

Characterization of a novel envelope protein (VP281) of shrimp white spot syndrome virus by mass spectrometry

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The primary structure of a novel envelope protein from shrimp white spot syndrome virus (WSSV) was characterized using a combination of SDS–PAGE and mass spectrometry. The resulting amino acid sequence matched an open reading frame (ORF), ORF1050, of the WSSV genome ORF database. ORF1050 contained 843 nt, encoding 281 aa, and was termed the *vp281* gene. Computer-assisted analysis showed that both the *vp281* gene and its product shared no significant homology with other known viruses. However, they shared striking identity/similarity with another WSSV structural protein, VP292, at both the nucleotide and amino acid sequence level, suggesting that *vp281* and *vp292* might have evolved by gene duplication from a common ancestral gene. WSSV VP281 cDNA was cloned into a pET32a(+) expression vector containing a T7 RNA polymerase promoter to produce (His)₆-tagged fusion proteins in *Escherichia coli* strain BL21. Specific mouse antibodies were raised using the purified fusion protein (His)₆-VP281. Western blot analysis showed that the mouse anti-(His)₆-VP281 antibodies bound specifically to VP281 of WSSV, without cross-reactivity with VP292. The transmission electron microscope immunogold-labelling method was used to localize VP281 in the WSSV virion as an envelope protein. The cell attachment 'Arg–Gly–Asp' motif in VP281 indicated that this protein might play an important role in mediating WSSV infectivity.

Introduction

White spot syndrome virus (WSSV), a shrimp virus with high morbidity, has been reported in the cultured penaeid shrimp worldwide since the early 1990s (Zhang *et al.*, 1994; Wongteerasupaya *et al.*, 1995; Nadala *et al.*, 1998). This causative virus agent has a wide host range in crustaceans, such as crabs and crayfish, besides its known infectivity of most species of penaeid shrimps (Wang *et al.*, 1998). Due to the lack of a suitable cell culture system, the crayfish *Cambarus clarkii* has been used as an alternate host for virus propagation (Huang *et al.*, 2001 and references therein).

The characteristic feature of WSSV is its bacilliform shape and a long, tail-like envelope extension (Durand *et al.*,

1997; Huang *et al.*, 2001). The full genome sequence of WSSV (~ 300 kb) reported recently contains approximately 180 putative open reading frames (ORFs) (van Hulten *et al.*, 2001; Yang *et al.*, 2001). More than 80% of these putative ORFs, and their products, share no homology to any known genes or proteins in GenBank (Yang *et al.*, 2001). The classification status of WSSV is still unknown due to the lack of molecular information.

At least 24 protein bands (5 major and about 20 minor bands) were present in the WSSV SDS–PAGE profile using Coomassie blue R-250 staining. Proteomic approaches were utilized to analyse these bands and 18 WSSV structural protein genes were identified (Huang *et al.*, 2002). Of the identified WSSV structural proteins, VP28, VP26/P22, VP19 and VP466 were associated with the viral envelope, whereas VP15 and VP24 were associated with the viral nucleocapsid (Zhang *et al.*, 2002; Huang *et al.*, 2002; van Hulten *et al.*, 2002); VP26 was reported originally to be a WSSV nucleocapsid protein (van Hulten *et al.*, 2000a). VP28, VP26/P22 and VP24 may have evolved by gene duplication due to the high amino acid

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identity of these proteins (van Hulten *et al.*, 2000b). VP28 was found to play an important role in the systemic infection of shrimp by WSSV (van Hulten *et al.*, 2001). In this study, we report a novel WSSV protein, VP281, identified by mass spectrometry. The transmission electron microscope (TEM) immunogold-labelling method was employed to localize VP281 in the WSSV virion. Homology search and computer-assisted prediction of VP281 domains were also performed in this investigation.

Methods

■ Preparation of WSSV virions and nucleocapsids. The WSSV isolate in this study originated from *Penaeus chinensis* (Ningbo, China) and was grown in an alternate host, the crayfish (*C. clarkii*). Virus purification was carried out as described previously (Huang *et al.*, 2001). The purity of the isolated virus sample was examined under a TEM (JEOL 100 CXII). For purification of virus particle fractions, WSSV virions were treated with Triton X-100 for 15 min at room temperature, loaded onto a 20–50% continuous CsCl gradient and centrifuged for 48 h at 110 000 *g* using a Beckman SW41-Ti rotor. Viral nucleocapsid and envelope bands were collected individually by side puncture and later diluted with 1 × TN buffer (0.02 M Tris–HCl, 0.4 M NaCl, pH 7.4) (1:10). Subsequently, the proteins were sedimented at 120 000 *g* for 45 min. The pellet was resuspended in 1 × TN buffer.

