Characterization of White Spot Syndrome Virus Envelope Protein VP51A and Its Interaction with Viral Tegument Protein VP26

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Received 14 June 2008/Accepted 17 September 2008

In this study, we characterize a novel white spot syndrome virus (WSSV) structural protein, VP51A (WSSV-TW open reading frame 294), identified from a previous mass spectrometry study. Temporal-transcription analysis showed that vp51A is expressed in the late stage of WSSV infection. Gene structure analysis showed that the transcription initiation site of vp51A was 135 bp upstream of the translation start codon. The poly(A) addition signal overlapped with the translation stop codon, TAA, and the poly(A) tail was 23 bp downstream of the TAA. Western blot analysis of viral protein fractions and immunoelectron microscopy both suggested that VP51A is a viral envelope protein. Western blotting of the total proteins extracted from WSSV virions detected a band that was close to the predicted 51-kDa mass, but the strongest signal was around 72 kDa. We concluded that this 72-kDa band was in fact the full-length VP51A protein. Membrane topology assays demonstrated that the VP51A 72-kDa protein is a type II transmembrane protein with a highly hydrophobic transmembrane domain on its N terminus and a C terminus that is exposed on the surface of the virion. Coimmunoprecipitation, colocalization, and yeast two-hybrid assays revealed that VP51A associated directly with VP26 and indirectly with VP28, with VP26 acting as a linker protein in the formation of a VP51A-VP26-VP28 complex.

Viral structural proteins, especially the envelope proteins, are important, not only because they are involved in virion morphogenesis, but also because they are the first molecules to interact with the host. The structural proteins often play vital roles in cell targeting, virus entry, assembly, and budding (1, 2, 21, 22, 24), as well as triggering host antiviral defenses (26). In the case of white spot syndrome virus (WSSV) (genus *Whispovirus*, family *Nimaviridae*) (37), a double-stranded DNA virus that has caused severe mortality and huge economic losses to the shrimp farming industry globally for more than a decade (5, 19), proteomic methods have helped to identify a total of 58 structural proteins, over 30 of which are currently recognized as envelope proteins (13, 31, 44, 47). Some of the WSSV envelope proteins involved in shrimp infection have been identified (12, 14, 34, 36, 41, 43), and these envelope and other WSSV structural proteins have been used in various studies, including RNA interference-based gene knockdown to silence viral structural-protein gene expression (8, 27, 45), DNA and protein vaccination to elevate host immunity (25, 29, 36, 39), and antibody neutralization techniques that neutralize the virus by preventing envelope proteins from interacting with host cell receptors (12, 34, 41, 43).

In the present paper, we characterize and investigate the functionality of a WSSV structural protein that was first reported by Tsai et al. (32). This protein, designated VP51A, corresponds to open reading frame 294 of the WSSV-TW isolate, and its gene encodes a polypeptide of 486 amino acids (aa) with a theoretical mass of 51 kDa. A method was recently established to assign the WSSV structural proteins to one of three morphologically distinct substructures in the virion: the nucleocapsid, the intermediate tegument layer that surrounds the nucleocapsid, and the outer viral envelope (31). We use the same method here to show that VP51A is an envelope protein, and we support this conclusion with an in vivo immunogold assay. Some WSSV structural proteins are known to interact with other structural proteins. For example, VP28 interacts with VP24 and VP26 (43, 44), and VP24 interacts with another WSSV envelope protein, WSV010 (3). Identification of these relationships among structural proteins is a significant step toward understanding the function of each protein and is potentially helpful in developing effective anti-WSSV strategies. We show here that VP51A forms a complex with two other major WSSV structural proteins, VP26 and VP28.

MATERIALS AND METHODS

**Virus.** The WSSV-TW strain was isolated from a batch of WSSV-infected *Penaeus monodon* shrimp collected in Taiwan in 1994 (18, 38), and it was used as the template for amplification of the vp51A, vp26, and vp28 coding regions in all of the following experiments.

