Characterization of an envelope protein (VP110) of White spot syndrome virus

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A protein of 110 kDa (termed VP110) from the envelope fraction of White spot syndrome virus (WSSV) was identified by SDS-PAGE and mass spectrometry. The resulting amino acid sequence matched an open reading frame (wsv035) containing an Arg–Gly–Asp (RGD) motif in the WSSV genome database. To validate the mass-spectrometry result, the C-terminal segment of the wsv035 open reading frame was expressed in Escherichia coli as a fusion protein, which was used to produce specific antibody. Analysis by Western blotting and immunoelectron microscopy demonstrated that VP110 was an envelope protein of WSSV. An interaction analysis was performed between VP110 and the host cells, using a fluorescence assay and a competitive-inhibition assay. The results showed that VP110 was capable of attaching to host cells and that adhesion could be inhibited by synthetic RGDT peptides, suggesting that the RGD motif in the VP110 sequence may play a role in WSSV infection.

INTRODUCTION

White spot syndrome virus (WSSV), the sole member of the genus Whispovirus of the family Nimaviridae, is currently the most virulent viral pathogen in the shrimp-farming industry around the world, causing high mortality and resulting in catastrophic economic losses. Studies have shown that WSSV is an enveloped virus with a large, double-stranded, circular DNA genome (~ 300 kb) containing approximately 180 putative open reading frames (ORFs), most of which have no homology with any known genes or proteins in public databases (Chang et al., 1996; Chen et al., 1997; Chou et al., 1995; van Hulten et al., 2001a; Wang et al., 1995; Wongteerasupaya et al., 1995; Yang et al., 2001). Functional studies of WSSV, in particular of the structural proteins, have been the focus of recent attention. In the past few years, the major structural proteins of the virion of WSSV have been identified by combining SDS-PAGE with mass spectrometry (MS) or two-dimensional electrophoresis with MS (Huang et al., 2002b; Tsai et al., 2004; Zhang et al., 2004).

To date, over 30 structural proteins of WSSV have been found. Among these, some envelope proteins, including VP28, VP26, VP281, VP68, VP292, VP124, VP39, VP187 and VP31, have been confirmed by Western blot analysis and immunoelectron microscopy (IEM) (Huang et al., 2002a; Li et al., 2005, 2006; Zhang et al., 2002a, b, 2004; Zhu et al., 2005, 2006). Furthermore, VP28, VP281, VP68 and VP31 were suggested to be involved in systemic infection by WSSV by an in vivo neutralization assay (Li et al., 2005; van Hulten et al., 2001b; Wu et al., 2005). In addition, VP26, a linker protein between the envelope and the nucleocapsid fraction, has been suggested to help the viral nucleocapsid to move towards the nucleus by interacting with actin (Xie & Yang, 2005). Tsai et al. (2004) found six Arg–Gly–Asp (RGD) motif-containing structural proteins, including the VP110 protein, but their function remains unknown. Increasing evidence indicates that the RGD motif of viral structural proteins plays a crucial role in the adherence process of viruses (Akula et al., 2002; Boonyakiat et al., 2001; Jackson et al., 2000; Mason et al., 1994). However, studies on the interaction of WSSV envelope proteins and the host cell have been limited. More in-depth research is needed to clarify the mechanism of infection, which may help to discover potential therapeutic targets or methods for prevention and treatment of this disease.

In this study, we focused on identification of the WSSV structural protein VP110 by Western blotting and IEM, as well as studying the interaction of VP110 with the host cell by using a fluorescence assay and a competitive-inhibition assay.

METHODS

Identification by MS. The WSSV isolate used in this study originated from WSSV-infected shrimps (Penaeus japonicus) and was proliferated in an alternative host, the crayfish (Procambarus clarkii). Virions of WSSV were prepared as described previously (Xie et al., 2005) and their purity was examined by transmission electron microscopy (JEOL 100 cxII). Envelope and nucleocapsid fractions were obtained by treatment with Triton X-100. In brief, WSSV virions were mixed with an equal volume of 0.5% Triton X-100 and incubated for 1 h at room temperature. The nucleocapsids were...
purified by centrifugation at 20,000 g for 20 min at 4 °C. The envelope fraction was collected and used for the following experiments, whilst the pellet (nucleocapsid) was subjected to a second round of Triton X-100 extraction to ensure that viral envelopes had been removed completely.