■ Identification and characterization of VP281. Mass spectrometry analysis of WSSV VP281 was described in a previous study (Huang *et al.*, 2002). Briefly, purified WSSV virions were separated using 12% SDS–PAGE with a Protean II Electrophoresis system (Bio-Rad). Protein bands were visualized using Coomassie brilliant blue R-250 staining. The target protein, band 11, was excised and subsequently ‘in-gel-digested’ using sequencing-grade, modified porcine trypsin (Promega). After a brief centrifugation step, the supernatant was vacuum-dried and redissolved in 0.5% trifluoroacetic acid and 50% acetonitrile (Shevchenko *et al.*, 1996). For MALDI-TOF mass spectrometry (matrix-assisted laser desorption ionization mass spectrometry–time of flight analysis), the matrix used was a saturated solution of α -cyano-4-hydroxycinnamic acid in 0.5% trifluoroacetic acid and 50% acetonitrile. The sample and the matrix (1:1, vol/vol) were loaded on the target plate and MALDI-TOF spectra of the peptides were obtained with a Voyager-DE STR Biospectrometry Workstation mass spectrometer (PerSeptive Biosystems). Analysis was performed in positive ion reflector mode with an accelerating voltage of 20 kV and a delayed extraction of 150 ns. Typically, 180 scans were averaged. Trypsin autoprolysis products were used as internal calibrants. Data mining was performed using MS-FIT software against the WSSV ORF database. For Nano-ESI (nano-electrospray ionization) mass spectrometry (Q-TOF) analysis, the in-gel-digested sample was desalted using C18 ZipTip (Millipore) and dried. After dissolving in 2 μ l of 50% acetonitrile and 0.5% formic acid, the sample was loaded into a metal-treated glass capillary. The capillary was then mounted on the nanoflow Z-spray source of a Q-TOF-2 mass spectrometer (Micromass). Flow rates usually varied from 8 to 16 nl/min. Instrument operation, data acquisition and analysis were performed using MASSLYNX/BIOLYNX software, version 3.5 (Micromass). Data searches against the WSSV ORF database were performed using Global Server (Micromass).

■ Homology analysis. An homology search of the *vp281* gene and its deduced amino sequences was performed against GenBank/EMBL, SWISSPROT and PIR databases using FASTA and BLAST programs. Protein

motifs were analysed by PROSITE release 16 database (Hofmann *et al.*, 1999). The identified ORF and its deduced amino acid sequence were analysed using the DNASIS and PROSIS software (Hitachi Software Engineering), respectively. Alignments of the amino sequences were conducted in CLUSTAL x (Thompson *et al.*, 1997) and edited in GeneDoc (Nicholas *et al.*, 1997).

■ Expression and localization of VP281. The *vp281* gene was amplified using the synthesized forward (5′ CACCCATGGGTATGGC-GGTAACTTGA 3′) and reverse (5′ AGACTCGAGTTATGTCCA-ACAATTAAA 3′) primers, containing a *Nco*I and an *Xho*I site (bold), respectively. The amplified DNA and plasmid vector were digested with *Nco*I and *Xho*I, respectively. After purification and ligation of the DNA fragments, the *vp281* gene was inserted into the pET32a(+) vector downstream of a (His)₆-tag and expressed in pET32a(+)-BL21 as a (His)₆-tagged fusion protein. The resulting recombinant plasmid was named pET32a(+)-281. *Escherichia coli* BL21 competent cells were transformed with the recombinant pET32a(+)-281 plasmid and positive colonies containing transformants were screened by colony PCR; pET32a(+)-281 was confirmed by DNA sequencing. Expression and purification of (His)₆-tagged VP281 were performed following the instructions of the pET System Manual, 9th edition (Novagen). The purified VP281 fusion protein was subsequently confirmed by MALDI-TOF mass spectrometry.

■ Antibody preparation. The purified (His)₆-VP281 fusion protein was used to immunize 3- to 4-week-old Swiss Albino mice once every 2 weeks by intradermal injection over an 8-week period. Titres of the antisera were 1:20 000, as determined by ELISA (Harlow & Lane, 1988). Protein A Sepharose CL-4B was used to isolate anti-(His)₆-VP281 IgGs, according to the manufacturer’s instructions (Amersham Pharmacia).