**Temporal-transcription analysis of vp51A by RT-PCR.** Adult *P. monodon* shrimp (mean weight, ~20 g) were experimentally infected with WSSV by injection and subsequently collected at 0 (i.e., immediately before infection), 2, 4, 6, 12, 24, 36, 48, and 60 h postinfection (p.i.) according to a procedure described by Tsai et al. (31). Total RNAs were isolated from the gills of the sampled shrimp by using Trizol reagent (Invitrogen Corp.) according to the manufacturer’s instructions. The isolated RNAs were treated with DNase I (Roche) at 37°C for 1 h and then recovered by phenol-chloroform-isomyl alcohol extraction and ethanol precipitation. The RNAs were reverse transcribed with SuperScript II re-
verses transcriptase (RT) (Invitrogen Corp.) and an oligo(dT) anchor primer (Roche). The first-strand cDNA products were subjected to PCR with the vp51A primers WSSV294-F-122 and WSSV294-R-632 (Table 1). For comparison, the WSSV genomic-DNA sequences were compared with the WSSV genomic-DNA sequences. The RNA samples used for 5′ rapid amplification of cDNA ends (RACE) (6) using a commercial 5′ RACE kit (Roche) with an avian myeloblastosis virus RT. The RNA samples used for 3′ RACE were isolated from the gills of WSSV-infected P. monodon at 36 h p.i. and treated with DNase I as described above. The appropriate gene-specific primers used for 5′ RACE are listed in Table 1. The final amplification products were cloned into the pGEM-T Easy vector (Promega) and sequenced. The sequences of the inserts are listed in Table 1. The final amplification products were cloned into the pET-28b (+) (Novagen). The resulting plasmids, pET-28b/Vp51A139-250, pET-28b/vp51A251-486, and pET-28b/vp51A51-486 (containing the VP51A aa residues 139 to 250, 251 to 486, and 51 to 486, respectively), were transformed into BL21 Codon Plus Escherichia coli cells (Stratagene) and used for protein production. The transformed cells were grown overnight at 37°C in Luria-Bertani medium supplemented with 50 μg/ml kanamycin. The cells were then inoculated into fresh medium at a ratio of 1:50 and grown at 37°C for 1.5 to 2 h. Expression was induced by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and incubation was continued for another 1.5 to 3 h. The induced bacteria were spun down at 4°C, suspended in ice-cold 1× phosphate-buffered saline (PBS) containing 10% glycerol and a protease inhibitor cocktail tablet (Roche), and sonicated for 3 min on ice. The insoluble debris was collected by centrifugation, suspended in 1× PBS containing 1.5% sodium lauryl sarcosine, and solubilized by shaking at room temperature for 1 h. The supernatant was clarified by centrifugation and mixed with Ni-nitrilotriacetic acid-agarose beads (Qiagen) on a rotating wheel at 4°C for 16 h. The beads were then washed several times with ice-cold wash buffer (1 M NaCl, 10 mM Tris-HCl, pH 7.5) to remove unbound material. The fusion proteins were eluted directly from the beads with sodium dodecyl sulfate (SDS) sample buffer and then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) analysis. For polyclonal-antibody production from the VP51A midsequence (aa 139 to 250) and C-terminal (aa 251 to 486) fragments, the protein bands containing the fusion proteins were sliced from the gel, minced, mixed with Freund’s adjuvant, and inoculated into rabbits.

### Table 1. Primer sequences for 5′ RACE and RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
<th>Usage</th>
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<tbody>
<tr>
<td>vp51A</td>
<td>WSSV294-F-122</td>
<td>GGAAGAGATGATCGTACCAGGAT</td>
<td>vp51A RT-PCR</td>
</tr>
<tr>
<td>icl</td>
<td>icl-F</td>
<td>CTTGTTTCTTCCGTAGAGTGT</td>
<td>icl RT-PCR</td>
</tr>
<tr>
<td>dnapol</td>
<td>dnapol-F</td>
<td>GATCTTAAACATCTTTTGCCA</td>
<td>dnapol RT-PCR</td>
</tr>
<tr>
<td>vp28</td>
<td>vp28-F</td>
<td>CTGGTCTGCCGCTGTTGATTT</td>
<td>vp28 RT-PCR</td>
</tr>
<tr>
<td>actin 2</td>
<td>P1882-actin F</td>
<td>CCGCCATGAGTGGATGTT</td>
<td>actin RT-PCR</td>
</tr>
<tr>
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<td>vp51A SP1</td>
<td>GTAGGCCCTAAATCCTGGTGTGGT</td>
<td>vp51A 5′ RACE</td>
</tr>
<tr>
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<td>vp51A 5′ RACE</td>
</tr>
<tr>
<td>vp51A</td>
<td>vp51A SP3</td>
<td>TTGAGGATGTTGAGTCTGCA</td>
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</tr>
<tr>
<td>vp51A</td>
<td>vp51A SP4</td>
<td>TTGGGCCATATAAGAAGCAC</td>
<td>vp51A 3′ RACE</td>
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### Table 2. Primer sequences used for the construction of various expression plasmids