WSSV virions and envelope and nucleocapsid fractions were analysed by 12% SDS-PAGE (Laemmli, 1970). The target protein (termed VP110) of ~110 kDa was excised from the gel and subjected to in-gel digestion using trypsin, as described by Eckerskorn & Lottspeich (1989) and Rosenfeld et al. (1992). MS analysis (nano-electrospray ionization MS/MS) was performed on a 4700 Proteomic Analyser (Applied Biosystems). The resulting MS data were searched against the NCBI nr database.

**Rapid amplification of cDNA ends (5' and 3' RACE).** The 5' and 3' ends of the cDNA encoding the vp110 gene were obtained by using a commercial 5'3' RACE kit (Roche) according to the manufacturer’s recommendations. The RNA sample was extracted from WSSV-infected crayfish 24 h post-infection by using the SV Total RNA Isolation system (Promega) and then treated with RNase-free DNase. For 5' RACE, this RNA was reverse-transcribed by using a random-hexadecamers primed and a poly(A) tail was added to the cDNA products by using terminal transferase in the presence of random-hexadecamers and dATP. The gene-specific primer 5sp1 (5'-AGTG-3') and an oligo(dT) anchor primer supplied with the kit were used for PCR. For 3' RACE, first-strand cDNA was synthesized using the oligo(dT) anchor primer. The primer 3sp1 (5'-ACTCTGGTCTTTCCCGAAGAA-3') and the anchor primer were used for PCR. Nested RACE-PCR was performed using primer 5sp2 (5'-CGATGGTGCGCTCACC-3') or 3sp2 (5'-CAATGTAACTCCCGGAAAGT-3') together with the anchor primer to reamplify the 5' and 3' RACE products, respectively. PCR products were purified on a 1.5% agarose gel and subcloned into the vector pMD18-T (TaKaRa). Ten randomly selected clones with inserts were sequenced and compared with the genomic DNA sequence of WSSV.

**Expression and purification of recombinant protein.** A sequence of 636 bp encoding the VP110 C-terminal segment ranging from aa 760 to 972 was amplified with the specific primers 5'-CTCGGATCCCTACGGACCTTATGCTGCTAC-3' and 5'-CGAGAATTCGCTGCTATTTTTGGCAAAAT-3' (restriction-enzyme sites underlined). The PCR product was digested with BamHI and EcoRI and cloned into vector pET-GST (Gene Power Laboratory Ltd) downstream of a 6×His tag. The recombinant plasmid was transformed into *E. coli* strain BL21 (DE3) competent cells. The expressed fusion protein [glutathione S-transferase (GST)-rVP110c] was purified by using Ni-NTA metal-affinity chromatography under denaturing conditions according to the instructions of the QiAexpressionist system (Qiagen).

**Preparation of antisera and Western blot analysis.** The GST–rVP110c protein or viral envelope fraction was used as antigen to immunize mice by intradermal injection four times, each with an interval of 10 days. The antigen (30 μg) was mixed with an equal volume of Freund’s complete adjuvant (Sigma) for the first injection. Subsequently, three injections were given using antigen mixed with an equal volume of Freund’s incomplete adjuvant (Sigma). Four days after the last injection, mice were exsanguinated, serum was collected and the titres of anti-VP110 antibody and antigen were determined by ELISA. Specific antiserum of high titre was stored in aliquots at ~80 °C until analysed.

Proteins from virions and from the envelope and nucleocapsid fractions were separated by SDS-PAGE (12% gel) and transferred to PVDF membrane (Amersham Biosciences) by semi-dry blotting at a constant current of 0.5 mA cm⁻² for 1.5 h at room temperature. The membrane was immersed in blocking buffer [1% BSA, 20 mM Tris/ HCl (pH 7.2), 1% NaN₃, 0-0.5% Tween 20] at room temperature for 1 h, followed by incubation with the specific antiserum (1:2000) for 1 h. Subsequently, alkaline phosphatase-conjugated goat anti-mouse IgG (Promega) was added at a dilution of 1:7500 and signals were detected by using a substrate solution containing 4-chloro-1-naphthol and X-phosphate (Promega).

