Lipid of white-spot syndrome virus originating from host-cell nuclei

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The hypothesis that white-spot syndrome virus (WSSV) generates its envelope in the nucleoplasm is based on electron microscopy observations; however, as yet there is no direct evidence for this. In the present study, the lipids of WSSV and the nuclei of its host, the crayfish Procambarus clarkii, were extracted and the neutral lipid and phospholipid contents were analysed by high-performance liquid chromatography, thin-layer chromatography and gas chromatography/mass spectrometry. Phosphatidylcholine (PC) and phosphatidylethanolamine comprised 62.9 and 25.8 %, respectively, of WSSV phospholipids, whereas they comprised 58.5 and 30 %, respectively, of crayfish nuclei phospholipids. These two phospholipids were the dominant phospholipids, and amounts of other phospholipids were very low in the total WSSV and crayfish nuclei phospholipids. The data indicate that the phospholipid profile of WSSV and crayfish nuclei are similar, which is in agreement with the model that the lipids of WSSV are from the host-cell nuclei. However, the fatty acid chains of PC were different between the WSSV virions and crayfish nuclei, and the viral neutral lipid component was also found to be somewhat more complicated than that of the host nuclei. The number of species of cholesterol and hydrocarbon in virus neutral lipid was increased compared with that in host-cell nuclei neutral lipid. It is suggested that the differences between WSSV and its host are either due to selective sequestration of lipids or reflect the fact that the lipid metabolism of the host is changed by WSSV infection.

INTRODUCTION

White-spot syndrome virus (WSSV), the only member of the novel genus Whispovirus and the family Nimaviridae (Mayo, 2002a, b), is a double-stranded DNA virus (Wang et al., 1995; Wongteerasupaya et al., 1995). The virus is an important pathogen of the cultured penaeid shrimp and can also infect most species of crustaceans (Chang et al., 1998; Chen et al., 2000; Corbel et al., 2001; Hameed et al., 2001; Lo et al., 1996). Electron microscopy observations of virus suspensions and sections has revealed rod-shaped and enveloped virions, which assemble in the nucleus of an infected cell by an unknown process (Wongteerasupaya et al., 1995). Although there has been considerable progress in recent decades towards determining and understanding the virus genome sequence (Chen et al., 2002; van Hulten et al., 2001; Yang et al., 2001) and structural proteins (Huang et al., 2002; Li et al., 2007; Tsai et al., 2004; Xie et al., 2006), little is known about the fundamental chemical composition of WSSV.

Lipids are present in the envelopes of many kinds of virus. The viral lipid matrix is an essential constituent for viral biological activity because treatment of virions with lipid solvents, detergents or lipase inactivates their infectivity (Barbanti-Brodano et al., 1971). The marked sensitivity of WSSV to detergents such as Triton X-100, NP-40 and octyl glucopyranoside indicates that WSSV may contain lipids (Tsai et al., 2006; Xie & Yang, 2006; Xie et al., 2006).

To date, the intranuclear membrane morphogenesis of WSSV is unknown. Unlike paramyxoviruses or herpesvirus, there is no evidence that the WSSV nucleocapsid associates with the plasma or nuclear membranes. WSSV seems most similar to the baculovirus occlusion-derived virus (ODV) form, because they both assemble in the host-cell nuclei and are released by causing disintegration of the host cells. However, no polyhedron structures or intranuclear microvesicles have been observed in WSSV-infected host-cell nuclei. The intranuclear microvesicle has been referred to as the precursor of the ODV envelope, as this intermediate structure originates from the inner nuclear membrane (Braunagel et al., 1996; Stoltz et al., 1973). The absence of this structure implies a different membrane morphogenesis from that of the baculovirus ODV.
Recently, a simple and efficient method of WSSV purification from infected crayfish tissues has been developed in our laboratory (Xie et al., 2005, 2006). The results of the present study indicate that WSSV contains lipids. The supporting data include a demonstrated sensitivity to lipid solvents and lipid analysis of the purified virus. The extensive analysis results show that the phospholipid composition of WSSV virions and host-cell nuclei is similar. This observation, when combined with previous electron microscopy results of sectioned infected tissues, indicates that WSSV lipid is derived from the host-cell nuclei. In addition, the differences in neutral lipid profiles and the fatty acid chains of phosphatidylycholine (PC) between WSSV and its host-cell nuclei are discussed.