<table>
<thead>
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<th>Construct</th>
<th>Primer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence (5′–3′)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Tag</th>
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<tbody>
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<td>CGCGGATCCCGGATGATACGAGACGATTTTGAG</td>
<td>His</td>
</tr>
<tr>
<td>pET-28b/Vp51A251-486</td>
<td>F</td>
<td>CCCAAGCTTATATATTTGTTGCTGCAATTC</td>
<td>R</td>
</tr>
<tr>
<td>pET-28b/Vp51A51-486</td>
<td>F</td>
<td>CCCAAGCTTATATATTTGTTGCTGCAATTC</td>
<td>R</td>
</tr>
<tr>
<td>pcDNA3/Vp51A</td>
<td>F</td>
<td>CCCAAGCTTATATATTTGTTGCTGCAATTC</td>
<td>R</td>
</tr>
<tr>
<td>pDHsp/Vp51A-FLAG-His</td>
<td>F</td>
<td>CCTCGGCTTGTTGGCTGCAATATAAATTTTG</td>
<td>Flag/V5</td>
</tr>
<tr>
<td>pDHsp/Vp526-FLAG-His</td>
<td>F</td>
<td>CCCAAGCTTATATATTTGTTGCTGCAATTC</td>
<td>V5</td>
</tr>
<tr>
<td>pGBK-Vp51A</td>
<td>F</td>
<td>TCCCGGGCAATTCTGGCATAAGCG</td>
<td>c-Myc</td>
</tr>
<tr>
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<td>F</td>
<td>CGCCGCTGAGGTCTGCTGACGAC</td>
<td>HA&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>pGAD-Vp28</td>
<td>F</td>
<td>CGCCGCTGAGGTCTGCTGACGAC</td>
<td>HA</td>
</tr>
</tbody>
</table>

<sup>a</sup> F, forward; R, reverse.

<sup>b</sup> The restriction enzyme cutting sites are underlined.

<sup>c</sup> HA, hemagglutinin.
Fractionation of virion proteins by detergent treatment at different NaCl concentrations. Adult crayfish, Procambarus clarkia, were challenged with WSSV, and the virions were purified from the hemolymph of the infected crayfish as described by Tsai et al. (32). The purified virus suspension was treated with 1% Triton X-100 in different concentrations (0, 0.1, 0.5, and 1 M) of NaCl solution, and the soluble and insoluble portions were then fractionated by centrifugation as described previously (31). The intact untreated-virion suspension served as a control. The proteins in each of the eight resultant fractions and in the intact purified virion control were resolved by Western blot analysis using the anti-VP51A C-terminal-fragment antibody. The bound antibodies were then stripped out of the membrane, and the membrane was reprobed with antibodies to the WSSV envelope protein VP26, to the tegument protein VP26, and to the nucleocapsid protein VP51C (31, 40). An additional Western blot analysis was performed on the intact purified virion proteins using the anti-VP51A midsequence fragment antibody as the probe.

Western blot analysis. Protein samples were resolved by SDS-PAGE. After separation, the proteins were transferred to polyvinylidene difluoride membranes (MBS). The membranes were incubated in blocking buffer containing primary antibodies for 1 h at room temperature. Next, the membrane was washed three times with 0.5% Tween 20 in Tris-buffered saline and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody. After three more washes, the proteins were visualized by use of a chemiluminescence reagent (Perkin-Elmer, Inc.).

Localization of VP51A by immunoelectron microscopy (IEM). Following the method of Tsai et al. (31), aliquots (10 μl) of purified virion suspension were adsorbed to Formvar-supported, carbon-coated nickel grids (200 mesh) for 5 min at room temperature. After the excess solution was removed, the grids were either prefixed for 5 min with 4% paraformaldehyde and 1% Triton X-100 simultaneously in 50 mM Tris buffer (to remove the virus envelope) or were left unfixed and were incubated with incubation buffer containing antibodies for 1 h at room temperature. Next, the membrane was washed three times with 0.5% Tween 20 in Tris-buffered saline and then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody. After three more washes, the proteins were visualized by use of a chemiluminescence reagent (Perkin-Elmer, Inc.).

To investigate the topology of VP51A, VP52, and VP26 in the WSSV virion, we followed the method of Zhao and Yuan (48) and treated aliquots (5 μg of total protein) of purified virions with trypsin (5 μg/ml; Promega) in 100 μl of buffer (50 mM Tris-HCl [pH 7.5], 1 mM CaCl2, 100 mM NaCl) at 37°C for 2 h. Trypsin digestion was terminated by phenylmethylsulfonyl fluoride at a concentration of 0.5 mM and then adding 1/50 volume of protease inhibitor. In some samples, prior to trypsin digestion, Triton X-100 was added to a final concentration of 1% to dissolve the viral envelope and expose the internal structure to the protease. Samples were analyzed by Western blotting using antibodies to VP51A, to the envelope protein VP26, and to the tegument protein VP26.

