JOURNAL OF VIROLOGY, Mar. 2006, p. 3021–3029 Vol. 80, No. 6
0022-538X/06/$08.00+0 doi:10.1128/JVI.80.6.3021–3029.2006

Identification of the Nucleocapsid, Tegument, and Envelope Proteins of the Shrimp White Spot Syndrome Virus Virion

Jyh-Ming Tsai,[1] Han-Ching Wang,[1] Jiann-Horng Leu,[1] Andrew H.-J. Wang,[2] Ying Zhuang,[3] Peter J. Walker,[4] Guang-Hsiung Kou,[1]* and Chu-Fang Lo[1]*

Institute of Zoology, National Taiwan University, Taipei, Taiwan[1]; Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan[2]; Department of Biological Sciences, National University of Singapore, Singapore[3]; and CSIRO Livestock Industries, Queensland Bioscience Precinct, St. Lucia, Australia[4]

Received 15 September 2005/Accepted 5 December 2005

The protein components of the white spot syndrome virus (WSSV) virion have been well established by proteomic methods, and at least 39 structural proteins are currently known. However, several details of the virus structure and assembly remain controversial, including the role of one of the major structural proteins, VP26. In this study, Triton X-100 was used in combination with various concentrations of NaCl to separate intact WSSV virions into distinct fractions such that each fraction contained envelope and tegument proteins, tegument and nucleocapsid proteins, or nucleocapsid proteins only. From the protein profiles and Western blotting results, VP26, VP36A, VP39A, and VP95 were all identified as tegument proteins distinct from the envelope proteins (VP19, VP28, VP31, VP36B, VP38A, VP51B, VP53A) and nucleocapsid proteins (VP664, VP51C, VP60B, VP15). We also found that VP15 dissociated from the nucleocapsid at high salt concentrations, even though DNA was still present. These results were confirmed by CsCl isopycnic centrifugation followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and liquid chromatography-nanoelectrospray ionization-tandem mass spectrometry, by a trypsin sensitivity assay, and by an immunogold assay. Finally, we propose an assembly process for the WSSV virion.

White spot syndrome virus (WSSV) is a widespread and disastrous viral pathogen of cultured shrimp that also attacks crabs and crayfish as well as many other crustaceans (3, 10, 16, 22). The WSSV virion is an enveloped particle of approximately 275 by 120 nm with an olivaceous-to-bacilliform shape, and it has a nucleocapsid (300 by 70 nm) with periodic striations (22, 25). The most obvious feature of WSSV is the presence of a thread-like extension at one end of the virion (2, 25), which gives this virus the family name *Nimaviridae* (13).

A virion is a complex assembly of macromolecules exquisitely suited for the protection and delivery of viral genomes. Its structural proteins are particularly important, since these proteins are the first molecules to interact with the host, and they therefore play critical roles in cell targeting as well as in the triggering of host defenses. Recently, thanks to the introduction of proteomic methods, the total number of known WSSV structural proteins increased to 39 (5, 17).

Immunogold electron microscopy (IEM) has been used to identify VP28, VP26, VP31, VP51C, VP36B, VP41A, VP12B, and VP180 as envelope proteins (4, 5, 8, 9, 28–30) and VP664 as a nucleocapsid protein (7). Other studies (1, 6, 18–20) that combined detergent treatment and Western blotting confirmed and expanded most of these results (VP28, VP19, and VP73 as envelope proteins; VP24, VP15, and VP35 as nucleocapsid proteins) but also identified VP26 as a nucleocapsid protein. Here for the first time we embark on a systematic study of the structural proteins of WSSV that not only allows us to resolve the question of VP26's location but also reveals the existence of a previously unreported component of the WSSV virion. This component is an intermediate layer between the envelope and the nucleocapsid. We refer to this proteinaceous layer as the tegument. This name was borrowed from two other kinds of DNA virus (herpesvirus and baculovirus) whose viral particles consist of four morphologically distinct components (DNA core, nucleocapsid, tegument, and envelope [14, 15]). We further show that VP26 is in fact a tegument protein and reconcile this conclusion with the earlier conflicting reports.

MATERIALS AND METHODS

Virus. The virus used in this study, WSSV Taiwan isolate (11), was originally isolated from a batch of WSSV-infected *Penaeus monodon* shrimps collected in Taiwan in 1994 (22). The entire genome sequence of this isolate is deposited in GenBank with accession number AF440570.

Proliferation and purification of intact WSSV virions. Hemolymph was collected from experimentally WSSV-infected shrimps (*P. monodon*; mean weight, 20 g), diluted 1:4 with phosphate-buffered saline, and frozen with liquid nitrogen as described previously (26). From this virus stock, a sample (0.5 ml) was centrifuged (1,500 × *g* for 10 min), and the supernatant was filtered (0.45-μm filter) and injected (0.2 ml; 1:20 dilution in phosphate-buffered saline) intramuscularly into healthy crayfish, *Procambarus clarkii*, between the second and third abdominal segments. Between 5 and 7 days later, hemolymph was collected from the infected crayfish and centrifuged at 1,500 × *g* for 10 min. The supernatant was layered on the top of a 35% (wt/vol in 50 mM Tris buffer, pH 7.5) sucrose solution and centrifuged at 89,000 × *g* using a 28 SA rotor in a Hitachi ultracentrifuge (SCP85H2) for 1 h at 4°C. The virus pellet was resuspended with Tris buffer and then subjected to a 35 to 65% stepwise sucrose gradient ultracentrifugation (89,000 × *g* for 2 h in a 40 ST rotor). The collected virus bands were then mixed with Tris buffer and repelleted at 89,000 × *g* for 1 h at 4°C. The resulting pellet was again dissolved in Tris buffer. To check for quality and quantity, virus samples were negatively stained with 2% sodium phosphotungstate and examined under a transmission electron microscope (JEOL JEM1010). Purified virus samples were prepared in this way for all the subsequent experiments.

* Corresponding author. Mailing address: Institute of Zoology, National Taiwan University, Taipei 106, Taiwan, Republic of China. Phone: 886-2-23633562. Fax: 886-2-23638179. E-mail for Guang-Hsiung Kou: ghkou@ntu.edu.tw. E-mail for Chu-Fang Lo: gracelow@ntu.edu.tw.