■ Western blot analysis. Purified WSSV virions, viral envelope and nucleocapsid fractions were subjected to 12% SDS–PAGE. Proteins were then transferred onto nitrocellulose membranes (Bio-Rad) in electroblotting buffer (25 mM Tris, 190 mM glycine, 20% methanol) at a constant voltage of 70 V for 3 h. The membrane was immersed in blocking buffer (3% BSA, 20 mM Tris, 0.9% NaCl, 0.1% Tween-20, pH 7.2) at 4 °C overnight, followed by incubation with polyclonal mouse anti-(His)₆-VP281 IgGs or mouse anti-(His)₆-VP292 IgGs (1:1000) for 3 h, respectively. Subsequently, HRP-conjugated goat anti-mouse IgGs (Sigma) were used and detection was performed with a substrate solution (4-chloro-1-naphthol, Sigma).

■ Localization of VP281 by immunoelectron microscopy (IEM). WSSV virions and nucleocapsids were mounted onto Formvar-coated or carbon-stabilized nickel grids, respectively. Grids were then blocked with 2% AURION BSA-C (Electron Microscopy Sciences) for 1 h and then incubated with the primary antibodies, purified polyclonal mouse anti-(His)₆-VP281 IgGs (1:1000 dilution in 1% AURION BSA-C), for 2 h. After washing three to four times with 1 × PBS, the grids were incubated with goat anti-mouse IgGs conjugated to 15 nm colloidal gold (Electron Microscopy Sciences) for 1 h at room temperature. Grids were washed a further two times with 1 × PBS and briefly stained with 2% phosphotungstic acid (PTA, pH 7.0) for 1 min. Specimens were examined under a TEM (JEOL 100 CXII). For control experiments, pre-immune mouse serum and mouse anti-(His)₆ antibodies were used to replace the primary antibodies indicated above.

Results

Identification of WSSV VP281 by mass spectrometry

Purified WSSV virions were separated by SDS–PAGE. More than 24 bands can be visualized using Coomassie