Comunmunoprecipitation. Full-length WSSV vp51A, vp26, and vp28 genes were inserted into V5- or FLAG-tagged vectors containing the heat-inducible Drosophila heat shock protein 70 promoter (pDHsp/V5-His and pDHsp/FLAG-His) (11) by PCR cloning using WSSV genomic DNA as the template. The primers used for PCR are listed in Table 2. For DNA transfection, Sf9 insect cells were seeded onto a six-well plate (8 × 10^5 cells/well) and grown in Sf-900 II serum-free medium (Invitrogen Corp.) at 27°C. At 6 h after the heat shock, the cells were washed with 1× PBS and lysed in 100 μl of NP-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40) supplemented with a protease inhibitor cocktail tablet. The lysate was centrifuged at 12,000 × g for 5 min, and an aliquot of the supernatant (10 μl) was reserved for Western blot analysis to confirm the expression of the transfected genes. The remaining supernatant (90 μl) was then incubated with 15 μl of anti-VP26 (VP26) and anti-VP28 (VP28) antibodies (1:100) overnight at 4°C. The unbound proteins were then washed five times in 150 μl of NP-40 lysis buffer. Aliquots of the total cell lysates and immunoprecipitated complexes were separated by 15% SDS-PAGE and transfected to a polyvinylidene difluoride membrane. V5-tagged fusion proteins were detected with rabbit anti-V5 antibody and goat anti-rabbit IgG-HRP conjugate (Sigma). FLAG-tagged VP51A and VP26 proteins were detected with mouse anti-FLAG monoclonal antibody (Sigma) and goat anti-mouse IgG-HRP conjugate (Sigma).

Yeast two-hybrid assay. Protein-protein interaction assays were performed using a commercial yeast two-hybrid system, Matchmaker 3 (Clontech), according to the manufacturer’s protocol. The bait plasmid, pGBK-VP51A, was constructed by cloning the PCR-amplified, full-length VP51A gene into the SmaI/BamHI sites of pGBK7 (Clontech) in frame with the GAL4 DNA binding domain. The prey plasmids, pGAD-P26 and pGAD-P26, were constructed by cloning the PCR-amplified, full-length VP26 and VP28 genes, respectively, into the EcoRI/XhoI sites of pGAD-T7 (Clontech). Following transformation, the AH109 cells were plated on selective agar. The proteins were tested for autoactivation by cotransformation the empty vector and selective agar. The proteins were tested for autoactivation by cotransformation the empty vector and selective agar. The proteins were tested for autoactivation by cotransformation the empty vector and selective agar. The proteins were tested for autoactivation by cotransformation the empty vector and selective agar. The proteins were tested for autoactivation by cotransformation the empty vector and selective agar. The proteins were tested for autoactivation by cotransformation the empty vector and selective agar.
and adenine (Ado) in the presence of 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal) (Sigma).

Indirect immunofluorescence assays of WSSV-infected shrimp hemocytes. Hemolymph was collected from healthy *P. monodon* shrimp and from WSSV-infected shrimp at 72 h p.i. using a syringe that contained cold modified Alsever solution (15). The hemocytes were placed on cover glasses, washed with PBS, and fixed in paraformaldehyde (4% in PBS) for 10 min at 4°C. After acetone treatment (3 min on ice), the hemocytes were incubated with blocking buffer (as described above) for 16 h at 4°C. The hemocytes were then washed with 1% Triton X-100 (Triton) in PBS-diluted polyclonal rabbit anti-VP51A C-terminal antibody, 300× PBS-diluted polyclonal rat anti-VP26 antibody, or both (16 h at 4°C). Next, the cells were washed with PBS containing 0.1% Triton X-100 and incubated with Alexa Fluor 488 dye-conjugated donkey anti-rabbit IgG or Alexa Fluor 594 dye-conjugated donkey anti-rat IgG antibody (1:500 in PBS, Jackson ImmunoResearch), or both for 2 h at room temperature. Counterstaining of the nucleus was performed with DAPI. After being washed three times with PBS (10 min each), the cover glasses were wet mounted and the fluorescent signals were examined as described above.

**RESULTS**

**Temporal-transcription analysis of vp51A**. The expression profiles of vp51A in the gills of *P. monodon* at various stages of WSSV infection were analyzed by RT-PCR (Fig. 1). The vp51A transcript was first detected at 6 h p.i. By comparison, the immediate-early gene ie1 and the early gene dnapol were both transcribed as early as 2 h p.i. and continued to be found to 60 h p.i. The transcript of the WSSV envelope protein gene vp28 was also detected at 2 h p.i. The actin control confirmed the quantity of the total RNA templates (Fig. 1E), and another control using a WSSV genomic-DNA-specific primer pair, IC-F2/IC-R3 (16), derived from an intergenic region of the WSSV genome, confirmed that there was no WSSV DNA contamination (data not shown). From these data, we conclude that vp51A is a gene that is expressed in the late stage of WSSV infection.

