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VP90 of white spot syndrome virus interacts with VP26 and VP28

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Summary. – Identification of structural protein relationships is likely to be important for virus assembly and anti-WSSV strategies. In this paper, VP90 of white spot syndrome virus (WSSV) was characterized. Temporal-transcription analysis showed that VP90 is expressed in the late stage of WSSV infection. Far-western and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) assay showed that VP90 interacts with VP26 and VP28, two major envelope proteins. Far-western blot with recombinant VP26 (rVP26), N-terminal end of VP28 (rVP28N) and C-terminal end of VP28 (rVP28C) provided further evidence for interaction of rVP90 and rVP26 or rVP28. These results suggest that VP90 is anchored to the envelope through interacting with VP26 and VP28.

Keywords: WSSV; rVP90; rVP26; rVP28

Introduction

White spot syndrome virus (WSSV) is a virulent shrimp pathogen responsible for high mortality in cultured shrimps, raising major concerns in the aquaculture industry. It causes up to 100% mortality within 3 to 10 days after infection, resulting in major economic losses to the shrimp farming industry (Lightner, 1996). WSSV belongs to a new virus family *Nimaviridae*, under a new genus *Whispovirus* (www.ncbi.nih.gov/ ICTVdb/Ictv/index.htm), which shares a low homology with other known DNA viruses (Mayo, 2002; van Hulten *et al*., 2001). It is an enveloped virus with a 305 kb double-stranded circular DNA genome and approximately 180 open reading frames (van Hulten *et al*., 2001; Yang *et al*., 2001). Over 50 structural protein genes and several non-structural protein genes have been characterized (Zhang *et al*., 2004; Tsai *et al*., 2006; Xie *et al*., 2006b; Li *et al*., 2008). Envelope proteins are important for virus infection, assembly, and budding (Chazal et al., 2003). In previous studies, results had shown that the function of envelope proteins was involved in virus infection and some WSSV structural proteins interact with other structural proteins (Chen *et al*., 2007; Xie *et al*., 2006a; Chang *et al*., 2008; Liu *et al*., 2009). Identification of structural protein interactions is a significant step toward understanding the function of each protein and is potentially helpful in developing effective anti-WSSV strategies (Chang *et al*., 2008).

The VP90 is one of the newly identified envelope proteins in WSSV genome at position 188173–190743 nt (Xie *et al*., 2006b). The open reading frame (wsv327) contains 2568 bp, presumably encodes a protein of 856 aa, with a theoretical M_r of about 90 kDa and is therefore referred to as VP90 in this study. VP90 contains cell attachment motif of RGD which is considered to be critical in interacting with integrin. Until now, there is little information concerning VP90. In this study, the segment of VP90 containing RGD motif was expressed and the interactions between VP90 and other structural proteins were identified. The exploration of the biochemical interactions of WSSV structural proteins might help to elucidate the molecular mechanisms of virion morphogenesis.

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Abbreviations: WSSV = white spot syndrome virus; MALDI = matrix-assisted laser desorption ionization; MS = mass spectrometry; rVP26 = recombinant VP26; rVP28N = recombinant N-terminal end of VP28; rVP28C = recombinant C-terminal end of VP28; rVP90 = recombinant VP90; DIG = digoxigenin; hpi = hours post infection

Materials and Methods

Virus. The infection of healthy crayfish *Procambarus clarkii* and the purification of virus were performed as described previously (Xie *et al.*, 2005).

Plasmid constructs. The N-terminal end of VP90 (wsv327, 245–1002 bp) containing RGD motif was amplified by PCR from WSSV genomic DNA. The forward primer 5′-CCCTCGAGATGAGGAGTGTCTACAGAGA-3′ and the reverse primer 5′-AAAAGCTTTCCTGCAGATGGGGATT-3′ were used to amplify VP90 gene. The amplified DNA and plasmid vector were digested with *Sac*I and *Hind*III (MBI, USA), respectively. After purification and ligation of the DNA fragments, the VP90 gene was inserted into the pBAD/gIII A vector (Invitrogen, USA) among *Sac*I (5′-end) and *Hind*III (3′-end) restriction sites upstream of a (His)′-tag to generate the plasmid pBAD/gIII A-VP90.