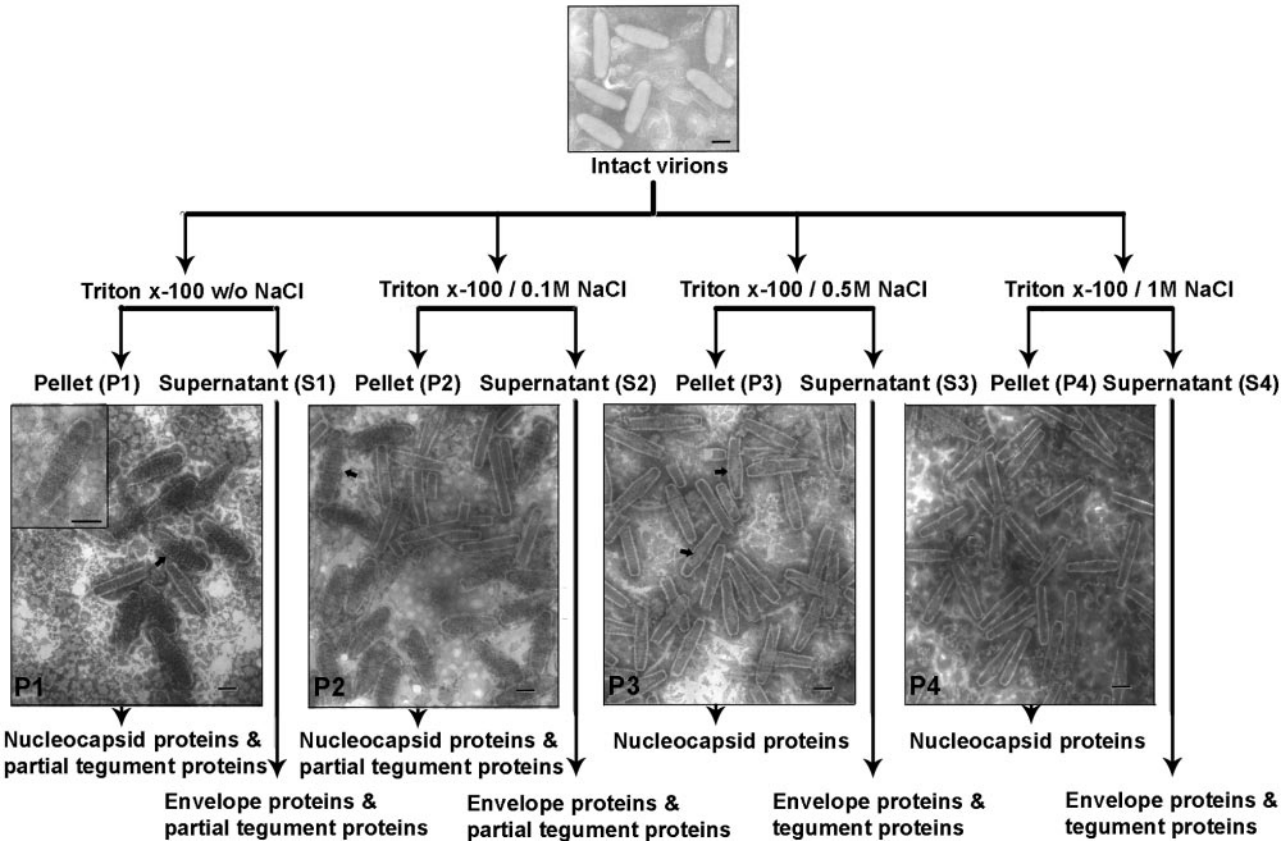

FIG. 1. Preparation of the eight virion protein fractions by treatment of the purified virions with Triton X-100 at different NaCl concentrations. The original location of the proteins in each of the four insoluble fractions (P1 to P4) and four soluble fractions was inferred from the electron micrographs and from other evidence (see text). Arrows in electron micrographs P1 and P2 indicate olive-shaped particles that exhibit a characteristic banded pattern, which can be seen more clearly in the P1 inset. Arrows in electron micrograph P3 indicate the condensed fibrillar structures inside the nucleocapsids. Scale bars = 100 nm.

Fractionation of virion proteins by detergent treatment at different NaCl concentrations. Using a modification of the procedure originally described by Walker et al. (21), a purified virus suspension was divided into five equal parts. One part was set aside as a control, while the other four parts were treated with 1% Triton X-100 at room temperature for 30 min at different salt concentrations (0 M, 0.1 M, 0.5 M, and 1 M NaCl). The four Triton X-100-treated parts were then layered on top of a 35% sucrose cushion and separated into two fractions, supernatant and pellet, by centrifugation at 30,000 × *g* for 1 h (27). The insoluble pellets were dissolved in an equal volume of Tris buffer, and the proteins in each of the eight resultant fractions (and in the intact purified virion control) were separated by 8 to 18% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After separation, the SDS-PAGE gels were either stained with Sypro Ruby stain or else transferred to polyvinylidene difluoride membranes (Millipore), blocked with 3% skim milk in Tris-buffered saline (50 mM Tris, 0.5 M NaCl, pH 7.5) plus 0.5% Tween 20 at 4°C overnight, and then subjected to Western blotting.

Identification of the proteins in each fraction by Western blot analysis. To identify the proteins in the SDS-PAGE gels, polyclonal antibodies against specific WSSV structural proteins were generated either in rat (for VP26) or in rabbit (for 14 other proteins) by using the expressed recombinant proteins in *Escherichia coli* as described previously (7). The polyvinylidene difluoride membranes were then incubated with diluted primary antibody for 1 h at room temperature, and anti-rabbit or anti-rat immunoglobulin G antibody conjugated to horseradish peroxidase was used as the secondary antibody. A chemiluminescence system (Perkin Elmer, Inc.) was used for detection.

Identification of proteins in the purified nucleocapsid. To confirm the identity of the nucleocapsid proteins, a purified virus sample in Tris buffer (1 ml) was treated with 1% Triton X-100 for 30 min at room temperature. This reaction mixture (1 ml) was then layered on top of a 40% (4 ml) to 50% (6 ml) discontinuous CsCl gradient (wt/vol in Tris buffer, pH 7.5) and centrifuged to equilibrium at 37,000 rpm (172,000 × *g*) for 20 h (40 ST rotor). Two visible bands, at buoyant densities of 1.28 and 1.40 g CsCl/ml, respectively, were collected separately, diluted in Tris, and repelleted at 89,000 × *g* for 1 h; the presence of nucleocapsids was then checked for by electron microscopy. The topmost fraction (1 ml; buoyant density, 1.15 g CsCl/ml) and bottom pellet (dissolved in TE buffer) were also collected. In these samples, the presence of WSSV DNA was detected as described previously (12); that is, WSSV genomic DNA was extracted by protease K and cetyltrimethylammonium bromide treatments, followed by phenol-chloroform extraction and ethanol precipitation.

The proteins in these samples were then separated by 8 to 18% gradient SDS-PAGE and stained with Sypro Ruby. Protein bands were manually excised from the gels and identified by liquid chromatography (LC)-nanoelectrospray ionization (nanoESI)-tandem mass spectrometry (MS/MS) as described previously (17).