**Mapping the 5’ and 3’ ends of the vp51A transcript by RACE**. The 5’ and 3’ ends of the vp51A transcripts were determined by the RACE method. The 5’ RACE products were cloned into the pGEM-T Easy vector, and seven clones were randomly chosen for sequencing with the results shown in Fig. 2A. Two of the randomly selected 5’ RACE products had their 5’ termini at nucleotide residue −135 (G) and five at −128 (G) (relative to the A in the translation initiation codon, ATG, which is defined as +1 [A]) (Fig. 2A). These results suggest that for WSSV vp51A, there are two candidate transcription initiation sites, located at nucleotide residues −135 (G) and −128 (G), respectively. The sequence around −128 (G) did not match any of the known WSSV or baculovirus start site consensus motifs, and its biological meaning (if any) remains unclear. Conversely, the sequence around the transcriptional initiation site at −135 (G) (ATGAG) was a close match to the WSSV late-gene transcriptional initiation site consensus motif (ATTAAG) (20). We also note that there was an A/T-rich region upstream of this site. In the 3’ region of vp51A, the poladenylation signal (AATAAA) overlapped with the stop codon. Sequence analysis of the cloned 3’ RACE products revealed that the poly(A) tail addition site was located 22 bp downstream of the poladenylation signal. Like many other WSSV genes (20), vp51A also had oligo(T) stretches downstream of the poly(A) tail addition site (Fig. 2B).

**Localization of VP51A in the virion**. In this study, we used a protein fractionation method developed by Tsai et al. (31) to determine whether VP51A is located in the envelope, the tegument, or the nucleocapsid. Using Western blot analysis of the eight different fractions of the WSSV virion proteins, VP51A’s profile was compared to the profiles of known envelope, tegument, and nucleocapsid proteins (VP28, VP26, and VP51C, respectively) (33). The results show that in the 1% Triton X-100-treated preparations, VP51A was similar to the envelope protein VP28 in that it was almost completely soluble in both the presence and absence of NaCl. The provisional conclusion that VP51A was therefore an envelope protein was then confirmed using an immunogold assay and IEM observation. When a gold-labeled secondary antibody was used in conjunction with an anti-VP51A antiserum derived from a C-terminal-region fragment (aa 251 to 486), gold particles were observed on the intact WSSV virions (Fig. 4A) but not on the tegument layer (Fig. 4B) or on the nucleocapsid surface (Fig. 4C). Control experiments showed that no gold particles were found on the envelopes of WSSV virions when normal rabbit serum was used as the primary antibody (Fig. 4D). Collectively, these results confirmed that VP51A is a WSSV envelope protein.

**Molecular mass of the VP51A protein**. Western blot analysis of the WSSV virion proteins using an antibody to a VP51A C-terminal fragment (aa 251 to 486) detected not only the theoretically predicted signal around 51 kDa, but also a major band at ~72 kDa (Fig. 5A). This unexpected result was checked using an antibody against the midsequence region (aa 139 to 250) of the VP51A coding region. The midsequence antibody detected the major band at ~72 kDa, as well as several other, smaller bands (Fig. 5B) that presumably resulted from posttranslational processing. The major 72-kDa band was...
also detected using both V5 tagging (Fig. 5C, lane 2) and isotope labeling (Fig. 5D, lane 2). Figure 5E (lane 2) shows Western blots for a truncated form of \( vp51A \) (aa 51 to 486) that omitted the transmembrane domain in the N-terminal region and was expressed in \( E. coli \) cells. The molecular mass of this truncated, recombinant VP51A was about 68 kDa, which is a little lower than the molecular mass of the full-length VP51A that was overexpressed in Sf9 cells (Fig. 5E, compare lanes 2 and 3). Since no glycosylation can occur in the \( E. coli \) BL21 expression system, this result suggests that the 72-kDa band of VP51A was also not due to glycosylation. Although it is unusual for the structural proteins of animal viruses to remain unglycosylated, this finding for VP51A is consistent with previous reports that none of the WSSV structural proteins are glycosylated (35, 44). We therefore tentatively conclude that the discrepancy between the apparent molecular mass (72 kDa) and the calculated molecular mass (51.5 kDa) of VP51A must be due to the abundance of negatively charged residues (14%), which would have the effect of retarding the protein’s migration through the gel (4, 7).

