions using pre-immune mouse serum as the primary antibody. Bars, 100 nm.

Association of VP24 and VP28

In order to characterize the functional properties of VP24, a far-Western experiment using biotinylated rVP24 was performed. The viral structural proteins were separated by SDS-PAGE and transferred to a membrane, renatured and subsequently incubated with biotinylated rVP24 or biotinylated GST as a negative control. After detection using AP-conjugated streptavidin, we observed a prominent band corresponding to VP28, suggesting that there is an interaction between VP24 and VP28 (Fig. 5a). No signal was detected in the GST control.

To further confirm the VP24–VP28 interaction, coimmunoprecipitation analysis was performed. As shown in Fig. 5(b), VP28 was immunoprecipitated with the anti-VP24 antibody. The results of the Western analysis (Figs 2 and 3) showed that there was no cross-reactivity of anti-VP24 antibody with VP28, so VP28 was pulled down by interacting with VP24.

VP24 antibody neutralization *in vivo*

The anti-VP24 IgG was used in an *in vivo* neutralization assay. A constant amount of WSSV was incubated with various amounts of anti-VP24 IgG and injected into crayfish. The results (Fig. 6) showed that the positive-control group (WSSV only, group 1) displayed 100% mortality by day 7 post-infection, whereas the negative-control group (0.9% NaCl, group 6) showed no mortality. Addition of anti-GST IgG (group 2) resulted in a small initial delay of mortality, which reached 100% by day 9. This could be due to compounds injected in crayfish stimulating the host-defence system. The groups injected with various amounts of anti-VP24 IgG mixed with virions exhibited 100% mortality by days 12, 15 and 19 for groups 3–5, respectively. The above experiments were performed once again and the same result could be obtained. Therefore, it could be

concluded that the infection of WSSV was truly delayed or neutralized by the antibody against VP24.

DISCUSSION

WSSV contains five major structural proteins, VP28, VP26, VP24, VP19 and VP15. In previous studies, VP28, VP26 and VP19 were shown to be associated with the viral envelope (van Hulten *et al.*, 2001b, 2002; Zhang *et al.*, 2002a, b; Xie & Yang, 2005) and VP15 with the nucleocapsid (van Hulten

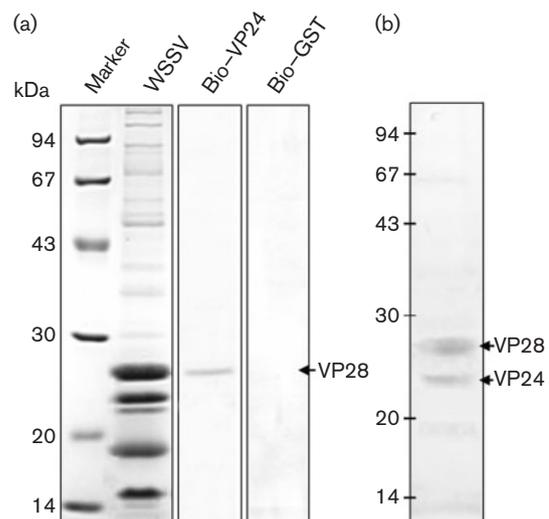


Fig. 5. (a) Far-Western analysis using biotinylated rVP24. The left panel shows the gel samples of viral structural proteins, whilst the middle and right panels show a far-Western analysis of an equivalent gel with biotinylated rVP24 (Bio-VP24) and biotinylated GST (Bio-GST), respectively. VP28 is indicated by an arrow. (b) VP28 is immunoprecipitated with anti-VP24 antibody. VP28 and VP24 are indicated by arrows.

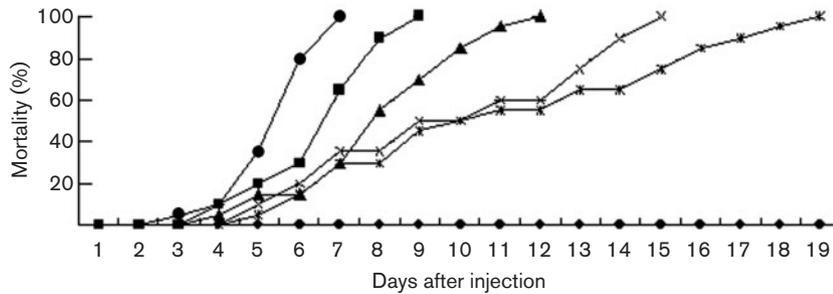


Fig. 6. Neutralization of WSSV infection in crayfish using anti-VP24 IgG. Group 1, WSSV positive control (●); group 2, virus incubated with anti-GST IgG (20 μ g) (■); groups 3–5, virus incubated with various amounts of anti-VP24 IgG [5 μ g (▲), 10 μ g (×) or 20 μ g (*), respectively]; group 6, negative control (◆). In all groups, virions injected into crayfish were equivalent to 10^6 copies.