**METHODS**

**Purification of WSSV virions.** WSSV inoculum was derived from *Litopenaeus vannamei* shrimp in Xiamen, PR China, and the purification process was performed according to previously described procedures (Xie et al., 2005, 2006). In brief, virus was amplified by infecting healthy adult crayfish (*Procambarus clarkii*). Five to six moribund crayfish at 7 days post-infection were used to produce purified WSSV by homogenization and three cycles of differential centrifugation (Avanti J-E Centrifuge System; Beckman). The virions were suspended in an appropriate volume of TM buffer [50 mM Tris/HCl (pH 7.5), 5 mM MgCl$_2$] containing 0.05% sodium azide and stored at 4°C until use. The purity of the virus preparation was evaluated by negative-staining transmission electron microscopy (Joel JEM-1230).

**WSSV infectivity assay.** Healthy crayfish that had been shown by PCR not to be infected with WSSV (Xu et al., 2001) were cultured in groups of 20 individuals per aquarium. Virus concentration was quantified by the method described by Zhou et al. (2007). Prior to injection, 2 ml virion suspension (10$^6$ virions µl$^{-1}$) was mixed thoroughly with the same volume of petroleum ether. The upper phase was removed and the lower phase was injected intramuscularly (100 µl per crayfish; group 2). A negative control (0.9% NaCl; group 2) was included in the injection regimen. Crayfish mortality caused by WSSV was monitored daily. Two dead individuals from each group were examined by PCR for confirmation of WSSV infection.

**Preparation of membrane-depleted nuclei.** Nuclei for chemical studies were isolated using a procedure modified from Lawson et al. (1969). The muscles of seven healthy crayfish were finely minced with scissors and homogenized in 5 vol s lysis buffer [20 mM Tris/HCl (pH 7.4), 130 mM NaCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$] containing protease inhibitors (1 mM PMSF, 1 mM benzamidine, 1 mM Na$_2$S$_2$O$_3$) using a mechanical homogenizer (IKA T10 basic). The homogenate was transferred into an ice-cold Dounce homogenizer (pre-treated with 1% BSA) and dounced with three strokes using a type B pestle. Subsequently, the mixture was filtered through 40 µm nylon sieve (BD Falcon). The resulting suspension was centrifuged for 5 min at 300 g and the pellet of nuclear debris was resuspended in 0.32 M sucrose in TM buffer [50 mM Tris/HCl (pH 7.5), 25 mM KCl, 5 mM MgCl$_2$]. The final suspension was added to the top of 8 vols 2 M sucrose in 1 mM CaCl$_2$. A white pellet of packed nuclei was obtained by centrifuging for 1 h at 50,000 g (Optima Max-E Ultracentrifuge; Beckman). All procedures were carried out at 4°C. The isolated nuclei were stained with propidium iodide and monitored by phase-contrast microscopy (Olympus IX70 microscope).

**Extraction and separation of lipids from pure virus, host-cell nuclei and haemocytes.** The buffer of the preparation of isolated WSSV was removed by centrifugation at 16,000 g for 20 min at 4°C and the virus preparation was dried at 4°C and weighed (Metler Toledo AE 240 scales). The dried material (~5 mg) was extracted for 1 h at room temperature with 1 ml chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene as an antioxidant. The organic extract was dried under vacuum to remove the solvent and weighed. The residue was dissolved in 0.1 ml chloroform and stored at ~20°C (Kates, 1972). In total, seven batches of WSSV preparation were extracted.

The pooled lipids extracted from the seven batches were applied to a silicic acid column (2 × 0.9 cm), and the neutral lipids were eluted with chloroform and the phospholipids with methanol. The fractions of phospholipids and neutral lipids were dried under nitrogen and weighed. The extraction, separation and quantification of phospholipids from host-cell nuclei were performed using the same procedure.