rVP26 was cloned using the forward primer of 5′-ACACCATGGATACACGTGTTGGAAG-3′ and the reverse primer 5′- GCGTCTAGAGTCTTCTTCTTGATTTCGT -3′ (*Nco*I and *Xba*I restriction sites are underlined). The PCR products were digested with *Nco*I and *Xba*I and cloned into pBAD/gIIIA vector. N-terminal end of VP28 (VP28N) contains 94 N-terminal amino acids comprising the residues 28 to 121 of VP28. C-terminal end of VP28 (VP28C) consists of 83 C-terminal amino acids comprising the residues 122 to 204 of VP28. Briefly, VP28N and VP28C genes were synthesized by PCR. VP28N and VP28C genes were digested with *Nco*I and *Xba*I and ligated with pBAD/gIIIA vector to form recombinants pBAD/gIIIA-VP28N and pBAD/gIIIA-VP28C. TOP10 (Tiangen, China) competent cells were transformed with rVP90, rVP26, rVP28N, rVP28C plasmids respectively and positive colonies were screened by colony PCR and restriction analysis. Recombinant plasmids were confirmed by DNA sequencing.

Protein expression and labeling. Cultures of TOP10 competent cells carrying the recombinant plasmid were grown in LB medium (100 ml, 50 μg/ml ampicillin) at 37°C with shaking at 300 rpm for 3–4 hrs. Then L-arabinose (Sigma-Aldrich, China) was added to a final concentration of 0.02% and the cultures were incubated for another 5 hrs. Bacteria pellets were harvested by centrifugation at 10,000×g for 10 min, disrupted by sonication and inclusion bodies were acquired by another centrifugation. The pellets were resuspended in guanidine hydrochloride buffer (6 mol/l guanidine hydrochloride, 0.1 mol/l sodium phosphate, 0.01 mol/l Tris-Hcl, pH 8.0). The rVP90, rVP26, rVP28N, rVP28C were purified using a column of TALON Metal Affinity Resins (Clontech, USA) under denaturating conditions and again renatured by successive 12 hr incubation with 6, 4, 2, and 0 mol/l Guanidine-HCL in Tris buffer (20 mmol/l Tris-HCL, 150 mmol/l NaCl, 1 mmol/l EDTA, 25 mmol/l dethiothreitol, 0.1% Tween-20, 10% glycerol, pH 7.5). Purified rVP90 was labeled with digoxigenin (DIG, Roche, Sweden) (Liu *et al.*, 2009).

Western-blot analysis. rVP90, rVP26, rVP28N, rVP28C were separated by SDS-PAGE and blotted to PVDF membrane (Millpore, USA). The membrane was blocked with blocking buffer (2% BSA+PBS) at 4°C overnight. After washing, the membrane was incubated with anti-His antibody (1:10,000) conjugated with HRP for 1 hr at room temperature. The membrane was washed and developed with HRP-substrate kit (Thermos, USA).

Antibody preparation. To prepare the specific antibodies against VP90, the purified rVP90 fusion protein was used as antigen to immunize mice four times by intradermal injection. For the first injection, antigen (50 μg) was mixed with an equal volume of Freund's complete adjuvant (Sigma-Aldrich,China). After two weeks, the following three injections were conducted using 50 μg of antigen mixed with an equal volume of Freund′s incomplete adjuvant (Sigma) once every week. Four days after the last injection, mice were bled and the antisera were collected. The titers of the antisera were assessed by ELISA using horseradish peroxidase conjugated goat anti-mouse IgG (Promega, USA). For a negative control, antigen was replaced with 1 × PBS.

VP90 interactions. The purified WSSV virions were separated by SDS-PAGE, blotted to a PVDF membrane and renatured gradually at 4°C overnight in HEPES buffer (20 mmol/l HEPES, 100 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l dithiothreitol, 0.1% Tween 20, 10% glycerol, pH 7.5) containing 5% non-fat milk. After rinsing with Tris-buffered saline (TBS) containing 0.5% Tween 20, the membrane was incubated with DIG-labeled purified rVP90 for 1 hr at room temperature on a rocking platform. The membrane was washed 3 times with TBS containing 0.5% Tween 20. Then anti-DIG antibody conjugated with HRP was added. After rinsing with TBS, interacting proteins were detected by exposing the membrane to an HRP-substrate kit. DIG-labeled BSA was used as a control. For further confirmation of VP90 interaction with rVP26 and rVP28, rVP26 or rVP28N and rVP28C were separated by 12% SDS-PAGE and blotted to a PVDF membrane. The membrane was renaturated gradually at 4°C overnight in HEPES buffer (20 mmol/l HEPES, 100 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l DTT, 0.1%Tween 20,10% glycerol, pH 7.5) containing 2% non-fat milk. The blot was washed with TBS, and incubated with 20 μg of DIG-labeled VP90 in 2 ml of HEPES buffer containing 1% non-fat milk for 4 hrs at 4°C respectively. After washing, the membrane-bound VP90 proteins were incubated with anti-VP90 antibody followed by incubation in HRP-conjugated rabbit anti-mouse IgG (1:3,000 dilution) for 1 hr and visualized by using diaminobenzidine (DAB,Thermo, USA).