Trypsin treatment of purified virions. Following the method of Zhu and Yuan (33), we 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 $CaCl_2$, 100 mM NaCl) at 37°C for 2 h. Trypsin digestion was terminated by adding phenylmethylsulfonyl fluoride to a final concentration of 0.5 mM and then adding 1/50 volume of protease inhibitors (Roche). In some samples, prior to trypsin digestion, Triton X-100 was added to a final of concentration of 1% to remove the viral envelope and expose the internal (tegument/nucleocapsid) structure to the protease. All samples were analyzed by Western blotting.

Localization of VP26, VP28, and VP664 by IEM. Aliquots (10 μl) of purified virion suspension were adsorbed to Formvar-supported, carbon-coated nickel grids (150 mesh) for 5 min at room temperature. The grids either were prefixed for 5 min with 4% paraformaldehyde and 1% Triton X-100 simultaneously in 50 mM Tris buffer (to remove the virus envelope) or else were left unfixed and were

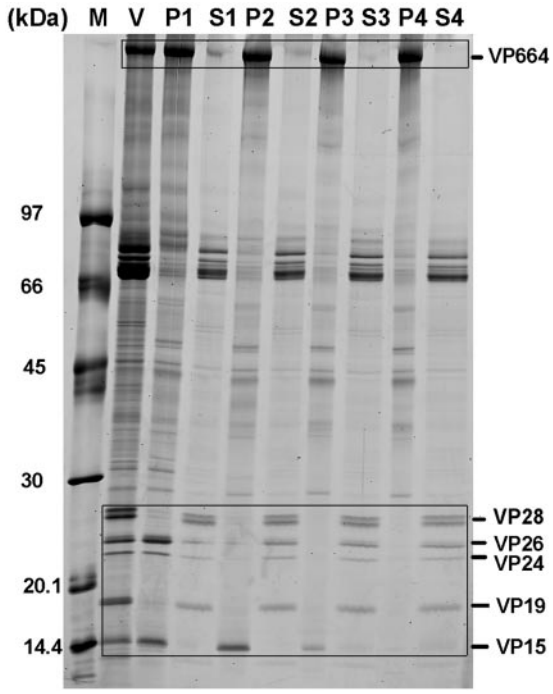

FIG. 2. Protein composition of detergent-treated WSSV virions. An 8 to 18% gradient SDS-PAGE gel was used and stained with Sypro Ruby stain. V is the untreated purified virus. Lanes P1 to P4 and S1 to S4 correspond to the fractions in Fig. 1. M is the protein molecular mass marker.

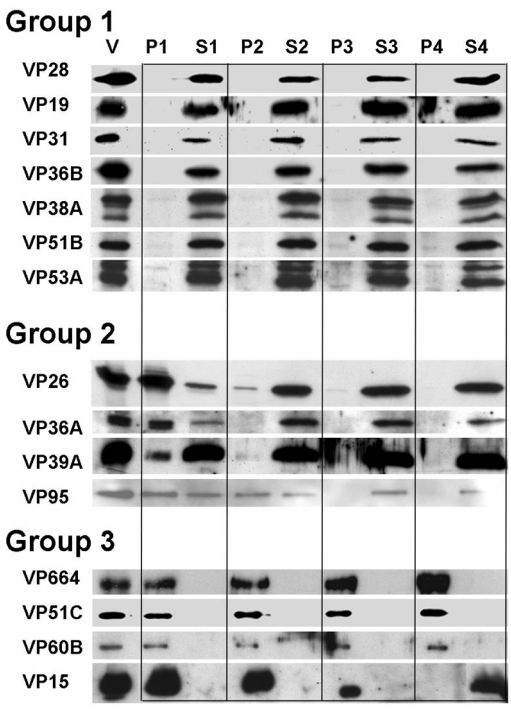

FIG. 3. Western blots of 15 of the protein bands from the SDS-PAGE whose results are shown in Fig. 2. The proteins were divided into three groups based on their reaction profiles.

incubated with incubation buffer only (0.1% Aurion Basic-c, 15 mM NaN_3, 10 mM phosphate buffer, 150 mM NaCl, pH 7.4). Other grids were loaded with purified nucleocapsid samples (5-min absorption at room temperature) prepared as described above using a CsCl gradient centrifugation. These nucleocapsid grids were left unfixed. All grids were then blocked with blocking buffer (5% bovine serum albumin, 5% normal serum, 0.1% cold water skin gelatin, 10 mM phosphate buffer, 150 mM NaCl, pH 7.4) for 30 min and incubated for 1 h at room temperature with a diluted primary antibody for VP26, VP28, or VP664. The primary antibodies were diluted 1:25 (1:40 for VP664) in incubation buffer. After incubation, and after several washes with incubation buffer, the grids for VP28 and VP664 were incubated with a goat anti-rabbit secondary antibody conjugated with gold particles (15-nm diameter; 1:20 dilution in incubation buffer) for 1 h at room temperature; for VP26, the second antibody was goat anti-rat. The grids were then washed extensively with incubation buffer, washed twice more with distilled water to remove excess salt, and negatively stained with 2% sodium phosphotungstate. Specimens were examined with a transmission electron microscope.

Ultrastructural observations by transmission electron microscopy. Following the method of Wang et al. (23), we used primary cell cultures from the lymphoid organ of *Penaeus monodon* to investigate morphogenesis of WSSV. Inoculated primary culture cells were harvested at 4 days postinoculation. Harvested cells were centrifuged at 175 × *g* for 10 min, and the resultant cell blocks were prefixed in 2.5% glutaraldehyde in 0.1 M cold phosphate buffer solution (pH 7.2) for 3 h at 4°C and then postfixed in 1% OsO_4 for 2 h at 4°C. The fixed samples were then dehydrated in an alcohol gradient series (from 70% to absolute alcohol) and embedded in Spurr-Epon. Ultrathin sections were cut on a Reichert OMU ultramicrotome and stained with uranyl acetate and lead citrate.

RESULTS

Classification of WSSV structural proteins as envelope proteins, tegument proteins, and nucleocapsid proteins. Figure 1 summarizes how the eight different fractions were prepared from the purified intact WSSV virions. Micrographs of the resulting pellets show that at zero or very low NaCl concentrations, there were many olive-shaped virus particles (indicated in Fig. 1, micrographs P1 and P2). However, while the original purified intact virions were relatively bright and had dark borders (Fig. 1, intact virions), the olive-shaped particles in P1 and P2 clearly showed a characteristic banded pattern (see inset) that suggested that these particles had lost their envelope. Figure 1 also shows several rod-shaped nucleocapsids in the salt-free P1 pellet. As the salt concentration increased, the proportion of rod-shaped nucleocapsids also increased, and at 0.5 M and 1 M NaCl, no olive-shaped particles were observed (Fig. 1, micrographs P3 and P4). Relatively bright components that are arranged along the long central axis of some nucleocapsids and appear to represent a packed fibrillar structure are also indicated in Fig. 1, micrograph P3.