**IEM.** WSSV virions or nucleocapsids were mounted onto carbon-stabilized copper grids (300 mesh) for 1 h. Grids were blocked with 3% BSA for 1 h, followed by incubation with anti-VP110 serum (diluted 1:200 in 3% BSA) for 2 h. After four washes with PBS, grids were incubated with goat anti-mouse IgG conjugated to colloidal gold (10 nm; Sigma) for 1 h. Grids were washed four times with PBS and stained with 2% phosphotungstic acid (pH 7.0) for 25 min. Specimens were examined by transmission electron microscopy (JEOL 100 CXII). For control experiments, anti-VP110 serum was replaced with normal mouse serum and treated as above.

**Biotin labelling of antibodies.** The IgG fraction was purified from anti-VP110 serum or anti-total envelope proteins serum by using rProtein A–Sepharose Fast Flow (Amersham Biosciences) according to the manufacturer’s instructions and then dialysed against PBS (pH 7.2). The concentration of protein was adjusted to 2 mg ml⁻¹ prior to labelling. A 10 mM stock solution of Sulfo-NHS-LC-Biotin (Pierce) was prepared immediately before use. Biotinylation of antibodies was performed by incubating purified IgGs with the stock solution of biotin in PBS for 30 min at room temperature (Sulfo-NHS-LC-Biotin: IgG molar ratio of 20:1), followed by the addition of 0.1 vol. 1 M Tris/HCl (pH 8.0) to stop the reaction. The biotinylated antibodies were then dialysed against PBS.

**Immunoprecipitation assay.** The viral envelope fraction was dialysed against PBS and the protein concentration was adjusted to 1 mg ml⁻¹. Total envelope proteins (50 μg) were incubated overnight at 4°C with 5 μl anti-VP110 serum or normal mouse serum (negative control). Subsequently, 5 μl protein A–Sepharose beads (Amersham Biosciences) was added to the mixture and incubated at 4°C for 1 h. The beads were collected by centrifugation and washed five times with 0.5 ml PBS. Bound proteins were dissociated by boiling in Laemmli sample buffer for 5 min. Released proteins were then separated by SDS-PAGE (12% gel) and transferred to a PVDF membrane, followed by incubation with biotinylated anti-VP110 antibody or anti-total envelope proteins antibody (diluted 1:2000) for 1 h. Western blotting was then carried out as described above.

**Fluorescence assay and competitive-inhibition assay.** Haemocytes extracted from healthy crayfish were seeded on 24-well poly-L-lysine-coated plates (10⁵ cells per well). After incubation for 30 min at room temperature, the serum was removed and residual binding sites were blocked with PBSB buffer (3% BSA in PBS) for 45 min. For the competitive-inhibition assay, cells were treated with an additional 30 min incubation step using synthetic RGDT or RDGT peptide (0.5 mg ml⁻¹; Shanghai Sangon) prior to the addition of total viral envelope proteins. Cells were then incubated with 0.1 ml viral envelope fraction containing native VP110 for 45 min at room temperature. Subsequently, the wells were washed three times with PBS and incubated with purified anti-VP110 serum (diluted 1:300 in PBSB) for 30 min, followed by immunostaining with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (diluted 1:200 in PBSB; Sino-American Biotechnology). Finally, stained cells were observed under a fluorescence microscope (Olympus IX70). As a control in the fluorescence microscopy, the primary antibody anti-VP110 serum was replaced with anti-VP124 serum prepared in our laboratory (Zhu et al., 2005). VP124 is also an envelope protein of WSSV, as confirmed by Western blotting and IEM, but has no RGD motif in its protein sequence.
RESULTS

MS analysis of VP110

The envelope and nucleocapsids of WSSV were separated by treating purified virions with Triton X-100 and separating the proteins from virions and from the envelope and nucleocapsid fractions by SDS-PAGE. An apparent band of ~110 kDa that was present only in the virions and in the envelope fraction was excised and subjected to in-gel digestion followed by sequencing using nano-electrospray ionization MS/MS. The results showed that all 26 peptide fragments from this target protein matched the putative product of WSSV ORF *wsv035* (GenBank accession no. AF332093) with 35% sequence coverage (data not shown). The *wsv035* ORF (nt 16983–14068) was presumed to encode a 972 aa protein with a theoretical molecular mass of 108 kDa. In fact, the result was similar to that reported by Tsai *et al.* (2004), who found that VP110 was a WSSV structural protein by MS of purified complete virions, although further characterization was not reported.