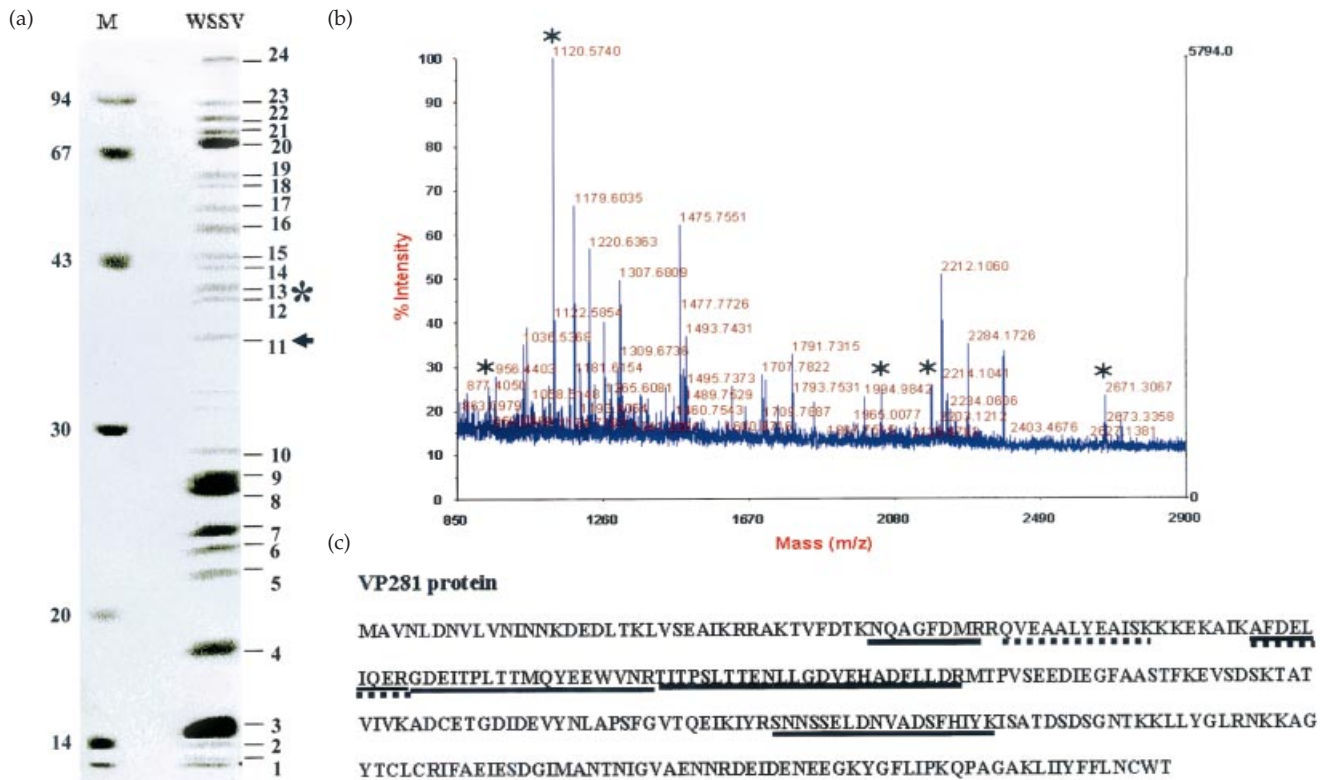


Fig. 1. Identification of the *vp281* gene by mass spectrometry. (a) SDS-PAGE of purified WSSV virions. VP281 (band 11) and VP292 (band 13) are indicated by an arrow and an asterisk, respectively. M: molecular mass marker proteins (kDa). (b) MALDI-TOF mass spectrometry spectrum of protein band 11. (c) Trypsin-treated peptides from MALDI-TOF and Nano-ESI mass spectrometry analyses that match the VP281 protein sequence within a mass accuracy of 100 p.p.m. are indicated by solid and dotted underlines, respectively.

brilliant blue R-250 staining (Fig. 1a). Trypsin digests of the reduced and alkaline-treated protein from band 11 was first analysed by MALDI-TOF mass spectrometry. The WSSV ORF database search with a list of trypsin-treated peptide masses identified one of the proteins as the product of the *vp281* gene (termed VP281). Five experimentally derived peptide masses were found to match the predicted peptide masses of VP281 within 100 p.p.m., covering 27% of its amino acid sequence (Fig. 1b, c). The trypsin-treated peptides of band 11 were subsequently sequenced by mass spectrometry using Nano-ESI mass spectrometry. The trypsin-treated peptides matching VP281 from the Nano-ESI mass spectrometry spectrum are shown in Fig. 1(c). After searching the WSSV ORF database, the resulting amino sequence was confirmed to be the product of the *vp281* gene.

The WSSV *vp281* gene sequence

The start and stop codons of the *vp281* gene were present at 141696 and 142538 nt, respectively, in the WSSV genome (Yang *et al.*, 2001). This 843 bp ORF presumably encoded a 281 aa protein (hence termed the *vp281* gene, GenBank accession number AF411634, and the resulting protein termed VP281), with a theoretical molecular mass of 31.5 kDa. A

typical TATA box was present at 46 nt upstream of the translation start site. The base sequence surrounding the methionine start codon (ACGATGG) of the *vp281* gene was in a favourable context for efficient eukaryotic translation initiation (PuNNATGPu) (Kozak, 1989). A polyadenylation signal (AATAAA) was present at 101 nt downstream of the translational stop codon of *vp281*. The putative protein is acidic, with an isoelectric point of 4.69 (Isoelectric Point Computation, EMBL). PREDICTPROTEIN (Bairoch *et al.*, 1997) predicted that VP281 has two potential N-linked glycosylation sites, N-(P)-ST-(P), and nine possible phosphorylation motifs (consensus pattern ST-X-RK or ST-XX-DE). In addition, a cell attachment sequence, Arg-Gly-Asp (RGD motif, PROSITE accession number PS00016), was found in VP281. The structure of the *vp281* gene and its deduced amino sequence are noted in Fig. 2(a).

Homology analysis

Homology searches and pairwise comparisons with the *vp281* gene and VP281 were conducted against GenBank/EMBL, SWISSPROT and PIR databases using BALST, FASTA and TFASTA but no apparent homology with any other known viral genes or proteins was found. However, significant homology