SDS-PAGE results for these fractions are shown in Fig. 2. Treatment with 1% Triton X-100 either with or without salt completely solubilized the major envelope proteins VP28 and VP19, while the major nucleocapsid protein, VP664, was always located predominantly in the pellet fraction, even at high NaCl concentrations. By contrast, the major structural proteins VP26 and VP24 were only partially solubilized in the salt-free treatment but were increasingly solubilized at higher salt concentrations. At low NaCl concentrations, the major structural protein VP15 was found in the pellet fractions (Fig. 2, lanes P1 and P2) as expected; interestingly, however, it became dissociated from the insoluble nucleocapsid particles at 0.5 M NaCl (Fig. 2, lanes P3 and S3), and at 1 M NaCl (Fig. 2, lanes P4 and S4), it was completely solubilized. Based on these data, we propose that the WSSV virion consists not only of an envelope

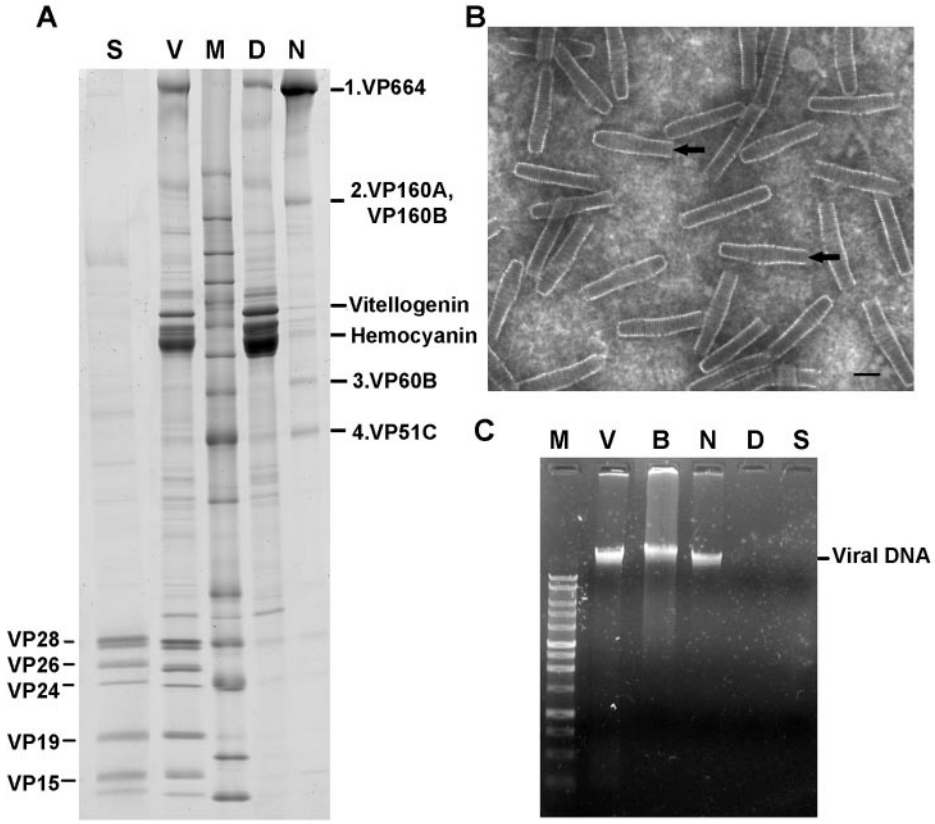

FIG. 4. (A) Sypro Ruby-stained 8 to 18% gradient SDS-PAGE gels of protein fractions purified on a CsCl gradient. Lane S is the topmost soluble protein fraction (buoyant density, 1.15 g/ml); lane N is the nucleocapsid band (buoyant density, 1.40 g/ml). Lane D represents the debris that banded at 1.28 g CsCl/ml. Lane V represents the total virions; lane M is the protein molecular mass marker. The numbered bands represent proteins that were identified by LC-MS/MS analysis. Vitellogenin and hemocyanin bands are indicated, and other known proteins in the supernatant are also identified. (B) Electron micrograph of negatively stained WSSV nucleocapsids purified from the nucleocapsid fraction of the CsCl equilibrium centrifugation. Scale bar = 100 nm. Many of the nucleocapsids appear to be open at one end (arrows). (C) Detection of viral DNA in the respective fractions. Lane headings are as in Fig. 4A, except for lane B, which represents the bottom pellet of the CsCl gradient; M, DNA marker.

and nucleocapsid but also includes an intermediate layer that we will refer to as the tegument. More evidence in support of the existence of this layer will be presented in the subsequent parts of this paper.

As further confirmation of the SDS-PAGE virion protein profiles, both the supernatants and the pellets were analyzed by immunoblotting of fifteen known proteins for which we have antibodies. Figure 3 shows how these 15 proteins fell into three groups based on their reaction profiles. Known envelope proteins (e.g., VP28, VP19) were in group 1, and known nucleocapsid proteins (e.g., VP664, VP15) were in group 3. The group 1 proteins always appeared solely in the supernatant, while the group 3 proteins (except for VP15) always appeared solely in the pellet. As did the SDS-PAGE results (Fig. 2), the Western blotting results suggest that VP15 was solubilized at high salt concentrations. We further note that the protein VP60B in group 3 is identified here as a nucleocapsid protein for the first time, while VP51C is now correctly identified as a nucleocapsid protein (and not an envelope protein, as was recently reported [5]). As for the group 2 proteins, these appeared in the pellet under detergent-only (i.e., salt-free) conditions but were increasingly solubilized as NaCl concentration was increased, and they appeared predominantly in the supernatants at higher NaCl concentrations. These results are consistent with the idea that group 2 proteins are tegument proteins.

SDS-PAGE protein profile of nucleocapsids purified by CsCl gradient, and identification of the nucleocapsid proteins by LC-nanoESI-MS/MS. Figure 4A shows the results of 8 to 18% gradient SDS-PAGE after purification of the nucleocapsids on a CsCl gradient. VP664 was much more abundant in the nucleocapsid band (lane N) than in the debris band (lane D). Transmission electron microscopy also showed that nucleocapsids were much more abundant in the nucleocapsid fraction (Fig. 4B) than in the debris fraction (data not shown). These results suggest that VP664 is the major protein component in WSSV nucleocapsids. We also note that morphologically, the nucleocapsids purified by CsCl gradient (Fig. 4B) very closely resemble the nucleocapsids that were obtained by treatment with Triton X-100 at high salt concentrations (Fig. 1, micrographs P3 and P4). The other known major structural proteins, VP28, VP26, VP24, VP19, and VP15, were all present in the soluble protein fraction with low buoyant density (1.15 g CsCl/ml; Fig. 4A, lane S). Since VP15 was not associated with the purified nucleocapsid fraction, we further tested for the pres-