MALDI-MS. The VP90-binding proteins were excised from Coomassie Brilliant Blue-stained gels. The gel pieces were sliced and allowed to dry before trypsin digestion. MALDI-MS was performed, and the MASCOT program was used to analyze the results. All the spectra of the test samples were acquired using the default mode. The data were searched by GPS Explorer using MASCOT as the search engine.

Transcriptional analysis. After WSSV infection, crayfish were randomly removed at 0, 12, 24, 48, and 72 hrs post infection (p.i.) and stored at -80°C. Total RNA was extracted from frozen gills

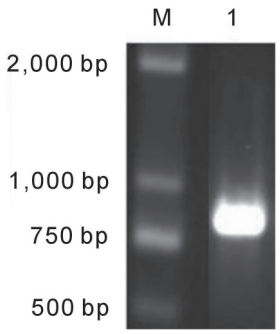

Fig. 1

Amplification of VP90 gene by PCR

PCR product (1) and DNA marker (M).

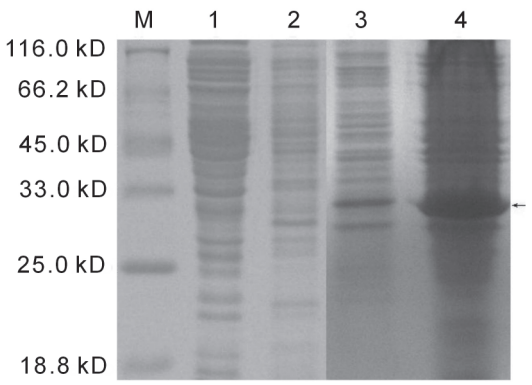

Fig. 2

SDS-PAGE analysis of rVP90 expression

Protein marker (M), negative controls (1–2), supernatant (3), inclusion bodies (4).

using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and digested with DNase (TaKaRa, China) before performing transcriptional analysis and direct PCR amplification. Primers specific for VP90 were used to perform RT-PCR and PCR (forward primer, 5′-AGACTCGAGAATGGACGGTGTGT-3′, reverse primer, 5′-GCCGAATTCCTTTCAAAAACACT-3′). Detection of β-actin mRNA (PCR product of 540 bp) was used as an internal control (forward primer, 5′-GTGGGCCGCTCTA GGCACCAA-3′; reverse primer, 5′-CTCTTTGATGTCACGCA CGATTTC-3′). VP28 was used as a positive control (forward primer, 5′-CTACTCGAGATGGATCTTTCTTTCACTC-3′; reverse primer, 5′- TATAAGCTTTCGGTCTCAGTGCCA-3′).

Results

VP90 expression

To validate VP90 expression, the C-terminal end of the wsv327 open reading frame was expressed in *Escherichia coli*. PCR amplification of the VP90 yielded a 773 bp DNA fragment (Fig. 1). The amplified VP90 gene was inserted into the vector. The positive plasmid containing the VP90 was transformed into *Escherichia coli* TOP 10 cells. Compared to non-transformants, SDS-PAGE analysis (Fig. 2) revealed that the expression of VP90 was correct. Analysis of SDS-PAGE estimated that the molecular mass was 32 kDa. Western blot analyses showed that mouse anti-(His)×6-antibodies bound specifically to VP90 (Fig. 3). Polyclonal antibodies against VP90 were prepared by using the purified recombinant protein. The specificity of the antibodies with 1:500 dilution, was tested with the rVP90. rVP26, rVP28N, and rVP28C were expressed in *E. coli* and recombinant proteins with His tags were confirmed by SDS-PAGE and Western blot assay (Chou *et al.*2011). The rVP26, rVP28N, and rVP28C were purified (Fig. 6a, Fig. 7a).