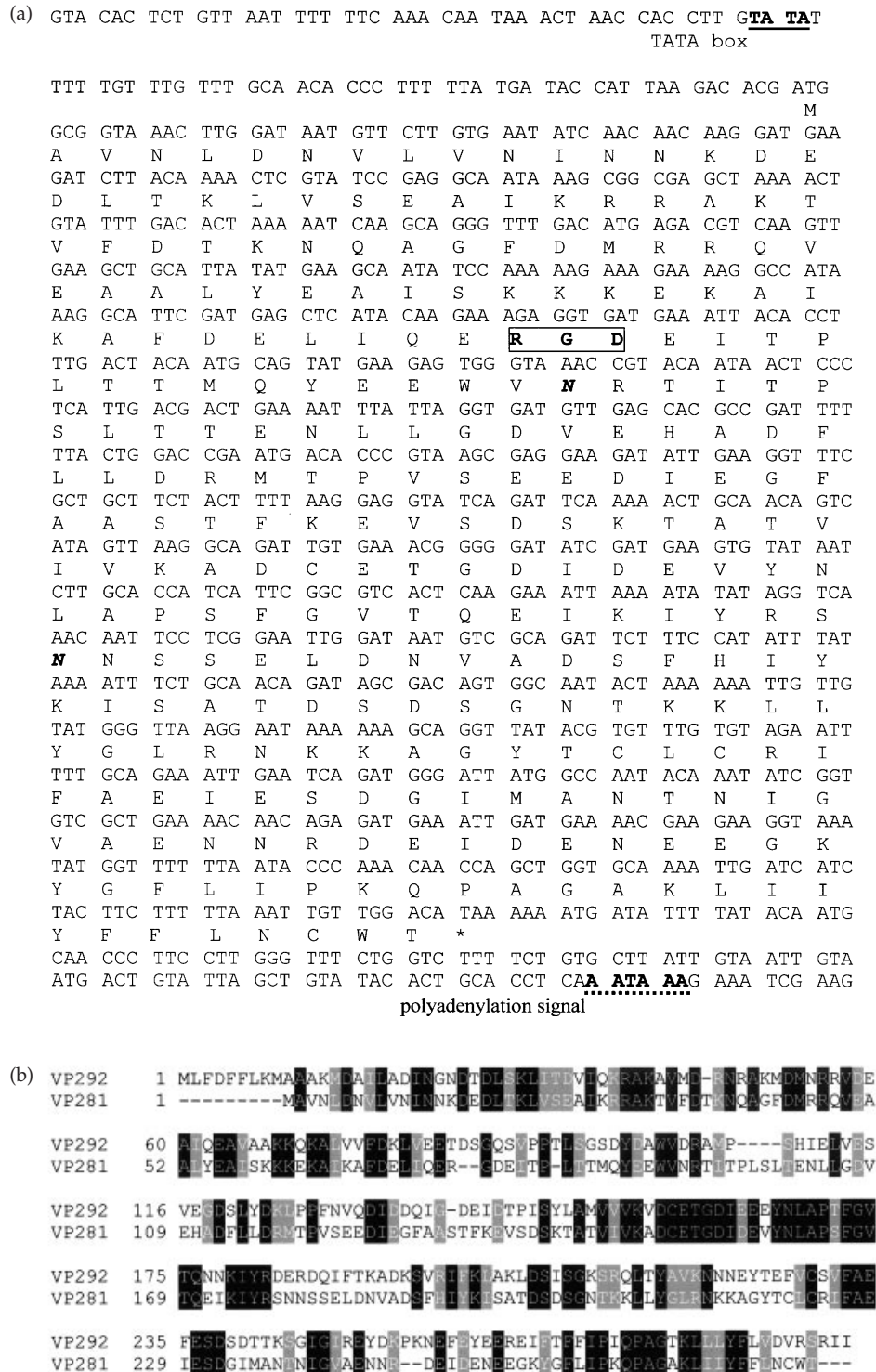


Fig. 2. Characterization of the *vp281* gene and its product, VP281. (a) WSSV *vp281* nucleotide sequence and the deduced VP281 protein sequence (one-letter code). The putative TATA box and polyadenylation signal (bold) are indicated by solid and dashed lines, respectively. Two potential *N*-linked glycosylation sites are indicated in bold italics; the putative cell attachment domain is in bold and boxed. (b) Alignments of the VP281 and VP292 amino acid sequences shows a considerable degree of homology. Sequence identity (black) and similarity (grey) between the two proteins are shown.

was found with the sequence of our newly identified WSSV VP292 protein. VP292 is encoded by ORF948 and has a putative molecular mass of 33 kDa. VP292 corresponds to band 13 (Fig. 1a) in the WSSV SDS-PAGE gel (ORF948 GenBank accession number is AF411636). VP281 and VP292 share an identity of 35 % and a similarity of 52 % in their amino acid sequences (Fig. 2b).