TABLE 1. Proteomic identification of WSSV nucleocapsid proteins

Band no.[a]	Name	WSSV ORF[b]	ORF size		VP size (mol mass [kDa])[d]	Sequence coverage (%)	Matched peptides	Reference or source
			Amino acids	Mol mass (kDa)[c]				
1	VP664	WSSV419	6,077	664	~664	19	118	17
2	VP160A	WSSV344	1,546	172.5	160	17	31	This study
2	VP160B	WSSV94	1,280	143.8	160	3	4	This study
3	VP60B	WSSV474	544	61.8	60	12	10	5
4	VP51C	WSSV364	466	51.9	51	17	12	5

[a] Band numbers are as shown in Fig. 4A.
[b] Based on the genome of the Taiwan isolate, GenBank accession no. AF440570.
[c] Predicted molecular mass based on the ORF.
[d] The sizes (molecular mass) of the virion proteins (VP) of the WSSV nucleocapsid were estimated after separation on SDS-PAGE gels.

ence of WSSV DNA in the purified nucleocapsids, and Fig. 4C confirms that the nucleocapsid fraction, shown in lane N, did in fact contain the viral DNA. Thus, since VP15 came to equilibrium in the soluble protein fraction at the top of the CsCl gradient, it must have been detached from the DNA during the purification procedures.

Figure 4A also suggests that the debris fraction proteins readily associate with vitellogenin and hemocyanin, both of which are soluble and would otherwise be expected to be found in the soluble protein fraction.

To identify the minor bands that were present in the nucleocapsid fraction (Fig. 4A, lane N), the protein bands in this lane were excised individually from the gel. Following trypsin digestion, the peptides of each band were sequenced using LC-nanoESI-MS/MS, and the sequences were matched against the NCBInr database. The positively identified proteins and their corresponding open reading frames (ORFs) in the viral genome are listed in Table 1. Two of these proteins, VP160A and VP160B, are newly reported structural proteins for WSSV. These results also confirm our earlier conclusion that VP51C and VP60B are both nucleocapsid proteins.

In purified virions, VP664 and VP28 were digested by trypsin, but VP26 was not. For this part of the study, VP28, VP26, and VP664 were selected as representative of the envelope, tegument, and nucleocapsid proteins, respectively. Figure 5 shows that when intact purified virions were treated with trypsin, the envelope protein VP28 was digested while VP26 (which we have identified as a tegument protein) was not, as expected. Surprisingly, however, the nucleocapsid protein VP664 was also digested, which means that the trypsin was somehow able to access the proteins in the nucleocapsid even when the virion remained enveloped. When the envelope was removed with Triton X-100, all three proteins were digested by trypsin. Repeated runs (data not shown) confirmed that all of these results were consistent.

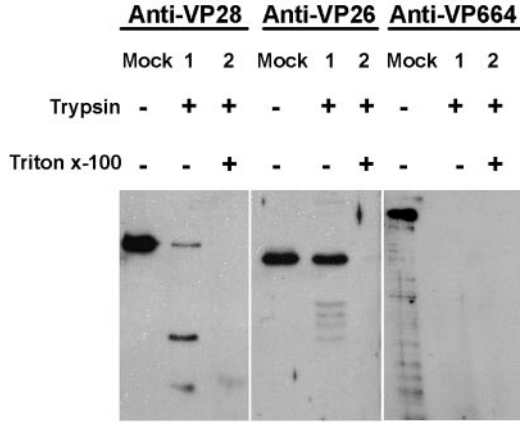

FIG. 5. Western blot analysis showing trypsin digestion of three representative proteins, VP28 (envelope), VP26 (tegument), and VP664 (nucleocapsid) in intact and detergent-treated virions.

Localization of VP26, VP28, and VP664 in the virion by IEM. Immunoelectron microscopy (Fig. 6) provided more evidence in support of our proposed tegument layer. VP28 was detected in the envelope of the intact virus but was not found when the envelope was removed, anti-VP664 antibodies reacted with the unenveloped virions and with the purified nucleocapsids, and VP26 was detected in the unenveloped virions but not in the intact virions or the purified nucleocapsids.

Observations of WSSV virion morphogenesis in vitro. Figure 7 shows WSSV virions at various stages of assembly in an LO primary cell culture 4 days after WSSV infection. First, the empty nucleocapsid forms (Fig. 7A) and the envelope grows up around it (Fig. 7B and C). Next, the fibrillar component (probably a complex of DNA and VP15 and perhaps other proteins) is folded or packed inside the nucleocapsid (Fig. 7D) and the virion becomes fatter (Fig. 7E). Finally, the open end of the nucleocapsid is closed, and the virion assumes its mature, olive-like shape (Fig. 7F). The dark areas of fibrillar component in Fig. 7D, E, and F correspond to the bright areas of condensed fibrillar structure in Fig. 1, micrograph P3. For reference, Fig. 7G shows a computer-enhanced electron micrograph of the partly assembled, enveloped nucleocapsid in Fig. 7D, and Fig. 7H shows a computer-enhanced electron micrograph of a complete, negatively stained virion.

DISCUSSION

VP26, VP24, and VP15 were all originally considered to be nucleocapsid proteins (18–20). Based on IEM evidence, Zhang et al. (30) subsequently argued that VP26 was an envelope protein, but the flattened condition of the virions shown in their micrograph raised the possibility that their sample preparation procedures may have disrupted the integrity of the virions. Here we attempted to settle this question. Referring to the herpesvirus model (32, 33) and based on the results of fractionation (Fig. 1 to 4), trypsin digestion of intact and unenveloped virions (Fig. 5), and IEM results (Fig. 6), we pro-

