VP292 of *White spot syndrome virus* Interacts with VP26

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Abstract Interactions between virus structural proteins are suggested to be crucial for virus assembly. Many steps in the process of white spot syndrome virus (WSSV) assembly and maturation remain unclear. In this paper, we discovered a new interaction of WSSV VP292. Temporal-transcription analysis showed that VP292 is expressed in the late stage of WSSV infection. Western blot and matrix-assisted laser desorption ionization MS assays showed that VP292 interacts with VP26, a major envelope protein. Far-western blot provided further evidence for interaction between VP292 and VP26. These results collectively demonstrated that VP292 anchors to the envelope through interaction with VP26.

Keywords White spot syndrome virus · VP292 · Characterization · Interact · VP26

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. It causes up to 100 % mortality within 3–10 days of infection, resulting in major economic losses to the shrimp farming industry [7]. WSSV belongs to a new virus family, *Nimaviridae*, under a new genus *Whispovirus* (www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm), which shares low

homology with other known DNA viruses [9, 12]. It is an enveloped virus with a 305 kb double-stranded circular DNA genome and approximately 180 open reading frames (ORFs) [12, 17]. Over 50 structural protein genes and several non-structural protein genes have been characterized [6, 10, 16, 18]. Envelope proteins are important for virus infection and assembly. In previous studies, it has been shown that the function of envelope proteins was involved in virus infection and some WSSV structural proteins interact with other structure proteins [1, 2, 8, 15].

The VP292 is one of the newly identified envelope proteins in WSSV genome at positions 130,566–131,441 (Accession No: AF411636) in the WSSV genome [18]. The ORF contains 876 bp, which presumably encodes a protein of 292 amino acids, with a theoretical molecular mass of about 33 kDa and was therefore referred to as VP292 in this study [11]. Until now, there is little information about physical properties and functions of VP292. In this study, VP292 was expressed and the interactions between VP292 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.

Materials and Methods

WSSV Envelop and Nucleocapsid Protein Prepared

Envelope and nucleocapsid fractions were obtained by treatment with Triton X-100. For the Triton X-100 extraction, intact virions were mixed with an equal volume of 1 % Triton X-100 as well as 0.15 M NaCl, and incubated at 4 °C for 30 min. The mixture was centrifuged at

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20,000×g for 20 min to separate the phases. Both phases were subjected to a second round of Triton X-100 extraction. Supernatant and pellet were concentrated by acetone precipitation at −20 °C and analyzed by SDS-PAGE. For interaction experiment, the pellet was rinsed with water to eliminate any residual supernatant solution and then resuspended in TN buffer.

Cloning, Expression and Purification of Recombinant VP292, VP26 and VP28

The entire VP292 gene was amplified from the genomic DNA of WSSV with forward primer 5'-TTACTCGAGATG TTGTTTGATTCT-3' and reverse primer 5'-GACAAG CTTTAATACGGGACCT-3' (*Xho*I and *Hind*III restriction sites underlined). The PCR product was digested with *Xho*I and *Hind*III and cloned into pBAD/gIII A vector, and the recombinant plasmid was transformed into *Escherichia coli* top 10 cells. The bacterial cultures were induced with 0.4 mM L-arabinose for 5 h at 37 °C and were then harvested. The desired recombinant protein was purified using a column of Ni-nitriloacetic acid resins under denatured conditions and renatured by successive 12 h incubations with 6, 4, 2 and 0 M guanidine-HCl in buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 25 mM dithiothreitol, 0.1 % Tween-20, 10 % glycerol, pH 7.5). Purified VP292 was labeled with digoxigenin (DIG) [8].

Recombinant VP26 was cloned using forward primer 5'-ACACCATGGATACACGTGTTGGAAG-3' and reverse primer 5'-GCGTCTAGA GTCTTCTTCTTGATTTCGT-3' (*Nco*I and *Xba*I restriction sites underlined). PCR product was digested with *Nco*I and *Xba*I and cloned into pBAD/gIII A vector. The other procedures were the same as above. Recombinant VP28 was constructed as previously reported [8].

SDS-PAGE and Western Blot Analysis

Expression cultures were subjected to 12 % SDS-PAGE analysis according to a published method [5]. For western blotting, the separated proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (MSI). The membranes were incubated in blocking buffer (1 % bovine serum albumin, 5 % skim milk, 50 mM Tris, 200 mM NaCl, pH 7.5) at 4 °C overnight, followed by incubation with monoclonal mouse anti-(His)'-HRP (1:3,000) (Invitrogen.com) for 2 h, respectively. Then the membrane was washed twice with 20 ml of PBST for 5 min with gentle agitation. Subsequently, detection was performed with a DAB (4-chloro-1-naphthol, Sigma) solution.

For identifying proteins interact with VP292, the viral 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 3 % non-fat milk. After washing, the membrane was incubated with anti-VP292 antibody. Then goat anti-mouse antibodies conjugated with HRP were added. The interacting proteins were detected by exposing the membrane to an HRP-substrate kit. Preimmune mouse serum was as the negative control. Positive bands were cut and identified by LC-MS/MS analysis.