Expression and purification of (His)₆-VP281 fusion protein

The WSSV *vp281* gene was cloned into the pET32a(+) vector and overexpressed as a (His)₆-tagged fusion protein. A band corresponding to the (His)₆-VP281 fusion protein was observed after inducing pET32a(+)-VP281 plasmid expression in *E. coli*. No protein bands were found at the same positions in both the induced and the uninduced pET32a(+) vector lanes (Fig. 3a). Recombinant (His)₆-VP281 was purified using affinity chromatography (Fig. 3a, lane 6) and the authenticity of the expressed protein was subsequently confirmed by mass spectrometry, MALDI-TOF (data not shown).

Western blot analysis

In Western blot analysis (Fig. 3b), purified mouse anti-(His)₆-VP281 IgGs reacted specifically with VP281 present either in the WSSV virion or in the purified viral envelope (equivalent to the band 11 of Fig. 1a). Results proved that VP281 was of WSSV origin rather than a protein linked superficially to the WSSV virion. Anti-VP281 antibodies did not react with the proteins of WSSV nucleocapsids, indicating this protein should belong to viral envelope. On the other hand, as shown in Fig. 3(b), no cross-reaction between VP281 antibodies and VP292 was observed and this result was substantiated further by a separate Western blot of the WSSV virion with the purified mouse anti-(His)₆-VP292 IgGs, in which it turned out that VP292 antibody specifically recognized VP292 (corresponding to band 13 of Fig. 1a) without cross-reactivity with VP281.

Localization of VP281 by IEM

Purified WSSV virions are shown in Fig. 4(a). Localization of VP281 in the WSSV virion was performed using the TEM immunogold-labelling method. Results demonstrated that the high-density gold particles were located specifically at the viral envelope (Fig. 4b); no apparent signals can be seen in the nucleocapsids (Fig. 4c). In a separate experiment, when mouse anti-(His)₆ antibodies were used instead of anti-(His)₆-VP281 IgGs, no gold labelling signals could be seen in the viral envelope (Fig. 4d). The pre-immune mouse antiserum was also used to replace the primary antibody but no labelling signals could be observed either in WSSV virions or in nucleocapsids (data not shown). From the above observations, it was concluded that VP281 is a WSSV envelope protein.