Structure of the *vp110* gene

The 5′ and 3′ regions of the *vp110* gene transcript were obtained by RACE. 5′ RACE analysis revealed that the transcription-initiation site was located at nt ~44 relative to the putative translation-initiation codon ATG, and a putative TATA box was found 27 nt upstream of the transcription-initiation site (Fig. 1). 3′ RACE analysis revealed that a poly(A) tail was located 58 nt downstream of the translation-termination codon (Fig. 1), although there was no obvious polyadenylation signal present (AATAAA). This result indicated that other undefined signal pathways that regulate WSSV *vp110* polyadenylation may exist. A cell-adhesion (RGD) motif, which has been described by Tsai *et al.* (2004), was located between aa 511 and 513 (Fig. 1).

Western blot analysis of VP110

Initially, we attempted to express full-length VP110 in *E. coli*. However, it could not be expressed successfully under all experimental conditions. Therefore, the C-terminal fragment of *vp110* was cloned into the pET-GST vector and expressed as a GST fusion protein (GST–rVP110c) in the BL21 (DE3) strain. Induced and non-induced samples were analysed by SDS-PAGE (12% gels). A band corresponding to the fusion protein (~55 kDa: GST (31 kDa) plus rVP110c (24 kDa)) was observed (data not shown). The expressed GST–rVP110c fusion protein was purified by using Ni-NTA affinity chromatography under denaturing conditions because of its insolubility. The purified protein was used to immunize mice and anti-VP110 serum was obtained.

To validate the MS results, the virions and envelope and nucleocapsid fractions were separated by SDS-PAGE.

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**Fig. 1.** Nucleotide sequence of WSSV *vp110* containing the 5′- and 3′-terminal regions and the deduced VP101 protein sequence (one-letter code). The primers used for 5′/3′ RACE (5sp1, 5sp2, 3sp1 and 3sp2) are underlined. The transcription-start signal is indicated by a solid arrow. The poly(A) revealed by 3′ RACE is indicated by a dashed arrow. The TATA box and the RGD motif (aa 511–513) are boxed.
in duplicate. One set of gels was stained directly with Coomassie blue R-250 (Fig. 2a), whilst the other set was transferred to a PVDF membrane. Western blot analysis showed that the anti-VP110 serum reacted only with the ~110 kDa protein in WSSV virions (Fig. 2b, lane 1) and in the envelope fraction (Fig. 2b, lane 2) and no reaction occurred with the nucleocapsid fraction (Fig. 2b, lane 3), indicating that the VP110 protein is present exclusively in the WSSV envelope fraction.

**Localization of VP110 by IEM**

In order to confirm the envelope association of the identified protein, an additional experiment was carried out using IEM. The results revealed that gold particles could be observed on intact WSSV virions when using anti-VP110 serum as the primary antibody (Fig. 3a), but not on the nucleocapsid surface (Fig. 3b). Control experiments showed that no gold particles were found on the envelopes of WSSV virions when normal mouse serum was used as the primary antibody (Fig. 3c). The results showed that VP110 is found in the envelope of WSSV.

**Interaction of VP110 with host cells**

Although the above results showed that VP110 is a viral envelope protein, the function of this protein remains undefined. Hence, further work is required to shed light on the role of VP110 in WSSV infection. In a fluorescent assay experiment, haemocyte cells from healthy crayfish were seeded onto 24-well plates and incubated with total envelope proteins of WSSV containing native VP110 protein (as we failed to express full-length VP110 in *E. coli*). When anti-VP110 serum was used as the primary antibody, a strong green fluorescent signal was detected on the surface of the cells (Fig. 4a), whilst no significant fluorescence was observed by using anti-VP124 serum (Fig. 4b). This result indicated that the VP110 can interact with as-yet-unknown cell-membrane proteins.