**Membrane topology of VP51A.** A hydrophobicity profile generated using Kyte-Doolittle hydrophobic plot analysis showed a

FIG. 2. Partial sequences of WSSV \( vp51A \) showing nucleotides and deduced amino acids for the 5’ (A) and 3’ (B) regions. The primers used for 5’ RACE and 3’ RACE (\( vp51A \) SP1, \( vp51A \) SP2, \( vp51A \) SP3, and \( vp51A \) SP4) are underlined. The bent arrows indicate the transcriptional start sites as revealed by sequencing seven randomly chosen 5’ RACE clones. The polyadenylation signal (AATAAA) is boxed and in boldface. The poly(A) addition site occurs 22 bp downstream of the polyadenylation signal and is indicated by a vertical arrow.

FIG. 3. Determination of VP51A’s location in the WSSV virion. Intact WSSV virions were subjected to detergent and NaCl treatment as indicated. After fractionation, the pellet (P) and supernatant (S) fractions were separated on SDS-PAGE and detected by Western blotting to produce profiles that are characteristic of envelope, tegument, and nucleocapsid proteins (31). Three representative WSSV structural proteins are shown for comparison. Lane V is the untreated purified virus.

FIG. 4. Localization of VP51A in the WSSV virion by immunogold assay using a rabbit anti-VP51A C-terminal antibody probe followed by a gold-labeled secondary antibody. (A to C) IEM images of purified WSSV virions (A), unenveloped virions (i.e., the tegument-nucleocapsid structure) (B), and the viral nucleocapsids (C). (D) IEM of WSSV virions, with preimmune rabbit serum used as the probe instead of the primary antibodies. The gold particle signals (arrows) were detected only on the enveloped virion. Scale bars, 100 nm.
high hydrophobicity in the N-terminal region of VP51A (data not shown), while sequence analysis of VP51A using the TMHMM program predicted that VP51A encodes a transmembrane helix between aa 2 and 24 (data not shown). To confirm these predictions, a recombinant VP51A fusion protein (rVP51A-V5) with a V5 tag on its C terminus was subjected to indirect immunofluorescence assays in transfected Sf9 cells. In cells that were treated with Triton X-100 to render them permeable to the anti-V5 antibody, the full-length rVP51A-V5 was detected both in the plasma membrane region and in the cytoplasm (Fig. 6A, top). In the nonpermeabilized cells, however, even though the rVP51A-V5 could no longer be detected in the cytoplasm, it was still detected on the outside surface of the plasma membrane (Fig. 6A, bottom). We conclude that the C-terminal region of the protein must therefore be located outside the cell membrane, because otherwise the V5 tag would not have been accessible to the anti-V5 antibody. This conclusion was further confirmed by conducting a similar assay for PmSTAT, a shrimp protein that is expressed in both the nucleus and cytoplasm of Sf9 cells (17). We found that in the absence of Triton X-100, no positive PmSTAT signals could be detected (data not shown). Taken together, these data suggest that VP51A is a type II transmembrane protein. A schematic of the proposed transmembrane topology of the V5-tagged VP51A is shown in Fig. 6B.

**Virion Topology of VP51A, VP26, and VP28.** The topology of these three structural proteins in the WSSV virion was investigated by using trypsin to distinguish between proteins that were accessible to proteolysis and those that were protected from digestion by the lipid bilayer. WSSV virions were either left untreated or treated with trypsin in the absence or presence of Triton X-100 to render them permeable to the anti-V5 antibody. rVP51A-V5 was visualized with rabbit anti-V5 antibody and Cy3-conjugated donkey anti-rabbit IgG antibody (Fig. 6B). Nuclei were visualized by counterstaining them with DAPI. Images c and g show the merged Cy3 and DAPI signals. The last column (d and h) shows the merged signal overlaid with the bright-field images of the corresponding cells. Scale bar, 10 μm. (B) Schematic of the proposed transmembrane topology of VP51A.
ence of the detergent Triton X-100, and the digested products were analyzed by Western immunoblotting using antibodies against the VP51A C terminus (aa 251 to 486) or against the full length of VP26 or VP28. As expected, the VP51A antibody recognized the VP51A 72- and 51-kDa proteins in the untreated virions (Fig. 7, lane 1). However, after digestion with trypsin in the absence or presence of Triton X-100, the 72-kDa protein was no longer detected, even though the 51-kDa protein was still present (Fig. 7, lanes 2 and 3). We note that the amount of the 51-kDa protein was only slightly decreased in the presence of Triton X-100 (Fig. 7, lane 3), which suggests that the 51-kDa VP51A protein was still protected from trypsin digestion even after the envelope was removed. Similar immunoblotting results were obtained with the antibody against the VP51A midsequence (data not shown). It is not clear why the 51-kDa VP51A was not degraded by trypsin digestion. The treated virions were also subjected to Western blotting to detect the envelope protein VP28 and the tegument protein VP26. VP28 was digested into two bands in the absence of Triton X-100 and completely digested in the presence of Triton X-100 (Fig. 7, lanes 4 to 6). VP26 was digested only in the presence of Triton X-100 (Fig. 7, lanes 7 to 9). Similar results for VP26 and VP28 were also demonstrated by Tsai et al. (31).