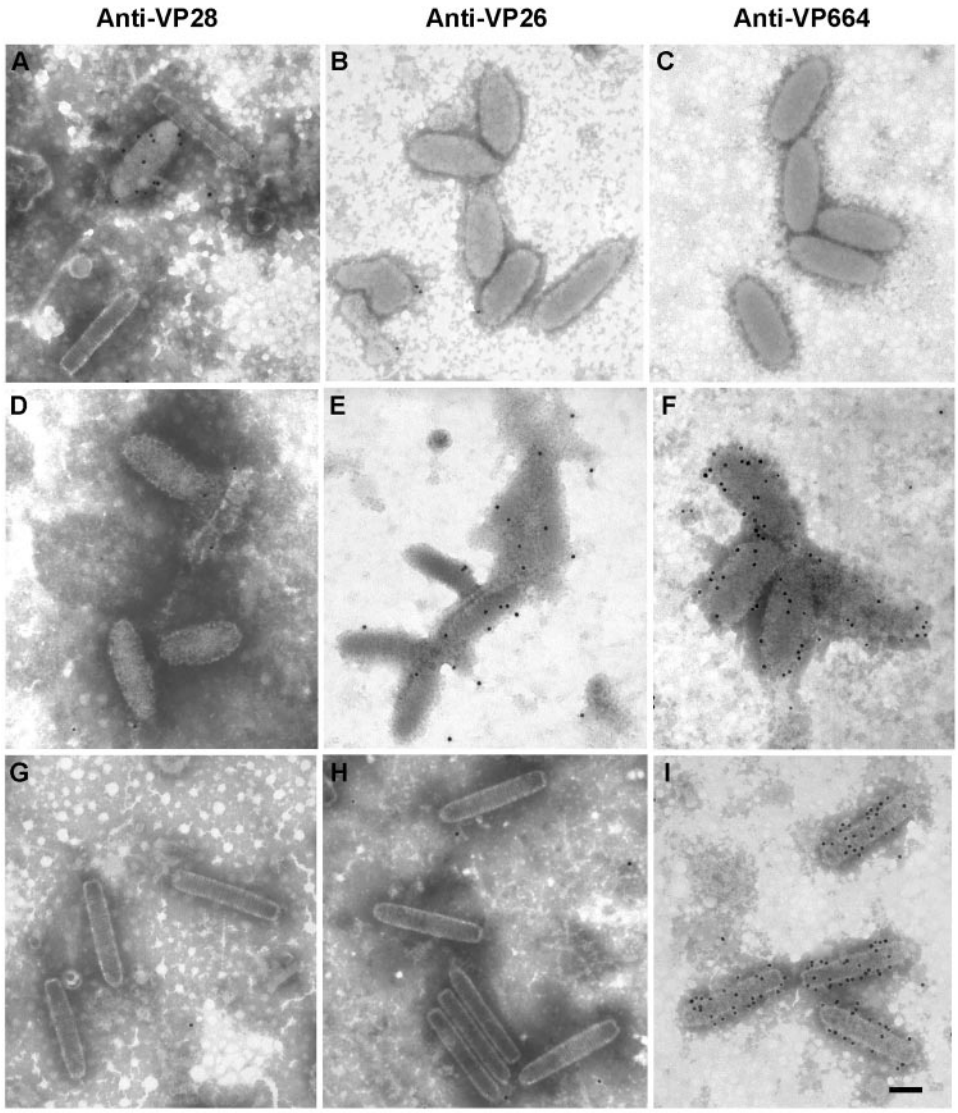

FIG. 6. Immunoelectron microscopy analysis of purified virus (row 1, enveloped virions; row 2, unenveloped virions; row 3, nucleocapsids) probed with VP28, VP26, and VP664 antibodies. The VP28 antibody bound only to the enveloped virion. The VP26 antibody bound only to unenveloped virions (i.e., the tegument-nucleocapsid structure) and not to the viral envelope or the nucleocapsid. The VP664 antibody bound both to unenveloped virions and to the nucleocapsids. Scale bar = 100 nm.

pose that VP26 is in fact a major tegument protein that is loosely associated with both the envelope and the nucleocapsid and is easily solubilized in salt-containing buffer. Based on the SDS-PAGE results (Fig. 1, 2, and 4) we also conclude that VP24 is likewise a tegument protein along with the other proteins in group 2 (Fig. 3).

In Fig. 3, VP28 and VP19 are in the envelope protein group (group 1), and VP664 is in the nucleocapsid protein group (group 3). This is consistent with previous reports (7, 19, 20). Figure 3 also shows for the first time that VP38A, VP51B, and VP53A are all envelope proteins, from which it follows that these proteins might be important in future virus-host interaction studies.

The role of VP15 is slightly more complex. VP15 is a basic DNA binding protein of WSSV, and it has been suggested that it may be involved in packing the viral genome in the nucleocapsid (24, 31). Even though VP15 was not found in the nucleocapsid fraction in Fig. 4A, this suggestion is still consistent with the CsCl gradient results. These results show that when VP15 was solubilized in the supernatant (Fig. 4A), the virus DNA was found in the nucleocapsid fraction and also in the bottom pellet but not in the debris fraction or the soluble protein fraction (Fig. 4C). We hypothesize that when VP15 is no longer present to ensure the orderly (folded, not helical) packing of DNA inside the nucleocapsid, the 300-kbp circular DNA might either remain tangled around the nucleocapsid (fraction N) or else break free completely so that the DNA gravitates to the bottom pellet while the lighter nucleocapsid proteins (empty capsids) form the upper band of debris.

Further support for this explanation is provided by the fibrillar structures that are seen in Fig. 1, micrograph P3.