VP90 gene transcription in vivo

The transcription analysis of VP90 was carried out by RT-PCR. RNA was extracted from shrimp tissues before infection (0 hr) and 12, 24, 48, 72, and 96 hrs after the WSSV challenge (Fig. 4). The vp90 gene specific transcript was first detected 96 hpi (hours post infection). A major structure gene vp28 detected 12 hpi was used as a positive control and crayfish β-actin gene as a loading control. Those results indicate that vp90 is a late gene.

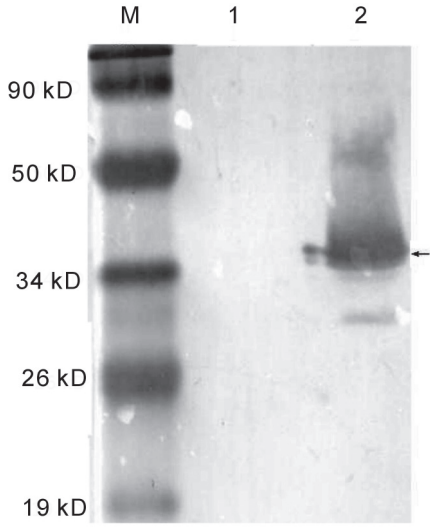

Fig. 3

Western blot analysis of rVP90 expression

Protein marker (M), negative control (1), inclusion bodies (2) (arrow indicates rVP90).

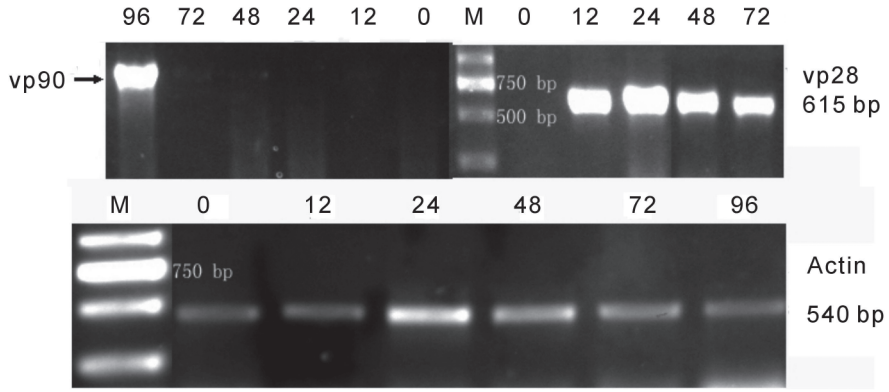

Fig. 4

VP90 gene trasncription *in vivo*

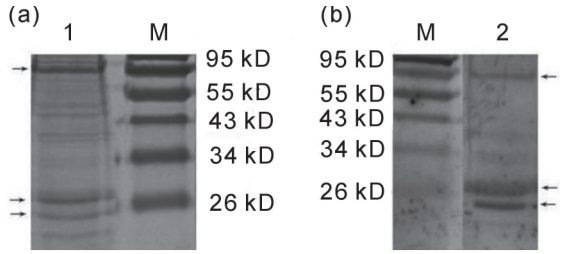

Fig. 5

rVP90 interacts with viral structural proteins

SDS-PAGE (a) and Far-western blot analysis (b) of the protein interactions. Protein marker (M) and the protein interactions (1 or 2).

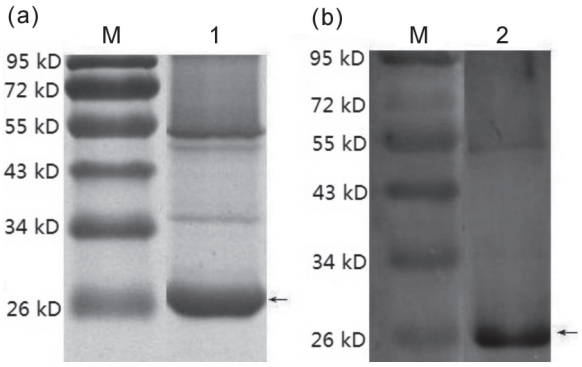

Fig. 6

Interaction of rVP90 with rVP26

SDS-PAGE (a) and Far-western blot analysis (b) of the interacting proteins. Protein marker (M) and the protein interactions (1 or 2).

