A novel envelope protein involved in White spot syndrome virus infection

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One open reading frame (designated vp76) from the White spot syndrome virus (WSSV) genome has the motif of a cytokine I receptor and has been identified as a structural protein. In this paper, vp76 was expressed in Escherichia coli and used to prepare a specific antibody to determine the location of the corresponding protein in the intact virion, the nucleocapsids and the envelope of WSSV. Western blotting with the VP76 antiserum confirmed that VP76 was an envelope protein of WSSV. To investigate the function of the VP76, WSSV was neutralized with the VP76-specific antiserum at different concentrations and injected intramuscularly into crayfish. The mortality curves showed that the VP76 antiserum could partially attenuate infection with WSSV, suggesting that VP76 is an envelope protein involved in WSSV infection.

INTRODUCTION

White spot syndrome virus (WSSV) is a major disease agent of shrimp and was first identified in southern Asia in the 1990s (Takahashi et al., 1994; Wang et al., 1995). WSSV now affects all major shrimp aquaculture industries worldwide. Moreover, its wide host range makes it a potentially serious pathogen for other crustaceans such as crab, crayfish and lobster (Chang et al., 1998; Corbel et al., 2001; Lightner, 1996; Lo et al., 1996).

The complete WSSV genome has been determined for three different geographic isolates with the genome size ranging from 292 to 305 kb in isolates from Thailand and China, respectively (van Hulten et al., 1996; Lo et al., 1996). Sequence analysis revealed a low level of homology between most WSSV ORFs and known genes from GenBank, and this virus has been assigned to the novel virus family Nimaviridae (http://www.ncbi.nlm.nih.gov/LCTvDb/lctv/fr-fst-g.htm). To date, most studies of viral genes have focused on viral structural proteins and conserved enzymes based on proteomic methods and sequence alignment (Tsai et al., 2000, 2004; van Hulten et al., 2000a, b, c, 2002; Witteveldt et al., 2001; Chen et al., 2002a, b; Huang et al., 2002a, b; Zhang et al., 2002a, b; Li et al., 2004; Leu et al., 2005). Gene function studies have been seriously hampered by the lack of WSSV-permissive cell lines. The functions of the few genes investigated have been determined either by in vivo neutralization or using a baculovirus expression system (van Hulten et al., 2001b; Chen et al., 2002a; Lin et al., 2002; Tseng et al., 2002).

In the WSSV genome, one open reading frame (ORF220 in the Chinese isolate, ORF112 in the Thai isolate), here designated vp76, has the conserved domain of a class I cytokine receptor (van Hulten et al., 2001a; Yang et al., 2001). The protein has been identified by proteomic analysis to be a structural protein (Huang et al., 2002b; Tsai et al., 2004). In this study, VP76 was further identified as an envelope protein by using SDS-PAGE and Western blotting, and its function was investigated by virus neutralization experiments with a VP76-specific antibody.

METHODS

Crayfish. The crayfish Procambarus clarkii, an alternative WSSV host, was used for virus proliferation. Crayfish were purchased from Wuhan market, Hubei Province, China, and each was approximately 59.5 g in weight and 13.6 cm in length. Animals were reared for 3–5 days and confirmed to be WSSV free by PCR with WSSV-specific primers before performing the experiments.

Virus proliferation and purification. Inoculum was prepared from WSSV-infected Penaeus monodon tissues (gills and pleopods) from Ningbo, China. Tissue was homogenized in TN buffer (0.02 M Tris/HCl pH 7.4, 0.4 M NaCl) at a ratio of 1:10 (w/w). After centrifugation at 1400 g for 15 min, the virus suspension was passed through a 0.22 μm filter and injected into healthy crayfish (at a dilution of 1:4 to 1:10). Seven days post-infection (p.i.), fresh haemolymph was collected from the moribund crayfish and immediately layered on to a 10–50 % (w/w) continuous sucrose gradient prepared with CN buffer (0.0272 M sodium citrate, 0.02 M NaCl, pH 7.4) and centrifuged at 110 000 g for 1 h. The virus band was removed and precipitated by centrifugation at 110 000 g at 4 °C for 45 min. The virus pellet was resuspended in TNE buffer (0.5 M Tris/HCl pH 7.4, 0.1 M NaCl, 0.01 M EDTA).