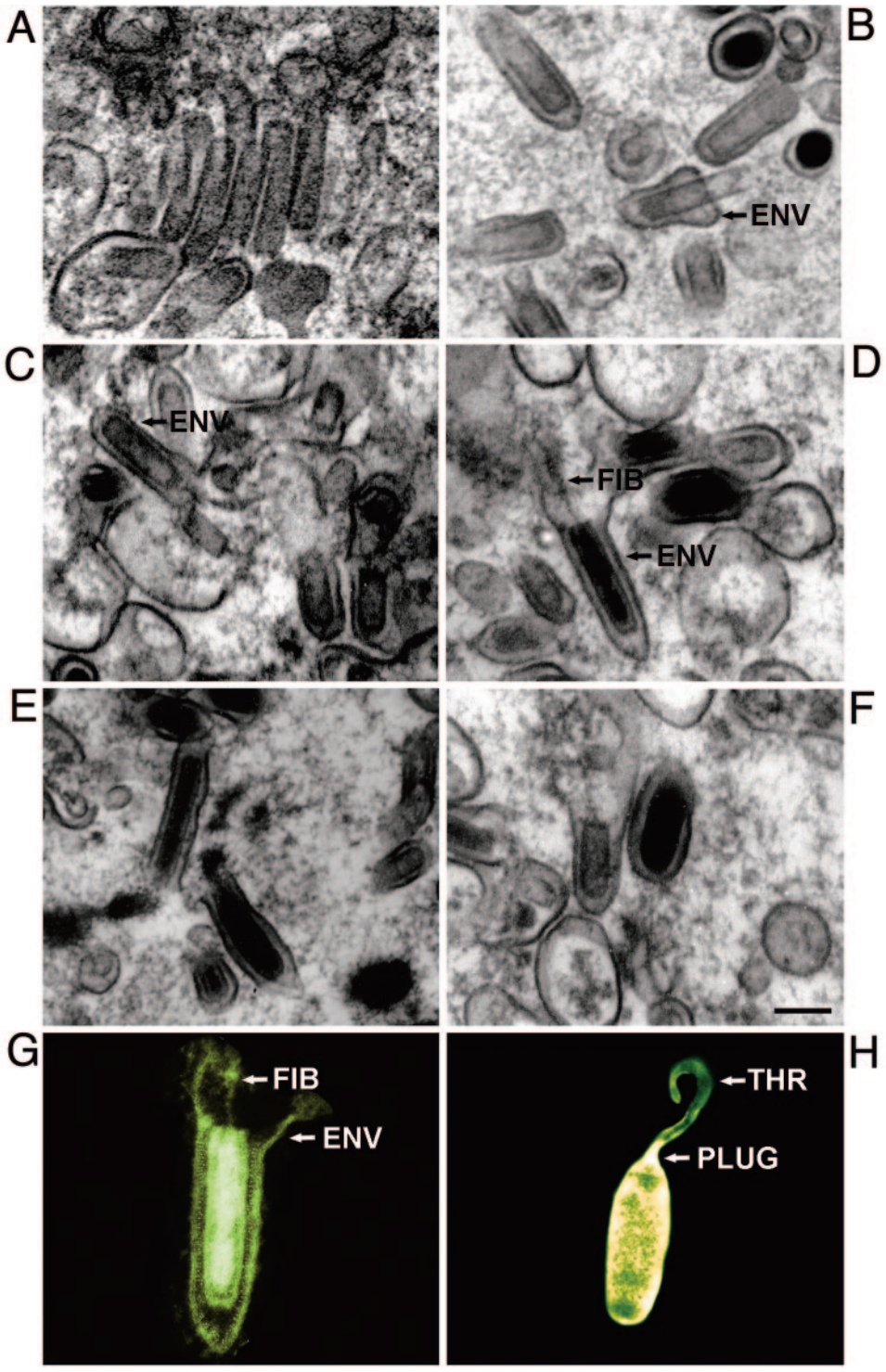

FIG. 7. (A to F) Thin-section electron micrographs showing successive stages of virion assembly in a primary culture of *Penaeus monodon* lymphoid organ cells infected with WSSV for 4 days. (G) Computer-enhanced image of the fibrillar component being packed inside the nucleocapsid shown in panel D. (H) Computer-enhanced image of an intact, negatively stained virion. ENV, envelope; FIB, fibrillar component; THR, characteristic thread-like extension of the WSSV virion; PLUG, distinct pluglike structure at the base of the thread. Scale bar = 100 nm.

The indicated structures, which we interpret as folded viral DNA, are less prevalent at 1 M NaCl, i.e., when VP15 is completely solubilized, because in the absence of VP15 the DNA would be more likely to have at least partly escaped and remained tangled half inside and half outside of the nucleocapsid. Conversely, the presence of VP15 may be essential for the DNA packing to proceed to completion, and considering the large quantity of DNA that must be

packed inside the mature virion, this may in turn account for WSSV's characteristic swollen olive-like shape. We therefore propose that while VP664 and VP15 both appear in the nucleocapsid, VP15 should in fact be considered as a core protein (as opposed to a major capsid protein such as VP664).

We proposed in a previous paper (7) that VP664 was a major nucleocapsid protein and that the banded pattern visible in WSSV nucleocapsids was due to VP664 molecules being arranged in a series of stacked disks. Here, the IEM data show not only that VP664 is part of the nucleocapsid but also that it was quite evenly distributed at intervals on the outer surface of the unenveloped virion (Fig. 6F; see also Fig. 5D in reference 7). This suggests that parts of the VP664 molecule must be exposed on the outside of the tegument-nucleocapsid structure. We therefore conclude that the tegument layer is not a completely impermeable structure but rather allows some domains of the VP664 molecules (possibly the globular knob domain) to project through to the outside. Furthermore, since Fig. 6E shows that the major tegument protein VP26 is also located on the outside of the tegument, we propose that VP26 may be inserted between the exposed VP664 domains and that this interleaving may increase the flexibility of the nucleocapsid and allow it to assume its characteristic olive-like shape in the mature virion. Consistent with this idea, we note that when VP26 is dissociated from the nucleocapsid at high NaCl concentrations, the nucleocapsid does in fact conform to a more rigid, rod-shaped structure (Fig. 6, G to I). We note too that the core protein VP15 is also dissociated at higher salt concentrations (Fig. 2), and that it too may be implicated in this reversion to a rod-shaped structure. If the presence of VP15 is required for complete packing of the DNA, then its removal may lead to disorganization of the folded core DNA and a collapse of the swollen olive-shaped virion (cf. Fig. 1, micrograph P2 versus P3).

While VP664 molecules evidently extend from the nucleocapsid through to the outside surface of the tegument, the converse is not true for VP26; that is, VP26 molecules are located exclusively in the tegument and do not extend inwards to the nucleocapsid. This helps to account for the unexpected result shown in Fig. 5 that even without treatment with detergent, VP664 is digested by trypsin, while VP26 is not: if we assume that trypsin digestion of envelope proteins such as VP28 also opens a hole in the envelope (perhaps by opening the pluglike structure shown in Fig. 7H, although this could not be directly verified by EM; data not shown), then trypsin would be able to penetrate to the inside of the nucleocapsid, where it could digest VP664. However, the tegument layer (where VP26 is located) would still not be immediately accessible and would therefore remain mostly intact (undigested).

ACKNOWLEDGMENTS

This investigation was supported financially by National Science Council grants (NSC93-2317-B-002-018 and NSC94-2317-B-002-011).

Proteomic mass spectrometry analyses were performed by the Core Facilities for Proteomics Research, located at the Institute of Biological Chemistry, Academia Sinica. We are indebted to Paul Barlow for his helpful criticism.