et al., 2002; Witteveldt *et al.*, 2005). VP24 was initially thought to be a nucleocapsid protein (van Hulst *et al.*, 2000b), but its localization was not confirmed by immunoelectron microscopy. Recent data in the literature showed that VP24 was present exclusively in the envelope fraction when high-purity virions were subjected to non-ionic-detergent treatment (Li *et al.*, 2005; Xie & Yang, 2005; Xie *et al.*, 2005), indicating that it might be an envelope protein. Furthermore, examination of the predicted protein sequence also suggested that VP24 is sufficiently hydrophobic to be envelope-associated (van Hulst *et al.*, 2000a). Therefore, we decided to undertake a detailed biochemical characterization of VP24.

Using our highly purified virus, we used a method described by van Hulst *et al.* (2000b) to separate viral envelopes from the nucleocapsids and obtained cleaner extraction, such that VP24 and the major envelope proteins, e.g. VP28 and VP19, were found exclusively in the envelope fraction (Fig. 2). However, we could not exclude the possibility that different methods and protocols used for separating the envelope or nucleocapsid protein might produce different results. Therefore, we used another detergent (Triton X-100) to extract envelope proteins, obtaining the same results. In addition, Triton X-114 extraction and immunoelectron microscopy confirmed that VP24 behaves as an envelope protein (Figs 3 and 4). According to our current data, this would mean that VP24 is probably envelope-associated.

To date, the function of VP24 in the life cycle of the virus is still unknown. Amino acid analysis of VP24 and VP28 indicated that these two proteins have about 43% amino acid identity (van Hulst *et al.*, 2000a) and it was reported that VP28 could bind to shrimp cells as an attachment protein and help the virus to enter the cytoplasm (Yi *et al.*, 2004). Therefore, we were originally interested in examining whether VP24 is involved in the process of virus entry, as is VP28. First, a cell-binding assay *in vitro* using biotinylated rVP24 was performed. The result indicated that VP24 could not bind to host-cell membranes (data not shown). Subsequently, in order to study further whether VP24 is involved in WSSV infection, an *in vivo* neutralization experiment with anti-VP24 IgG was conducted. The results revealed that infection by WSSV could be delayed significantly by anti-VP24 IgG, suggesting that VP24 plays a role in the WSSV infection. In addition, we have provided evidence for the

direct interaction of VP24 and VP28 and we further postulate that VP24 and VP28 form a protein complex and participate in virus infection together.

Besides participation in virus infection, envelope proteins often play vital roles in virus assembly and budding (Chazal & Gerlier, 2003). Although during the last decade, intensive efforts have been undertaken for characterization of the structural genes, the initial steps in WSSV morphogenesis are poorly understood. Crucial to the understanding of the molecular events is the need to identify the many molecules involved. In this study, additional *in vitro* experiments provided evidence for the direct interaction of VP24 and VP28, the first time that the interaction between these structural proteins has been identified in WSSV. It was reported that protein–protein interactions are needed for viral morphogenesis of *Vaccinia virus* (VACV) (Szajner *et al.*, 2003). VACV G7L protein interacts with the A30L protein and the stability of each was dependent on its association with the other. Both structural proteins are required for the association of dense viroplasm with viral membranes. Although, at present, we could not conclude whether VP24 and VP28 are required for WSSV morphogenesis, the identification of the VP24–VP28 interaction might provide insight to further investigations of the mode of morphogenesis.

Further research is required to reveal the exact role of VP24 in WSSV infection or morphogenesis. Efforts to identify additional proteins associated with VP24 are in progress. We anticipate that further exploration of the functions of envelope proteins, including VP24, will facilitate a better understanding of the molecular mechanism underlying WSSV infection and assembly and may be helpful for the diagnosis and control of virus infection.