Separation of envelope and nucleocapsid. A volume of 250 μl resuspended virions was treated with 1 % NP-40, 10 mM Tris/HCl pH 8.5 for 2 h at room temperature with gentle agitation and then layered on to 6 ml 30 % (v/v) glycerol in 10 mM Tris/HCl pH 8.5 and centrifuged at 150 000 g for 60 min at 4 °C. The envelope protein was recovered from the top of the gradient, acetone
precipitated and dissolved in TNE buffer after being centrifuged at 4000 g for 30 min. The pelleted nucleocapsids were resuspended in TNE buffer.

**Polypeptide separation and transfer to membrane.** Viral polypeptides were analysed by SDS-PAGE as described by Laemmli (1970) and stained with Coomassie brilliant blue. Semi-dry blotting was performed to transfer the polypeptides on to PVDF membranes (Promega) using Tris/glycine buffer (48 mM Tris base, 39 mM glycine, 20 % methanol, pH 8.3).

**Transcriptional analysis of vp76.** After injection of WSSV, crayfish were randomly removed at 0, 4, 8, 12, 24 and 48 h p.i. and stored at −80 °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 vp76 were used to perform RT-PCR and PCR (forward primer, 5′-CTCTTAGGCAACAAG-3′; reverse primer, 5′-CTCTTTGATGTCAC-GGGATCC-3′). Detection of β-actin mRNA (PCR product of 540 bp) was used as an internal control (forward primer, 5′-GTGGGCGG-CCTCTAGGCAACAA-3′; reverse primer, 5′-CTCTTTGATGTCAC-GGACGATTTC-3′).

**Expression and purification of VP76 in Escherichia coli.** The vp76 gene was amplified by PCR using the forward primer 5′-CACCAGAATTCATGGCAGGGAATAG-3′, containing a BamHI site (shown in italic), and the reverse primer 5′-GGCGCATATGGCTTCTTAC-3′, which is downstream of the stop codon of the gene. After purifying the PCR product, the DNA was ligated into the pGEM-T Easy vector (Promega), digested with BamHI and SalI, and ligated into the pET28-b vector downstream of a (His)6 tag. The resulting plasmid was then transformed into E. coli BL21 competent cells and expressed. The recombinant plasmid, pET28-b-vp76, was confirmed by DNA sequencing. Expression and purification of VP76 were performed following the instructions of Sambrook et al. (1989).

**Preparation of VP76 antibody.** Purified (His)6-tagged fusion protein was used as antigen to immunize rabbits. Rabbits were induced for 1 week following injection of 10 μg Freund’s complete adjuvant (Sigma) and then immunized three times (at 2-week intervals) with the mixed purified fusion protein and Freund’s incomplete adjuvant (50 μg each). Antiserum was collected at 15 days after the last immunization. Antiserum titres were determined by Western blotting.

**Western blotting.** The transferred membrane was blocked overnight in 3 % (w/v) BSA in TBS (0-2 M NaCl, 50 mM Tris/HCl pH 7.5) and then incubated with VP76 polyclonal antiserum diluted 1:500 in Solution 1 (Can Get Signal Immunoreaction Enhancer Solution; Toyobo) for 1 h at room temperature. Anti-rabbit antibody conjugated to alkaline phosphatase was diluted at a concentration of 1:1000 in Solution 2 (Can Get Signal Immunoreaction Enhancer Solution; Toyobo) and detected using BCIP and NBT.

**Virus quantification and antibody neutralization.** Virus concentration was quantified by real-time PCR with SYBR Green chemistry. The concentration of virions that resulted in 100 % mortality was used as the positive control. The antibody neutralization experiment was divided into six groups. Except for the negative control in which 10 crayfish were injected with 200 μl TN buffer (group 6), all other groups were injected with 100 μl virions (equivalent to 106 copies) that had been incubated with 100 μl of TN buffer (group 1), with pre-immune rabbit antiserum (group 2) or with various dilutions of VP76 antiserum (group 3, diluted 1:10 in TN buffer; group 4, diluted 1:5; group 5, undiluted). All sera were incubated for 30 min at 56 °C before being incubated with virions. Two and three replicates were performed for group 1 and groups 3–5, respectively, to confirm the consistency of the experiment.

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**RESULTS**

**Transcriptional analysis of the vp76 gene**

RT-PCR with gene-specific primers was used to analyse transcription of the vp76 gene at 0, 4, 8, 12, 24 and 48 h p.i., respectively. At 24 h p.i., vp76 mRNA could be detected by RT-PCR as a band of 806 bp (Fig. 1).