Interaction of VP90 with virions, VP26, and VP28

For analysis of interaction between VP90 and virion, the viral proteins were separated by SDS-PAGE and blotted to PVDF membranes which were then incubated with DIG-labeled VP90. After detecting with HRP-conjugated anti-DIG antibody, three prominent immunoreactive bands corresponding to 26 kD, 28 kD, and 75 kD were seen in the viral fractions (Fig. 5). No band was seen in the control. To identify the interacting proteins, protein bands were cut out from SDS-PAGE gels and digested by trypsin. By searching peptide mass fingerprint NCBI BLAST database, the proteins were identified as VP26 and VP28. Five obtained tryptic peptide masses were found to be matching with WSSV VP26 with the amino acid sequence coverage of 31%. Eleven obtained tryptic peptide masses were found to be matching to WSSV VP28 with the amino acid sequence coverage of 51%.

To further analyze the interaction of VP26 and VP28 with VP90, modified far-western assays were performed with rVP28N, rVP28C, and rVP26 constructed in our laboratory.

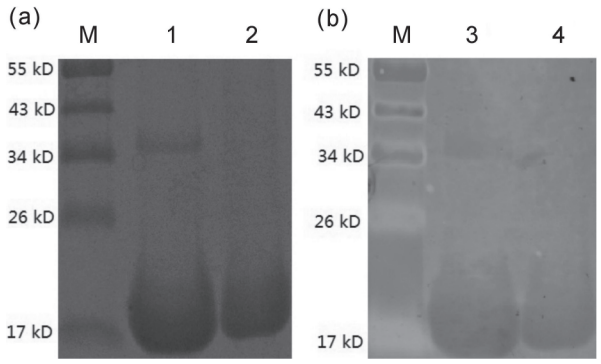

Fig. 7

Interaction of rVP90 with rVP28N and rVP28C

SDS-PAGE (a) and Far-western blot analysis (b). Protein marker (M), purified rVP28N (1) and rVP28C (2), rVP90-rVP28N interaction (3) and rVP90-rVP28C interaction (4).

Results showed that rVP90 interacted with rVP26 (Fig. 6), rVP28N, and rVP28C (Fig. 7).

Discussion

VP90 is a low-abundance envelope protein encoded by ORF wsv327 (Yang *et al.*, 2001). Sequence alignment indicated that VP90 has no homology with any other known protein. Previous study has demonstrated that VP90 is present in the envelope fraction of WSSV (Xie *et al.*, 2006b). Computer analysis showed that there were multiple putative glycosylation and phosphorylation sites in both, N-terminal and C-terminal end of VP90. The predicted VP90 encoding protein contains a predominant transmembrane domain and RGD motif, which suggests a possible involvement of VP90 in WSSV infection and binding to other viral envelope protein. VP90 interaction with VP26 and VP28 indicated that VP90 is associated with the membrane hydrophobic phase by membrane-spanning components. In addition, VP90 was found to contain cell attachment domain RGD (159–161 aa) which plays an important role in mediating cell recognition and infectivity of a variety of pathogens. Cell attachment assay showed that VP90 can attach to shrimp hemocytes, suggesting an involvement of VP90 in WSSV infection. Further studies will be need to demonstrate the role of VP90 RGD domain in initiating WSSV infection and how it interacts with other proteins.

Interaction between structural proteins is common in enveloped viruses. More reports have showed that VP24, VP26, and VP28 as link proteins in WSSV assembly. For example, WSV010 interacts with VP24 (Chen *et al.*, 2007), VP24 with VP28 (Xie *et al.*, 2006), VP51A with VP26 (Chang *et al.*, 2008), VP38 with VP24 (Jie *et al.*, 2008), VP37 with VP26 and VP28 (Liu *et al.*, 2009). Moreover, four major envelope proteins VP19, VP24, VP26, and VP28 of WSSV can form a multi-protein complex (Zhou *et al.*, 2009). Wan reported that VP26 acts as a linker protein to link the viral envelope and nucleocapsid by binding to nucleocapsid protein VP51 (Wan *et al.*, 2008). Further study showed that VP51A interacted directly not only with VP26 but also with VP19 and VP24. In 3D model, VP24 acts as a core protein that directly associates with VP26, VP28, VP38A, VP51A, and WSV010 to form a membrane-associated protein complex (Chang *et al.*, 2010).

The 3D model of membrane protein complex formed by WSSV structural proteins indicated that VP28 is exposed on the outer surface of virion (Chang *et al.*, 2010). We inferred that VP90 is anchored to the surface of virion by interacting with VP28. Recent research indicated that WSSV interacts with shrimp cells through more than one cell receptor. This research further supports the idea of envelope complex acting as an "infectome" for cell recognition, attaching, and penetrating into the cell and will enhance our understanding of WSSV assembly and potential targets for the design of antiviral drugs.

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