Haemolymph from crayfish was collected into an anticoagulant solution (0.14 M NaCl, 0.1 M glucose, 30 mM citric acid, 26 mM sodium citrate, 10 mM EDTA, pH 4.6) on ice. Approximately 20 ml haemolymph sample was centrifuged at 200 g for 10 min at 4°C and then washed twice with PBS. The haemocyte pellet was air dried at 4°C and stored at ~20°C. Finally, the lipids of five haemocyte samples were extracted and measured.

**Thin-layer chromatography (TLC).** The phospholipids were separated on silica gel 60 F254 TLC plates (10 × 10 cm; Merck). One-dimensional chromatograms were developed with chloroform/methanol/water (14:6:1, v/v) (Barnes et al., 1987; Rozemond, 1967). Separated phospholipids were detected by exposure of the chromatograms to iodine vapour. Identification of the spots was confirmed by comparison with commercially obtained phospholipids standards (Sigma-Aldrich).

**High-performance liquid chromatography (HPLC).** The phospholipids of WSSV and host-cell nuclei were each dissolved in 0.5 ml acetonitrile and 20 µl was injected into an HPLC column ( Dionex P680 HPLC Pump with an Agilent 8450/HP UV Detector and Kromasil C18 250 × 4.6 mm column). The mobile phase was a mixture of acetonitrile and water (80:20, v/v) at a flow rate of 1 ml min$^{-1}$. Individual phospholipid classes were identified and quantified by comparing retention times and peak area with those of standards (Sigma-Aldrich). All reagents were HPLC-grade and obtained from Sinopharm Chemical Reagent Co.

**Gas chromatography/mass spectrometry (GC-MS).** Neutral lipids from the purified virions and host-cell nuclei were dried under a stream of nitrogen and the residue was dissolved in 100 µl ether. Neutral lipids were separated and identified by GC-MS (TRACE GC coupled to a TRACE MS$^+$; Thermo Finnigan). Chromatography was performed utilizing a DB-1 capillary column (30 m × 0.25 mm; film thickness 0.25 mm). Helium was used as the carrier gas at a flow rate of 11 ml min$^{-1}$. The injection and detection temperatures were set at 250°C and the column temperature was programmed from 80 to 150°C at 10°C min$^{-1}$ and then from 150 to 280°C at 8°C min$^{-1}$.

Components were identified by comparison of their mass spectra with those in the NIST98 GC-MS library and in the literature (Adams, 1995), as well as by comparison of their retention indices with literature data (Davies, 1990).

The assay was repeated three times with good agreement between assays, and the results of the final assay were reported.
RESULTS

Infectivity assay

If WSSV contains lipids, its infectivity may be maintained by a combination of lipid ordering. To investigate the loss of virus infectivity after treatment with petroleum ether, an animal test was performed using crayfish. The results (Fig. 1) showed that the positive-control group (WSSV only; group 1) displayed 82% mortality by day 7 post-infection and 100% by day 9, whereas the negative-control group (0.9% NaCl; group 3) showed no mortality. Treatment with petroleum ether (group 2) significantly delayed mortality, which reached 29% by day 7 and 100% by day 13. The experiment was repeated with the same result. Therefore, it was concluded that treatment with petroleum ether causes a reduction in the infectivity of WSSV, indicating the presence of lipids in WSSV.

Lipid analysis

The total lipid content of WSSV and host haemocytes was determined gravimetrically. The results showed that WSSV contains lipid and that the viral lipid extract comprised approximately 22% of the dry weight of the virus. However, by contrast, lipids comprised only 10.9% of the host haemocytes.

Electron micrograph analysis of ultrathin sections of infected crayfish tissues has shown that WSSV completes its assembly in the nucleus (Chou et al., 1995; Durand et al., 1997; Wang et al., 1995). Thus, if WSSV contains lipids, these lipids must derive from the host nucleus. We isolated crayfish cell nuclei and compared their lipid composition with that of WSSV (Table 1). The neutral lipid fraction comprised 27.4 and 22.5% of the total lipids in WSSV virions and crayfish cell nuclei, respectively, whereas the phospholipid fraction accounted for 72.6 and 77.5% of the total lipids, respectively. Moreover, the neutral lipid-to-phospholipid ratios were similar in both groups (0.38 in WSSV and 0.29 in host-cell nuclei).