Antibody Preparation

To prepare the specific antibody against VP292, the purified VP292 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). After 2 weeks, the following three injections were conducted using 50 µg antigen mixed with an equal volume of Freund's incomplete adjuvant (Sigma) once a week. Four days after the last injection, mice were euthanized and antisera were collected. The titre of the antisera were assessed by ELISA using horseradish peroxidase conjugated goat anti-mouse IgG (Promega). For negative control, antigen was replaced with 1× PBS.

Transcriptional Analysis of VP292

After injection of WSSV, crayfish were collected at 0, 12, 24, 48 and 72 h p.i. and stored at 28 °C. Total RNA was extracted from frozen gills using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and digested with DNase before performing transcriptional analysis and direct PCR amplification. Primers specific for VP292 were used to perform RT-PCR and PCR (forward primer, 5'-ATGTTGTTTGATTCT-3', reverse primer, 5'-TAATACGGGACCT-3'). Detection of b-actin mRNA (PCR product of 540 bp) was used as an internal control (forward primer, 5'-GTGGGCCGCTCTAGGCACCAA-3'; reverse primer, 5'-CTCTTTGATGTCACGCACGATTTC-3'). VP28 was used as positive control (forward primer, 5'-CTACTCGAGATGGATCTTTCTTTCACTC-3'; reverse primer, 5'-TATAAGCTTTCGGTCTCAGTGCCA-3').

Far-Western Assay to Identify Proteins That Bind to VP292

For the far-western blotting assay, recombinant VP26 or VP28 were separated by 12 % SDS-PAGE and transferred to a PVDF membrane. The membrane was renatured gradually at 4 °C overnight in HEPES buffer containing 2 % non-fat milk. After washing 3 times with PBST, the membrane was incubated with DIG-labeled VP292 protein (20 µg/ml) in binding buffer (10 mM Tris-HCl, pH 6.5,

Fig. 1 PCR amplification of VP292 gene (GelA), SDS-PAGE (GelB) and western blotting (GelC) analysis of recombinant VP292 using anti-His monoclonal antibody, Gel A PCR result. Lane 5 VP292 gene, lane M DNA marker. Gel B and Gel C SDS-PAGE and western

blot result. Lane 1 induced rVP292, lane 2 vector control, lane M protein marker, lane 3 and lane 4 western blot analysis of VP292. Lane 3 vector control, lane 4 recombinant VP292 (arrow indicated)

5 mM CaCl_2 , 10 mM MgCl_2 , containing 0.02 % skim milk and 1 % Triton X-100 for 2 h. After washing, the membrane-bound VP292 proteins were incubated in HRP-conjugated-DIG antibody (1:3,000 dilution) for 1 h, and then the signals were visualized using an HRP-substrate kit. DIG-labeled BSA was used as a control.

Mass Spectrometry Analysis

CBB-stained protein spots were excised from gels. Gel pieces were minced and allowed to dry before trypsin digestion. MALDI-TOF-MS/MS was performed, and the MASCOT program was used to analyze the results. The MALDI mass spectrometer (MS) was an ABI-4700-TOF-TOF proteomics analyzer (Applied Biosystems, Framingham, MA, USA) instrument. All spectra of the test samples were acquired using the default mode. Data were searched by GPS Explorer using MASCOT as the search engine.

Results

Expression of VP292 in *E. coli*

PCR amplification of the VP292 gene yielded an 876-bp DNA (Fig. 1) fragment with expected sequence. The amplified VP292 gene was inserted into the vector pBADgIII/A. Positive insertion in the recombinant plasmids were screened by double digestion with *Xho*I and *Hind*III and two expected fragments were showed. After DNA sequence of the construct was confirmed to be correct and in frame with pBADgIII/A, plasmid pBADgIII/A-VP292 containing VP292 gene was transformed into *E. coli* Top 10 cells by selection for resistance to ampicillin. SDS-PAGE (Fig. 1) revealed that VP292 was

expressed compared to no-transformant. From SDS-PAGE, the molecular weight of VP292 protein was estimated to be ~41 kDa. Western blot results showed that mouse anti-(His) × 6-antibodies bound specifically to VP292 (Fig. 1). Polyclonal antibody against VP292 was prepared using the purified recombinant protein. The specificity of the antibody was assayed with the recombinant protein VP292 and the dilution of the polyclonal antibody was 1:500.

Temporal Analysis of VP292 Gene transcription

The transcription analysis of VVP292 was carried out by RT-PCR. Total RNAs were extracted from shrimp tissues before infection (0 h) and at 12, 24, 48, 72 and 96 h after WSSV challenge (Fig. 2). Specific transcript of VP292 gene was first detected at 96 hpi (hours post infection). A major structure gene VP28 detected from 12 hpi was used as a positive control and crayfish β -actin gene as a loading control. This result indicated that VP292 was a late gene.