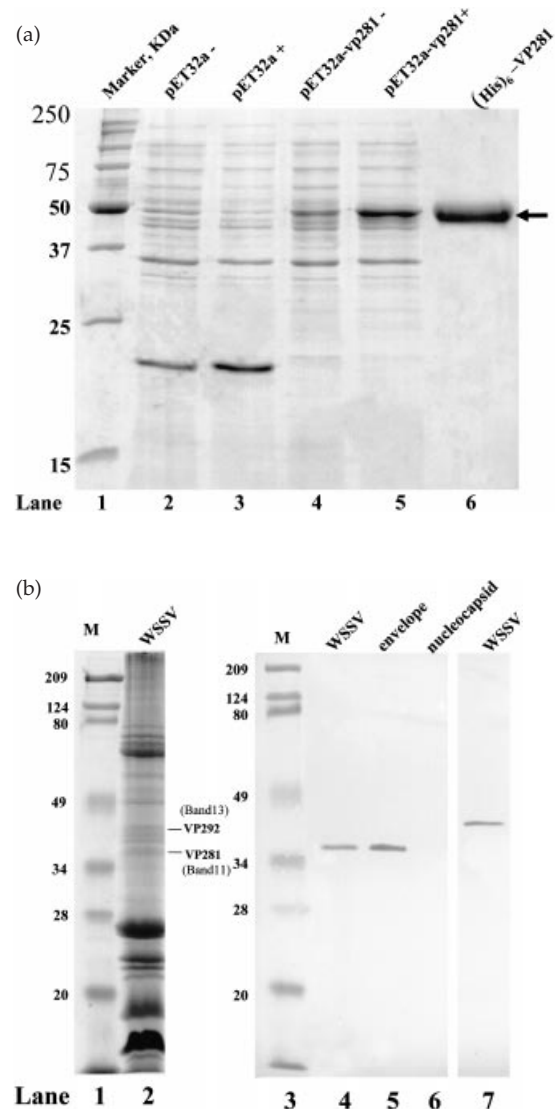


Fig. 3. Expression of VP281 in *E. coli* and Western blot analysis. (a) Expression of VP281 in *E. coli* BL21 cells. Coomassie brilliant blue-stained 12 % SDS-PAGE gel with broad range molecular mass protein markers (lane 1); pET32a(+) [BL21 cells transformed with pET32a(+) vector] either uninduced (lane 2) or induced (lane 3); pET32a(+)-VP281 [recombinant pET32a(+)-vp281 plasmid] either uninduced (lane 4) or induced (lane 5); purified (His)₆-VP466 protein (lane 6, arrow). (b) Western blot of the SDS-PAGE of WSSV virions with anti-VP281 IgGs. Shown are Coomassie brilliant blue-stained 12 % SDS-PAGE gels with pre-stained broad range protein molecular mass markers (Bio-Rad) (lanes 1 and 3) and purified WSSV virions (lane 2). Western blot of the SDS-PAGE of intact WSSV (lane 4), purified viral envelope (lane 5) and viral nucleocapsids (lane 6) with anti-VP281 anti-mouse IgGs; a separate Western blot of the SDS-PAGE of intact WSSVs with anti-VP292 anti-mouse IgGs (lane 7) is also shown.

Discussion

The estimated molecular mass of the protein in band 11 in the WSSV SDS-PAGE gel is 37 kDa (Fig. 1a), which is greater than the theoretical molecular mass (31.5 kDa) of the *vp281* gene product. This difference indicated that some post-translational modifications might have occurred. Computer-