The major phospholipids were also found at similar relative concentrations. Complete separation of the phospholipids in WSSV and host-cell nuclei was achieved by one-dimensional TLC (Fig. 2). The retention factor (RF) is the ratio of the distance a particular spot travels relative to the distance travelled by the solvent. Fig. 2 shows that WSSV and host-cell nuclei both had two phospholipids whose RF values were close to those of PC and phosphatidylethanolamine (PE). However, the concentra-

Table 1. Lipid composition of WSSV and crayfish cell nuclei

<table>
<thead>
<tr>
<th>Component</th>
<th>Virus</th>
<th>Nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lipids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutral lipids (%) *</td>
<td>27.4</td>
<td>22.5</td>
</tr>
<tr>
<td>Phospholipids (%) *</td>
<td>72.6</td>
<td>77.5</td>
</tr>
<tr>
<td>Ratio of neutral lipids : phospholipids</td>
<td>0.38</td>
<td>0.29</td>
</tr>
<tr>
<td>Phospholipids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidylcholine (%) †</td>
<td>62.9</td>
<td>58.5</td>
</tr>
<tr>
<td>Phosphatidylethanolamine (%) †</td>
<td>25.8</td>
<td>30.0</td>
</tr>
</tbody>
</table>

*Results are given as the percentage of total lipids.
†Results are given as the percentage of total phospholipids. Phospholipids at low levels were not detected. Results are based on UV absorbance with 98–100% recovery of the standards.
tion of sphingomyelin (SM), which is generally a major phospholipid constituent of plasma membranes, was too low to be detected in WSSV and nuclei by TLC. It is interesting that the spot of nuclear PC had a higher RF value than that of standard PC. To confirm our conclusion that the major phospholipids of WSSV and host-cell nuclei are PC and PE and to measure their content, the phospholipids in WSSV and nuclei were analysed by HPLC. The results shown in Fig. 3 confirmed that PC and PE were the dominant components with a content of 62.9 and 25.8 %, respectively, of the total viral phospholipids, whereas they comprised 58.5 and 30.0 %, respectively, of the total host-cell nuclei phospholipids (Table 1). These data showed that the phospholipid profiles of WSSV and host-cell nuclei are similar.

The results of analyses of neutral lipids by GC/MS are shown in Table 2. As the boiling points of cholesterol and hydrocarbons are lower than those of glyceride and free fatty acids, GC/MS with direct neutral lipid extract could only detect cholesterol and hydrocarbons. As shown in Table 2, all of the viral hydrocarbons were saturated and most of the hydrocarbons in the cell nuclei were saturated except for one species that was unsaturated. The number of species of cholesterol and hydrocarbon in virus neutral lipid was increased compared with that in cell nuclei neutral lipid, with 15 types in the virus and 11 types in the cell nuclei. However, the six compositions that formed the dominant amount of cholesterol and hydrocarbon in virus neutral lipid and WSSV were consistent.

**DISCUSSION**

The infectivity of the virions of WSSV, as shown by the mortality of infected crayfish, was reduced after extraction with petroleum ether. It has been reported previously that WSSV envelope proteins can be separated from the virion by detergents (Tsai et al., 2006; Xie & Yang, 2006; Xie et al.,

![Fig. 3. Chromatogram of phospholipids from WSSV and crayfish cell nuclei, and comparison with phospholipid standards. (a) Separation of phospholipid standards. (b) Separation of extracts from whole WSSV virions and crayfish cell nuclei. The mobile phase was a mixture of acetonitrile and water (80 : 20, v/v) at a flow rate of 1 ml min⁻¹. Absorbance was measured at 210 nm.](image)