**FIG. 8.** VP51A interacts with VP26, but not with VP28. (A) Coimmunoprecipitation of V5-tagged VP26 or VP28 with FLAG-tagged VP51A from transfected cells. Sf9 cells were transfected with plasmids expressing V5-tagged VP26, V5-tagged VP28, FLAG-tagged VP51A, or empty plasmid (vector) as indicated. (a) At 6 h after heat shock, the cell lysates were harvested and separated by SDS-PAGE, and input expression was confirmed by Western blotting (blot) using either anti-V5 antibody or anti-FLAG antibody as a probe. The arrows indicate the expressed V5-tagged VP26, V5-tagged VP28, and FLAG-tagged VP51A. (b) The cell lysates were immunoprecipitated (I.P.) with anti-FLAG M2 affinity resins, and then the immunoprecipitated complexes were subjected to Western blot analysis with an anti-V5 antibody probe. (B) The yeast two-hybrid results confirmed that VP51A specifically interacted with VP26, but not with VP28. (a) Yeast growth on medium lacking both Leu and Trp indicated the presence of each respective pair of plasmids. (b and c) Yeast growth on low-stringency (−Leu/−Trp/−His) and high-stringency (−Leu/−Trp/−His/−Ade) media, respectively. The blue signal in image c is due to the presence of X-a-Gal. The positive signals represent protein-protein interactions. (C) Intracellular localizations of VP51A and VP26 in WSSV-infected *P. monodon* hemocytes by confocal microscopy. (a) VP51A was visualized using rabbit anti-VP51A antibody and Cy3-conjugated donkey anti-rabbit IgG antibody. (b) VP26 was visualized with rat anti-VP26 antibody and FITC-conjugated donkey anti-rat IgG antibody. (c) Nuclei were visualized by counterstaining them with DAPI. (d) Merged Cy3, FITC, and DAPI signals. Scale bar, 10 μm.
VP51A protein and the empty vector (pGBK-VP51A/pGADT7) did not induce reporter gene activation (Fig. 8B, b and c), from which we conclude that VP51A does not have autonomous activation ability. Growth on the low-stringency (SD/−Leu/−Trp/−His) and high-stringency (SD/−Leu/−Trp/−His/−Ade/X-a-Gal) plates was observed only when the yeast was transformed with pGBK-VP51A/pGAD-VP26 or with the positive control (Fig. 8B, b and c). Again, these results showed that VP51A interacts with VP26 but does not interact with VP28.

In order to assess the intracellular distribution of VP51A and VP26 in WSSV-infected P. monodon cells, hemocytes were collected at a late stage of infection (72 h p.i.). The virus-infected cells were fixed, permeabilized, stained for rabbit anti-VP51A and rat anti-VP26 antibodies, and analyzed by confocal microscopy. As shown in Fig. 8C, strong punctate signals from both VP51A (image a) and from VP26 (image b) were observed in the plasma membrane and cytoplasm, and the merged image shows that these signals were almost completely superimposed (image d). No signals were detected in the uninfected P. monodon hemocyte controls (data not shown). Clearly, this suggests that both VP51A and VP26 colocalized to the same subcellular locations.

VP26 links VP51A and VP28. A previous study showed that VP26 interacts with the most abundant envelope protein, VP28 (44), and this result was reconfirmed here by a coimmunoprecipitation assay with V5-tagged VP28 and FLAG-tagged VP26 expressed in Sf9 insect cells. The two images in Fig. 9A, a, show that both VP26-FLAG and VP28-V5 were successfully expressed in Sf9 cells. Complexes consisting of VP28-V5 plus VP26-FLAG were coimmunoprecipitated by anti-FLAG M2 affinity gel and detected by Western blotting with anti-V5 antibody (Fig. 9A, b, lane 2). As discussed above, we had already established that VP51A interacts with VP26 but not with VP28 (Fig. 8A and B), so we next designed a coimmunoprecipitation experiment to investigate the interactions among all three of these proteins. Expression of VP51A-FLAG, VP26-V5, and VP28-V5 in the cotransfected Sf9 cells was confirmed by Western blot analysis with anti-FLAG and anti-V5 antibodies (Fig. 9B, a). The putative VP51A-VP26-VP28 complex was immunoprecipitated by using an anti-FLAG M2 affinity gel against the FLAG epitope on VP51A, and the presence of both VP26 and VP28 in the complex was demonstrated by Western blotting using anti-V5 antibody (Fig. 9B, b). For the control, cells were cotransfected with an empty V5 vector instead of the VP26-V5 plasmid, with the result that neither VP26 nor VP28 was observed in the Western blot analysis (Fig. 9B, b, lane 1). We therefore conclude that VP26 plays a key role in the formation of a VP51A-VP26-VP28 ternary complex.