Fig. 2 Temporal analysis of VP292 gene transcription. RT-PCR was conducted using a VP292 specific primers, b VP28 specific primers, and c crayfish β -actin specific primers, respectively

Table 1 Matched amino acid sequences of VP26 (gil29292122) by ESI-MS/MS analysis

Start-end	Mr (calc)	Observed	Miss	Sequence
52–57	768.48	768.50	0.0179	VPIQRR
58–64	820.47	820.47	0.0012	AKVMSIR
60–67	947.51	947.53	0.0174	VMSIRGER
193–202	1186.67	1186.67	0.0117	NVIDIKDEIK
41–51	1313.60	1313.62	0.0269	SVVANYDQMMR
146–157	1336.58	1336.60	0.0133	GNTMSNTYFSSK

VP292 Interacts with VP26

For analysis of interaction between VP292 and virions, the viral proteins were separated by SDS-PAGE and transferred to PVDF membranes which were then incubated with purified VP292 following with anti-VP292 antibody. After detecting with HRP-conjugated anti-mouse IgG, two prominent bands corresponding to VP292 and VP26 were seen in the viral fractions (Fig. 3). No band was seen in the control. To identify the interaction, protein bands were cut out from SDS-PAGE gels and digested by trypsin. Peptide mass fingerprints were performed using MALDI-TOF MS and analyzed by the MS-FIT system. The band representing VP26 (Fig. 3) from SDS-PAGE gel showed identical mass spectra: 768.48, 820.47, 879.46, 947.51, 963.50, 1186.67, 1313.60, 1329.60, 1336.58 (Table 1). All mass spectra were matched with spectra of the VP26 of WSSV in the mass database with the amino acid sequence coverage of 46 %.

To further identify the interaction between VP26 and VP292, far-western assays were performed with recombinant VP26 and VP28. Results showed that VP292 interacted with recombinant VP26 (Fig. 4) and that it did not interact with recombinant VP28.

Discussion

VP292 protein is one of structural proteins of WSSV and it is present in the envelope fraction of WSSV [3, 16]. The amino acid sequence of this protein indicates a predicted molecular weight of 33 kDa, but on SDS gels it migrates at a position corresponding to 39 kDa. Transcription analysis showed that VP292 is a late gene, which did not show up until 96 hpi. Computer-assisted analysis showed that both the VP292 gene itself and its product shared no significant homology with other known viruses. However, they demonstrated striking similarity with VP37 at both the nucleotide and amino acid sequence level. Therefore we were interested in expression and further characterization of VP292. The expressed of VP292 showed greater molecular weight than the predicted molecular mass (42 kDa) of the

Fig. 3 Far-western blot analysis of VP292 interacting with WSSV structural proteins. **a** WSSV proteins were separated by SDS-PAGE, **b** WSSV were transferred on PVDF membranes overlaid with purified VP292. Positive band showed with VP292 and VP26 (Line 1, Arrow indicated positive band)

Fig. 4 Far-western blot analysis of VP292 interacting with VP26, **a** SDS-PAGE analysis of purified recombinant VP26, lane 1 purified of recombinant VP26. Line M protein marker. **b** Far-western-blotting assay of VP292 interact with VP26. Line M protein marker, lane 2 purified of VP26 interact with DIG-labeled VP292

VP292 gene product. This discrepancy is due to the addition of His-tag to the VP292 and post-translational modification to the protein. Western blot analysis showed that VP292 contained His-tag and it had been expressed successfully.

VP292 and VP37 share a sequence similarity of 52 %, suggesting that VP292 and VP37 might have evolved by gene duplication from a common ancestral gene. Such homologous genes have been found in WSSV VP28, VP26

and VP24 [11], and further studies demonstrated that VP24 interacts with VP28 and is involved in virus infection [15]. We have reported that VP37 interacts with VP28 and VP26 [8]. Until now, there is little information concerning the role of VP292. In this study, experimental results indicated that VP292 interact directly with VP26. Structure analysis of VP292 protein exhibits very low homology to VP26 protein and does not reveal any significant structural similarities with VP26. Both VP37 and VP292 interacting with VP26 suggested that the similar domain of VP37 and VP292 might interact with VP26.

VP26 is considered to be a tegument protein and it lies between the virus envelope and nucleocapsid [10]. VP26 was found to interact with viral nucleocapsid *in vitro* [14], as well as VP28 [16]. More evidences are accumulating to show that VP26 is an important linker protein between the envelope and envelope proteins, and envelope and nucleocapsid of virions [1, 13]. Given that VP292 lacks a predicted transmembrane domain, it is possible that VP292 anchors in the viral envelope through association with VP26. A previous study has shown that the major envelope proteins VP28, VP26, VP24 and VP19 form a complex framework. The other low-abundance envelope proteins bind to this framework by interacting with at least one of the four major envelope proteins [19]. For example, VP38 interaction with VP24 [4], VP37 interaction with VP26 and VP28 [8] and VP010 interaction with VP24 [2] have been reported by various groups. The finding that VP292 interacts with VP26 will improve our understanding of WSSV assembly and morphosis. Further study is required to map the VP292 recognition site for VP26 and to explore the structure-based interaction.

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