ACKNOWLEDGEMENTS

This investigation is supported by the National Natural Science Foundation of China (30330470, 40276038) and the Fujian Science Fund (2003F001).

REFERENCES

Bordier, C. (1981). Phase separation of integral membrane proteins in Triton X-114 solution. *J Biol Chem* **256**, 1604–1607.

- Chang, P.-S., Chen, H.-C. & Wang, Y.-C. (1998).** Detection of white spot syndrome associated baculovirus in experimentally infected wild shrimp, crab and lobsters by in situ hybridization. *Aquaculture* **164**, 233–242.
- Chazal, N. & Gerlier, D. (2003).** Virus entry, assembly, budding, and membrane rafts. *Microbiol Mol Biol Rev* **67**, 226–237.
- Chen, L.-L., Lo, C.-F., Chiu, Y.-L., Chang, F.-C. & Kou, G.-H. (2000).** Natural and experimental infection of white spot syndrome virus (WSSV) in benthic larvae of mud crab *Scylla serrata*. *Dis Aquat Organ* **40**, 157–161.
- Chen, L.-L., Wang, H.-C., Huang, C.-J. & 9 other authors (2002).** Transcriptional analysis of the DNA polymerase gene of shrimp white spot syndrome virus. *Virology* **301**, 136–147.
- Chou, H. Y., Huang, C. Y., Wang, C. H., Chiang, H. C. & Lo, C. F. (1995).** Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. *Dis Aquat Organ* **23**, 165–173.
- Corbel, V., Zuprizal, Z., Shi, C., Huang, Sumartono, Arcier, J.-M. & Bonami, J.-R. (2001).** Experimental infection of European crustaceans with white spot syndrome virus (WSSV). *J Fish Dis* **24**, 377–382.
- Huang, C., Zhang, X., Lin, Q., Xu, X., Hu, Z. & Hew, C.-L. (2002a).** Proteomic analysis of shrimp white spot syndrome viral proteins and characterization of a novel envelope protein VP466. *Mol Cell Proteomics* **1**, 223–231.
- Huang, C., Zhang, X., Lin, Q., Xu, X. & Hew, C.-L. (2002b).** Characterization of a novel envelope protein (VP281) of shrimp white spot syndrome virus by mass spectrometry. *J Gen Virol* **83**, 2385–2392.
- Laemmli, U. K. (1970).** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Li, L., Xie, X. & Yang, F. (2005).** Identification and characterization of a prawn white spot syndrome virus gene that encodes an envelope protein VP31. *Virology* **340**, 125–132.
- Li, H., Zhu, Y., Xie, X. & Yang, F. (2006).** Identification of a novel envelope protein (VP187) gene from shrimp white spot syndrome virus. *Virus Res* **115**, 76–84.
- Lo, C. F., Leu, J. H., Ho, C. H. & 9 other authors (1996).** Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. *Dis Aquat Organ* **25**, 133–141.
- Mayo, M. A. (2002).** A summary of taxonomic changes recently approved by ICTV. *Arch Virol* **147**, 1655–1656.
- Sahul Hameed, A. S., Yoganadhan, K., Sathish, S., Rasheed, M., Murugan, V. & Jayaraman, K. (2001).** White spot syndrome virus (WSSV) in two species of freshwater crabs (*Paratelphusa hydrodomous* and *P. pulvinata*). *Aquaculture* **201**, 179–186.
- Szajner, P., Jaffe, H., Weisberg, A. S. & Moss, B. (2003).** Vaccinia virus G7L protein interacts with the A30L protein and is required for association of viral membranes with dense viroplasm to form immature virions. *J Virol* **77**, 3418–3429.
- Tsai, J.-M., Wang, H.-C., Leu, J.-H., Hsiao, H.-H., Wang, A. H.-J., Kou, G.-H. & Lo, C.-F. (2004).** Genomic and proteomic analysis of thirty-nine structural proteins of shrimp white spot syndrome virus. *J Virol* **78**, 11360–11370.
- van Hulten, M. C. W., Goldbach, R. W. & Vlask, J. M. (2000a).** Three functionally diverged major structural proteins of white spot syndrome virus evolved by gene duplication. *J Gen Virol* **81**, 2525–2529.