To exclude the possibility that this interaction might be mediated by other proteins within the WSSV envelope fraction, we conducted immunoprecipitation assays to determine whether VP110 could interact with other proteins. As shown in Fig. 5, after the immune complexes formed by total envelope proteins and anti-VP110 or normal mouse antibody had been separated by SDS-PAGE, the results showed that only VP110 was detected by using either biotin-labelled anti-VP110 antibody (Fig. 5, lane 1) or biotin-labelled anti-total envelope protein antibody (Fig. 5, lane 2). In the negative control, no stained band was seen with biotin-labelled anti-total envelope protein antibody when
total envelope proteins were immunoprecipitated with normal mouse antibody (Fig. 5, lane 3). These results indicated that no reaction occurred between VP110 and the other viral envelope proteins, suggesting that the interaction of VP110 with the host cell is specific and is not mediated by other proteins.

In the face of these results, we hypothesized that a cell-adhesion (RGD) motif present in the VP110 sequence might participate in recognition of host cells. To test this hypothesis, a competitive-inhibition assay was performed using the synthetic peptides RGDT and RDGT. The results showed that no obvious fluorescent signal was observed on the cell surface when haemocyte cells pre-incubated with synthetic RGDT peptide were incubated with total envelope proteins (Fig. 4c). In contrast, distinct fluorescent signals were seen using the RDGT peptide under the same experimental conditions (Fig. 4d). These results indicated that the RGDT peptide could inhibit the attachment of VP110 to the cell membrane effectively, suggesting that the RGD motif in the VP110 sequence plays a vital role in WSSV infection.

**DISCUSSION**

Although the sequences of a number of WSSV envelope proteins have been published in recent years, little is known about their function. Here, we detected a low-abundance protein of ~110 kDa in the WSSV envelope fraction by Coomassie staining and this protein was found to match the wsv035 ORF product in the WSSV genome by MS/MS. As this protein was only found in the viral envelope fraction, it appeared to be an envelope-associated protein. In a previous study, the VP110 protein was identified as a structural protein by MS/MS (Tsai et al., 2004) and was found to contain a cell-adhesion (RGD) motif, but further location and functional identification were not pursued.

In this study, we found that the VP110 protein was present only in the viral envelope fraction and was located exclusively on the intact WSSV virion surface, as determined by Western blotting and IEM, indicating that VP110 is an envelope protein of WSSV. In subsequent experiments, we used immunofluorescence microscopy to show that the native VP110 protein contained in the total viral envelope proteins could attach to the host-cell surface. Moreover, the immunoprecipitation results indicated that this interaction was not mediated by other proteins, as no other proteins were found in the immune complex. However, these results were still insufficient for us to conclude that the RGD motif within the VP110 sequence was biologically and functionally active. In general, RGD-mediated interactions can be interrupted or inhibited by using short peptides encompassing the RGD sequence. To understand in more depth...
may participate in adhesion of the virus to the host cell via the RGD motif. More in-depth studies will help to clarify the role of VP110 in WSSV infection and to explore new therapeutic approaches, providing valuable information for the prevention and control of disease caused by WSSV.

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**Fig. 5.** Western blot analysis of immune complexes. Total viral envelope proteins were incubated with anti-VP110 serum or normal mouse serum (negative control). Complexes immunoprecipitated with anti-VP110 serum were separated by SDS-PAGE, transferred to a PVDF membrane and incubated with biotin-labelled anti-VP110 IgG (lane 1) (diluted 1:2000) or biotin-labelled anti-total envelope proteins IgG (lane 2). Complexes immunoprecipitated with normal mouse serum were separated and transferred to the membrane as above and incubated with biotin-labelled anti-total envelope proteins IgG (lane 3). Lane M, low-molecular-mass protein marker.

In general, viral envelope proteins, especially those containing the RGD motif, are thought to play a crucial role in cell binding. As expected, the interaction between VP110 and host cells was inhibited exclusively by the synthetic RGDT peptide, whereas the control RDGT peptide did not influence binding, indicating that the interaction is correlated tightly with the RGD motif within the VP110 sequence.

**REFERENCES**


Characterization of WSSV VP110