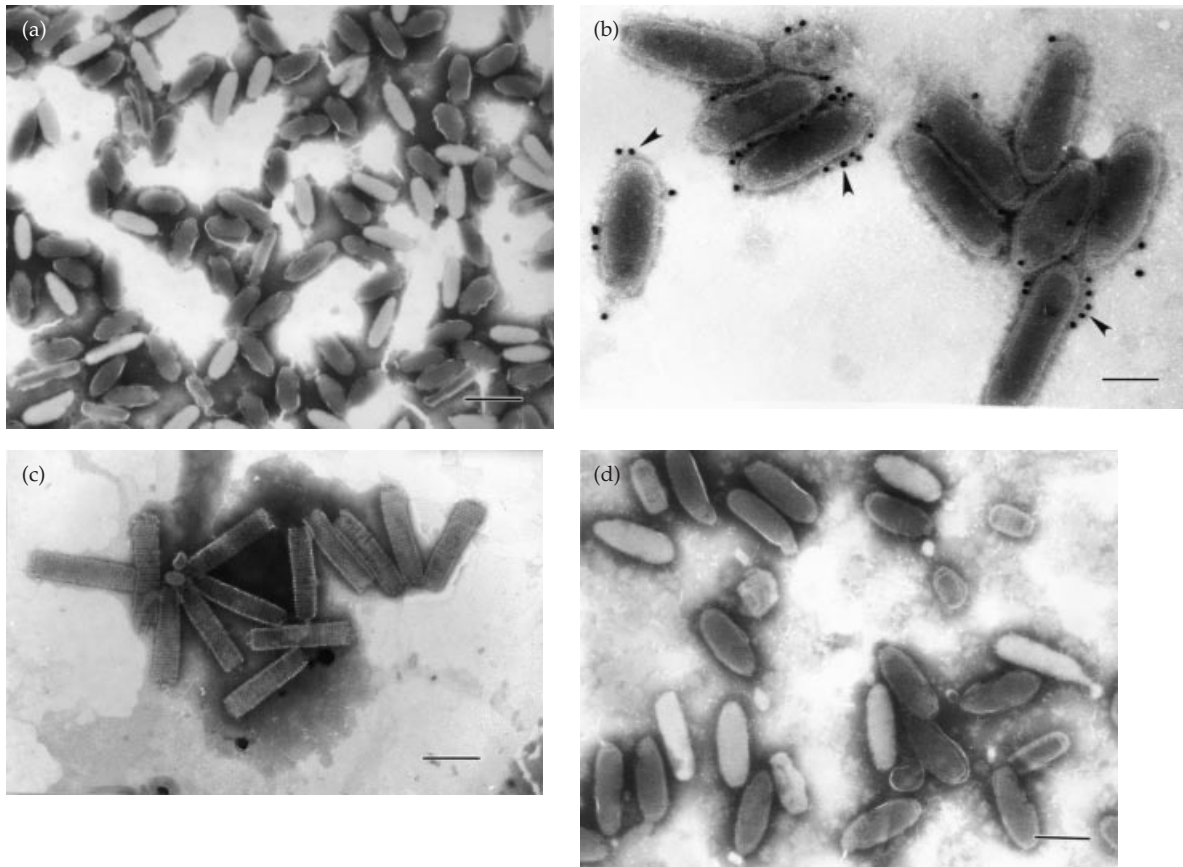


Fig. 4. Localization of VP281 in the WSSV virion. (a) Electron micrograph of negatively stained WSSV virions (2% PTA). Bar, 390 nm. (b) In the experimental groups, polyclonal mouse anti-(His)₆-VP281 IgGs were used as the primary antibody and goat anti-mouse IgGs as the secondary antibody. Secondary antibodies were conjugated with 15 nm colloidal gold particles. Gold particles were located specifically at the viral envelope (arrowheads). Bar, 135 nm. (c) No labelling can be seen in the purified WSSV nucleocapsids. Bar, 180 nm. (d) With mouse anti-(His)₆ IgGs as the primary antibody, no gold particles were found in the viral envelope. Bar, 210 nm.

assisted analysis revealed that there are two potential *N*-linked glycosylation sites and several possible phosphorylation sites in VP281. In addition, VP281 was found to contain an RGD motif (aa 75–77, Fig. 2a). This conserved RGD tripeptide, termed the cell attachment domain (Ruoslahti & Pierschbacher, 1986), was shown to play an important role in mediating recognition by cells and infectivity of a variety of pathogens, including adenovirus type 2, coxsackievirus A9, echovirus type 22, foot-and-mouth disease virus, human parechovirus type 1 and others (Mateu *et al.*, 1996; Boonyakiat *et al.*, 2001 and references therein). Further studies will be needed to demonstrate if the RGD domain in VP281 plays a similar role in initiating WSSV infection and how it interacts with other proteins.

Homology queries of the *vp281* gene and its product did not reveal data of significant similarity to any other viral proteins in the NCBI database library. However, it is closely related to VP292, another newly identified WSSV structural protein (Huang *et al.*, 2002), at the amino acid and nucleotide sequence level (Fig. 2b). Such homologous genes have been

found in WSSV VP28, VP26/P22 and VP24 (van Hulten *et al.*, 2000b) (and in some unknown ORFs in the WSSV genome database); these homologous genes were assumed to have evolved by gene duplication and were assigned to 10 gene families (van Hulten *et al.*, 2002). The newly identified *vp281* and *vp292* gene sequences were found to be identical to ORF127 and ORF118, respectively, as described by van Hulten *et al.* (2002), and were sorted into WSSV gene family 10 (van Hulten *et al.*, 2002). Our observations supported the proposition that VP281 and VP292 represented a new WSSV structural protein group that might have evolved by gene duplication from a common ancestral gene. Since characterization of VP292 is still in progress, it is unknown whether there are functional divergences between these two proteins. Gene duplication events are usually documented in RNA viruses and some large DNA viruses, such as closteroviruses (Boyko *et al.*, 1992), rhabdoviruses (Wang & Walker, 1993), simian haemorrhagic fever virus (Godeny *et al.*, 1998) and alphaherpesviruses. For RNA viruses, such events were due possibly both to the strict constraints on the size of the RNA

genome and to their rapid evolution (Boyko *et al.*, 1992); it was also presumed that the duplicated genes present in large DNA viruses might play important roles for the co-evolution of the virus and host in responding to pressures of selection (Davison, 1999 and references therein). Further research will be performed to investigate the biological importance of the presence of multiple duplicated genes with high identities in the WSSV genome.

So far a total of five WSSV envelope proteins has been identified: VP28, VP26/P22, VP19, VP466 and VP281 (Huang *et al.*, 2002; van Hulten *et al.*, 2002 and references therein). Compared to some known membrane proteins, VP281 lacks a predominant transmembrane region. The absence of transmembrane domains may suggest that this protein was produced in soluble form. Such forms were well documented in some membrane fusion proteins, pathogen receptors and some cell adhesion molecules, functioning to anchor the polypeptides to the membrane hydrophobic phase by means of locating aqueous activities or possibly interacting with some membrane-spanning components (Stevens & Arkin, 2000).

For enveloped viruses, *in vivo* neutralization experiments are conducted routinely to study the function of viral envelope proteins and to identify viral protein epitopes involved in the virus infection process, which might lead to preventive approaches to control virus diseases (Burton *et al.*, 2000; Schofield *et al.*, 2000). Of the identified WSSV envelope proteins, VP28 was found to be involved in the systemic infection of shrimp by WSSV and its polyclonal antiserum was capable of neutralizing virus infection (van Hulten *et al.*, 2001). Similar experiments can be conducted to elucidate if VP281 is also involved in such processes and to find out if its antiserum can play a role to prevent or inhibit WSSV infection of shrimp.

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