**VP76 expression and antibody preparation**

The expressed viral protein was 76 kDa (Fig. 2), which corresponded to the theoretical molecular mass of the fusion protein. The sensitivity and specificity of the rabbit antiserum against this fusion protein were analysed by Western blotting. Expressed protein transferred on to membrane was detected at an antiserum dilution of 1:800 (data not shown).

**Virus purification, SDS-PAGE and location of VP76**

After purification, large amounts of purified enveloped virions and nucleocapsids were obtained from the haemolymph of crayfish infected with WSSV (Fig. 3). SDS-PAGE showed that the envelope and the nucleocapsid proteins were well separated (Fig. 4a). Five major polypeptides were...
observed in intact virions: VP28, VP26, VP24, VP19 and VP15. There was no apparent 76 kDa band observed by SDS-PAGE. However, a weak 76 kDa band was detected in the intact virion and envelope protein fractions in the Western blot using the VP76 antiserum (Fig. 4b).

**VP76 antibody neutralization**

In the neutralization assay, the positive-control group injected with $10^6$ copies of virions resulted in 100% mortality at 12 days p.i. (Fig. 5, group 1). The group of crayfish injected with pre-immune antiserum mixed with virions exhibited 100% mortality by day 13 (Fig. 5, group 2). The groups injected with the various dilutions of VP76 antiserum mixed with virions exhibited 100% mortality by days 14, 17 and 22 for groups 3–5, respectively (Fig. 5), but with an obvious initial delay. The negative control (injected with TN buffer) showed no mortality (Fig. 5, group 6).

**DISCUSSION**

The *vp76* gene is 2025 nt and encodes a protein of 675 aa with a theoretical molecular mass of 76 kDa. Computer analysis has shown that this gene contains the conserved motif sequence of a class I cytokine receptor (gp130).
To identify the function of this protein further, \textit{vp76} was successfully expressed in \textit{E. coli} and the protein used to produce antibody. A Western blot with the expressed protein showed that the VP76 antibody was VP76 specific (data not shown). This antibody was used to hybridize with the WSSV structural proteins transferred on to membrane. The weak band detected in the intact virion and envelope fractions indicated that VP76 is a minor envelope protein (Fig. 4).

Transcriptional analysis showed that \textit{vp76} was transcribed at 24 h p.i. (Fig. 1). This suggests that \textit{vp76} is a late-expressed gene, which is consistent with a previous study by Huang \textit{et al.} (2002b), but is later than was found in a study by Tsai \textit{et al.} (2004). We suggest that this difference might be caused by the different virus copy numbers and hosts used in the infection experiments.

Neutralization experiments have often been used to study the role of virion proteins in infectivity. This method successfully characterized the function of VP28, a major envelope protein involved in WSSV infection (van Hulten \textit{et al.}, 2001b). In the present study, neutralization experiments showed that WSSV infection was partially neutralized by the VP76-specific antisem, suggesting that VP76 is involved in WSSV infection, although infection could not be completely blocked.

In the WSSV genome, there are numerous genes that encode homologues of eukaryotes, such as DNA polymerase, ribonucleotide reductase and thymidine, and thymidylate kinase. VP76 contains the conserved motif of a class I cytokine receptor (van Hulten \textit{et al.}, 2001a; Yang \textit{et al.}, 2001) and is involved in WSSV infection. This implies that WSSV has evolved mechanisms to evade the host defence system (for example, cytokines) in favour of virus infection and replication. Such virus mimicry mechanisms have been well studied in poxviruses and herpesviruses of animals and humans (Barry & McFadden, 1997; Kalvakolanu, 1999). However, as there is no available information on shrimp cytokines and cytokine receptors, the exact role of VP76 remains to be determined.

To date, about 39 structural proteins from WSSV have been identified or preliminarily identified using proteomic methods (van Hulten \textit{et al.}, 2000a, c, 2002; Huang \textit{et al.}, 2002b; Tsai \textit{et al.}, 2004). Some have been confirmed to be involved in WSSV infection, DNA binding and nuclear localization, or to be components of the envelope and nucleocapsid (van Hulten \textit{et al.}, 2001b; Zhang \textit{et al.}, 2001; Chen \textit{et al.}, 2002a; Li \textit{et al.}, 2004; Leu \textit{et al.}, 2005). VP76 is the second WSSV envelope protein shown to be involved in cellular infection.

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