- van Hulten, M. C. W., Westenberg, M., Goodall, S. D. & Vlask, J. M. (2000b).** Identification of two major virion protein genes of white spot syndrome virus of shrimp. *Virology* **266**, 227–236.
- van Hulten, M. C. W., Witteveldt, J., Peters, S., Kloosterboer, N., Tarchini, R., Fiers, M., Sandbrink, H., Lankhorst, R. K. & Vlask, J. M. (2001a).** The white spot syndrome virus DNA genome sequence. *Virology* **286**, 7–22.
- van Hulten, M. C. W., Witteveldt, J., Snippe, M. & Vlask, J. M. (2001b).** White spot syndrome virus envelope protein VP28 is involved in the systemic infection of shrimp. *Virology* **285**, 228–233.
- van Hulten, M. C. W., Reijns, M., Vermeesch, A. M. G., Zandbergen, F. & Vlask, J. M. (2002).** Identification of VP19 and VP15 of white spot syndrome virus (WSSV) and glycosylation status of the WSSV major structural proteins. *J Gen Virol* **83**, 257–265.
- Wang, C. H., Lo, C. F., Leu, J. H. & 7 other authors (1995).** Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon*. *Dis Aquat Organ* **23**, 239–242.
- Wang, Y.-C., Lo, C.-F., Chang, P.-S. & Kou, G.-H. (1998).** Experimental infection of white spot baculovirus in some cultured and wild decapods in Taiwan. *Aquaculture* **164**, 221–231.
- Witteveldt, J., Vermeesch, A. M. G., Langenhof, M., de Lang, A., Vlask, J. M. & van Hulten, M. C. W. (2005).** Nucleocapsid protein VP15 is the basic DNA binding protein of white spot syndrome virus of shrimp. *Arch Virol* **150**, 1121–1233.
- Wongteerasupaya, C., Vickers, J. E., Sriurairatana, S. & 7 other authors (1995).** A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*. *Dis Aquat Organ* **21**, 69–77.
- Wu, W., Wang, L. & Zhang, X. (2005).** Identification of white spot syndrome virus (WSSV) envelope proteins involved in shrimp infection. *Virology* **332**, 578–583.
- Xie, X. & Yang, F. (2005).** Interaction of white spot syndrome virus VP26 protein with actin. *Virology* **336**, 93–96.
- Xie, X., Li, H., Xu, L. & Yang, F. (2005).** A simple and efficient method for purification of intact white spot syndrome virus (WSSV) viral particles. *Virus Res* **108**, 63–67.
- Xu, L. M., Wang, W. & Yang, F. (2001).** Research of quantitative PCR technology of white spot bacilliform virus (WSBV). *High Technol Lett* **11**, 14–16 (in Chinese).
- Yang, F., He, J., Lin, X., Li, Q., Pan, D., Zhang, X. & Xu, X. (2001).** Complete genome sequence of the shrimp white spot bacilliform virus. *J Virol* **75**, 11811–11820.
- Yi, G., Wang, Z., Qi, Y., Yao, L., Qian, J. & Hu, L. (2004).** VP28 of white spot syndrome virus is involved in the attachment and penetration into shrimp cells. *J Biochem Mol Biol* **37**, 726–734.
- Zhang, X., Huang, C., Xu, X. & Hew, C. L. (2002a).** Transcription and identification of an envelope protein gene (p22) from shrimp white spot syndrome virus. *J Gen Virol* **83**, 471–477.
- Zhang, X., Huang, C., Xu, X. & Hew, C. L. (2002b).** Identification and localization of a prawn white spot syndrome virus gene that encodes an envelope protein. *J Gen Virol* **83**, 1069–1074.
- Zhang, X., Huang, C., Tang, X., Zhuang, Y. & Hew, C. L. (2004).** Identification of structural proteins from shrimp white spot syndrome virus (WSSV) by 2DE-MS. *Proteins* **55**, 229–235.
- Zhu, Y., Xie, X. & Yang, F. (2005).** Transcription and identification of a novel envelope protein (VP124) gene of shrimp white spot syndrome virus. *Virus Res* **113**, 100–106.
- Zhu, Y.-B., Li, H.-Y. & Yang, F. (2006).** Identification of an envelope protein (VP39) gene from shrimp white spot syndrome virus. *Arch Virol* **151**, 71–82.