REFERENCES

1. **Chen, L. L., J. H. Leu, C. J. Huang, C. M. Chou, S. M. Chen, C. H. Wang, C. F. Lo, and G. H. Kou.** 2002. Identification of a nucleocapsid protein (VP35) gene of shrimp white spot syndrome virus and characterization of the motif important for targeting VP35 to the nuclei of transfected insect cells. Virology **293:**44–53.
2. **Durand, S., D. V. Lightner, R. M. Redman, and J. R. Bonami.** 1997. Ultrastructure and morphogenesis of white spot syndrome baculovirus (WSSV). Dis. Aquat. Org. **29:**205–211.
3. **Flegel, T. W.** 1997. Major viral diseases of black tiger prawn (*Penaeus monodon*) in Thailand. World J. Microbiol. Biotechnol. **13:**433–442.
4. **Huang, C., X. Zhang, Q. Lin, X. Xu, and C. L. Hew.** 2002. Characterization of a novel envelope protein (VP281) of shrimp white spot syndrome virus by mass spectrometry. J. Gen. Virol. **83:**2385–2392.
5. **Huang, C., X. Zhang, Q. Lin, X. Xu, Z. Hu, and C. L. Hew.** 2002. Proteomic analysis of shrimp white spot syndrome viral proteins and characterization of a novel envelope protein VP466. Mol. Cell. Proteomics **1:**223–231.
6. **Huang, R., Y. Xie, J. Zhang, and Z. Shi.** 2005. A novel envelope protein involved in white spot syndrome virus infection. J. Gen. Virol. **86:**1357–1361.
7. **Leu, J. H., J. M. Tsai, H. C. Wang, A. H. Wang, C. H. Wang, G. H. Kou, and C. F. Lo.** 2005. The unique stacked rings in the nucleocapsid of the white spot syndrome virus virion are formed by the major structural protein VP664, the largest viral structural protein ever found. J. Virol. **79:**140–149.
8. **Li, L., X. Xie, and F. Yang.** 2005. Identification and characterization of a prawn white spot syndrome virus gene that encodes an envelope protein VP31. Virology **340:**125–132.
9. **Li, Q., Y. Chen, and F. Yang.** 2004. Identification of a collagen-like protein gene from white spot syndrome virus. Arch. Virol. **149:**215–223.
10. **Lo, C. F., C. H. Ho, S. E. Peng, C. H. Chen, H. E. Hsu, Y. L. Chiu, C. F. Chang, K. F. Liu, M. S. Su, C. H. Wang, and G. H. Kou.** 1996. White spot syndrome associated virus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. Dis. Aquat. Org. **27:**215–225.
11. **Lo, C. F., H. C. Hsu, M. F. Tsai, C. H. Ho, S. E. Peng, G. H. Kou, and D. V. Lightner.** 1999. Specific genomic DNA fragments analysis of different geographical clinical samples of shrimp white spot syndrome virus. Dis. Aquat. Org. **35:**175–185.
12. **Lo, C. F., J. H. Leu, C. H. Ho, C. H. Chen, S. E. Peng, Y. T. Chen, C. M. Chou, P. Y. Yeh, C. J. Huang, H. Y. Chou, C. H. Wang, and G. H. Kou.** 1996. Detection of baculovirus associated with white spot syndrome (WSSV) in penaeid shrimps using polymerase chain reaction. Dis. Aquat. Org. **25:**133–141.
13. **Mayo, M. A.** 2002. A summary of taxonomic changes recently approved by ICTV. Arch. Virol. **147:**1655–1656.
14. **Mettenleiter, T. C.** 2002. Herpesvirus assembly and egress. J. Virol. **76:**1537–1547.
15. **Miller, L. K.** 1997. The baculoviruses. Plenum, New York, N.Y.
16. **Nakano, H., H. Koube, S. Umezawa, K. Momoyama, M. Hiraoka, K. Inouye, and N. Oseko.** 1994. Mass mortalities of cultured kuruma shrimp, Penaeus japonicus, in Japan in 1993: epizootiological survey and infection trials. Fish Pathol. **29:**135–139.
17. **Tsai, J. M., H. C. Wang, J. H. Leu, H. H. Hsiao, A. H. Wang, G. H. Kou, and C. F. Lo.** 2004. Genomic and proteomic analysis of thirty-nine structural proteins of shrimp white spot syndrome virus. J. Virol. **78:**11360–11370.
18. **van Hulten, M. C. W., R. W. Goldbach, and J. M. Vlak.** 2000. Three functionally diverged major structural proteins of white spot syndrome virus evolved by gene duplication. J. Gen. Virol. **81:**2525–2529.
19. **van Hulten, M. C. W., M. Reijns, A. M. Vermeesch, F. Zandbergen, and J. M. Vlak.** 2002. Identification of VP19 and VP15 of white spot syndrome virus (WSSV) and glycosylation status of the WSSV major structural proteins. J. Gen. Virol. **83:**257–265.
20. **van Hulten, M. C. W., M. Westenberg, S. D. Goodall, and J. M. Vlak.** 2000. Identification of two major virion protein genes of white spot syndrome virus of shrimp. Virology **266:**227–236.
21. **Walker, P. J., K. A. Byrne, D. H. Cybinski, D. L. Doolan, and Y. Wang.** 1991. Proteins of bovine ephemeral fever virus. J. Gen. Virol. **72:**67–74.
22. **Wang, C. H., C. F. Lo, J. H. Leu, C. M. Chou, P. Y. Yeh, H. Y. Chou, M. C. Tung, C. F. Chang, M. S. Su, and G. H. Kou.** 1995. Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon*. Dis. Aquat. Org. **23:**239–242.
23. **Wang, C. H., H. N. Yang, C. Y. Tang, C. H. Lu, G. H. Kou, and C. F. Lo.** 2000. Ultrastructure of white spot syndrome virus development in primary lymphoid organ cell cultures. Dis. Aquat. Org. **41:**91–104.
24. **Witteveldt, J., A. M. Vermeesch, M. Langenhof, A. de Lang, J. M. Vlak, and M. C. W. van Hulten.** 2005. Nucleocapsid protein VP15 is the basic DNA binding protein of white spot syndrome virus of shrimp. Arch. Virol. **150:**1121–1133.
25. **Wongteerasupaya, C., J. E. Vickers, S. Sriurairatana, G. L. Nash, A. Akarajamorn, V. Boosaeng, S. Panyim, A. Tassanakajon, B. Withyachumnarnkul, and T. W. Flegel.** 1995. A non-occluded, systemic baculovirus that occurs in

the cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*. Dis. Aquat. Org. **21:**69–77.

26. **Wu, J. L., K. Suzuki, M. Arimoto, T. Nishizawa, and K. Muroga.** 2002. Preparation of an inoculum of white spot syndrome virus for challenge tests in *Penaeus japonicus*. Fish Pathol. **37:**65–69.
27. **Xie, X., H. Li, L. Xu, and F. Yang.** 2005. A simple and efficient method for purification of intact white spot syndrome virus (WSSV) viral particles. Virus Res. **108:**63–67.
28. **Zhang, X., C. Huang, X. Tang, Y. Zhuang, and C. L. Hew.** 2004. Identification of structural proteins from shrimp white spot syndrome virus (WSSV) by 2DE-MS. Proteins **55:**229–235.
29. **Zhang, X., C. Huang, X. Xu, and C. L. Hew.** 2002. Identification and localization of a prawn white spot syndrome virus gene that encodes an envelope protein. J. Gen. Virol. **83:**1069–1074.
30. **Zhang, X., C. Huang, X. Xu, and C. L. Hew.** 2002. Transcription and identification of an envelope protein gene (p22) from shrimp white spot syndrome virus. J. Gen. Virol. **83:**471–477.
31. **Zhang, X., X. Xu, and C. L. Hew.** 2001. The structure and function of a gene encoding a basic peptide from prawn white spot syndrome virus. Virus Res. **79:**137–144.
32. **Zhu, F. X., J. M. Chong, L. Wu, and Y. Yuan.** 2005. Virion proteins of Kaposi's sarcoma-associated herpesvirus. J. Virol. **79:**800–811.
33. **Zhu, F. X., and Y. Yuan.** 2003. The ORF45 protein of Kaposi's sarcoma-associated herpesvirus is associated with purified virions. J. Virol. **77:**4221–4230.