**DISCUSSION**

Our results suggest that the major VP51A protein species is unglycosylated and that its apparent mass of 72 kDa is in fact due to a high proportion of charged residues that retard the protein's migration in the gel. We note that two earlier studies identified only a single VP51A (or VP52A) band in the region of ~51 kDa, and neither study reported the major 72-kDa band. In one study (32), the 72-kDa region of the gel was dominated by a very high-intensity band of crayfish hemocyanin that obscured all of the neighboring bands. In the other study (44), the 72-kDa VP51A protein was not reported, even though the entire crayfish was used as the source material and the problem of hemocyanin contamination was solved by using a different purification method. We found, however, that by using the same source tissues and protocols as Xie et al. (44),
we were able to detect the 72-kDa VP51A in the purified virions, and in fact, Western blotting showed that the smaller species of VP51A (i.e., 51 kDa and others) (Fig. 5A and B) were consistently present as well (data not shown). Since the 51-kDa protein could be detected by both the anti-VP51A midsequence and C-terminal antibodies (Fig. 5A and B) and since the peptide sequences deduced from the tandem mass spectrometry data of Tsai et al. (32) matched only the C-terminal region of VP51A (J.-M. Tsai, personal communication), we hypothesize that the 51-kDa band was in fact produced by N-terminal truncation of the full-length protein. Further, since Western blotting consistently produced the same protein profiles regardless of the purification method that was used, we infer that VP51A is sensitive only to site-specific proteases and is otherwise not easily degraded. Lastly, we note that since the 72-kDa, 51-kDa, and some other, smaller VP51A proteins are all readily detected in the WSSV virion (Fig. 5A and B), it seems likely that all of these forms are essential components of the viral particle, and they may all participate at various stages of virus morphogenesis.

We note that there is similar evidence for the proteolytic processing of at least one other WSSV structural protein. Xie et al. (44) identified five different species of VP150 in purified WSSV virions, and the smallest of these proteins had the same apparent mass as VP53A of Tsai et al. (32). Since these two studies used different purification methods, and since VP53A and the 53-kDa species of VP150 were both identified as products of the same open reading frame (WSSV011 in the China isolate; WSSV017 in the Taiwan isolate), we hypothesize that WSSV VP150 may also be subjected to proteolytic processing by a specific virus or host protease.

The ability of WSSV to replicate successfully in a wide range of crustacean hosts suggests that a common crustacean protease might be involved in VP51A proteolysis. Furin is one possible candidate. This extensively studied cellular proprotein convertase is used by many cleavable viral envelope proteins (23, 28), and in WSSV VP150, we found two overlapping instances of the furin recognition motif RXX(K/R)RR near the N terminus (39RKKRRK41). However, our pilot experiments suggested that furin is not in fact involved in the proteolytic processing of the VP51A 72-kDa protein molecule (data not shown). Thus, for the moment, the cellular or viral factors involved in VP51A cleavage and the events governing the trafficking of the precursors remain unknown.

The protein domains exposed on the surfaces of viruses play fundamental roles in infection by binding to cell receptors, promoting cell fusion processes, or interacting with elements of the host immune system (1, 24, 26). Thus, the determination of a protein’s membrane topology is an important first step toward understanding its function. Topological predictions of a protein’s membrane topology is an important first step toward understanding its function. Thus, the determination of the precursor remains unknown.

The functional significance of this complex is unknown, but we note that VP26 and VP28 are both major WSSV structural proteins. VP28 is an envelope protein that is implicated in cell attachment during infection (12, 34, 46), and although the location of VP26 is still disputed (e.g., by Tang et al. [30]), most of the recent evidence (31, 42, 44) (Fig. 7) suggests that it is in fact a tegument or linker protein. In the virion, the hydrophobic N-terminal region of VP26 may be anchored in the envelope, while its C terminus is bound to the nucleocapsid (42). Based on the fact that VP26 binds with actin, Xie and Yang (42) further hypothesized that it may be instrumental in trafficking the WSSV nucleocapsid into the host nucleus via the cytoskeleton. Given the propensity of VP51A to form a complex with VP26 and VP28, it will be interesting to investigate the extent to which VP51A may contribute to the functionality of either or both of these two major WSSV structural proteins.

ACKNOWLEDGMENTS

This investigation was supported financially by National Science Council grants (NSC95-2313-B-212-006-MY2, NSC96-2317-B-002-015, and NSC96-2317-B-002-020). We are indebted to Paul Barlow for his helpful criticism.

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