**Table 2.** Cholesterol and hydrocarbon composition of WSSV and crayfish cell nuclei

<table>
<thead>
<tr>
<th>Component*</th>
<th>Area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WSSV</td>
</tr>
<tr>
<td>3-Ethyl-3-methyl heptane</td>
<td>0.60</td>
</tr>
<tr>
<td>5-Methyl dodecane</td>
<td>0.49</td>
</tr>
<tr>
<td>4,6-Dimethyl dodecane</td>
<td></td>
</tr>
<tr>
<td>2,6,11-Trimethyl dodecane</td>
<td>1.03</td>
</tr>
<tr>
<td>2,6,10-Trimethyl dodecane</td>
<td>0.46</td>
</tr>
<tr>
<td>Tetradecane†</td>
<td>0.92</td>
</tr>
<tr>
<td>Heptadecane</td>
<td>2.47</td>
</tr>
<tr>
<td>2,6,10,14-Tetramethyl pentadecane</td>
<td>1.02</td>
</tr>
<tr>
<td>Hexadecane</td>
<td>1.87</td>
</tr>
<tr>
<td>Eicosane†</td>
<td>1.66</td>
</tr>
<tr>
<td>Octadecane‡</td>
<td>2.08</td>
</tr>
<tr>
<td>2,6,10,14-Tetramethyl hexadecane†</td>
<td>3.56</td>
</tr>
<tr>
<td>Heneicosane†</td>
<td>2.26</td>
</tr>
<tr>
<td>2,6,11,15-Tetramethyl hexadecane</td>
<td>1.52</td>
</tr>
<tr>
<td>Dibutyl phthalate</td>
<td>2.11</td>
</tr>
<tr>
<td>2,6,10,15,19,23-Hexamethyl-(all-E)</td>
<td>1.44</td>
</tr>
<tr>
<td>2,6,10,14,18,22-tetracosahexaene‡</td>
<td></td>
</tr>
<tr>
<td>Tetracontane</td>
<td>1.28</td>
</tr>
<tr>
<td>Hexatriacontane</td>
<td>1.22</td>
</tr>
<tr>
<td>Cholesta-3,5-diene</td>
<td>9.31</td>
</tr>
<tr>
<td>Cholesterol†</td>
<td>64.25</td>
</tr>
<tr>
<td>Total§</td>
<td>94.58</td>
</tr>
</tbody>
</table>

*Compounds are listed in order of their elution on the DB-1 capillary column. †Compounds present in both WSSV and host-cell nuclei. ‡Unsaturated hydrocarbon. §Some compounds in trace amounts were not identified.
Together, these results indicate that lipids are present in the WSSV envelope and that the viral envelope can be removed by lipid solvents or detergents. In the present study, it was interesting that the mortality of infected crayfish still reached 100% after 13 days when WSSV virions were extracted with petroleum ether (Fig. 1). This result indicated that petroleum ether treatment reduced rather than eliminated the infectivity of WSSV virions, suggesting that the lipids in the viral envelope do not play crucial roles in viral infection.

Direct evidence for the presence of lipids in WSSV was obtained by lipid analysis. Total lipids were extracted and their concentrations determined gravimetrically. The results showed that the lipid content of WSSV was much higher than that of the crayfish haemocytes. In addition, previous electron microscopy of purified WSSV virions has revealed that most virus particles are intact, with no apparent cellular debris (Xie et al., 2005; Zhou et al., 2007). Thus, the lipids we extracted from WSSV virions cannot be due to contamination by the host cell.

Generally, viruses derive their lipids from the host cell. Comparative lipid analyses of paramyxoviruses, myxoviruses, rhabdoviruses and togaviruses have shown that the composition of the viral lipids resembles the lipid composition of the host-cell plasma membrane at which the viruses mature (Klenk & Choppin, 1970; Laine et al., 1972; Lenard & Comans, 1974). Quigley et al. (1971) found that six different RNA viruses grown in the same host had similar lipid composition profiles. To confirm the origin of viral lipids, we performed comparative lipid analyses of WSSV and crayfish cell nuclei. We chose uninfected crayfish muscle cells from which to isolate the nuclei for two reasons: (i) to avoid contamination by the virus and (ii) because it is easy to extract abundant nuclei from this cell type. TLC results showed that the RF value of the presumed PC spot in nuclei was a little higher than that of standard PC and PC in WSSV. Barnes et al. (1987) reported that the RF values of phosphatidylinositol (PI), phosphatidylycerine (PS) and lysophosphatidylcholine (LPC) were all lower than that of PC. In the present study, the same developing solvent was used and the RF values of the PI, PS and LPC standards were also lower than that of PC (data not shown). Thus, the spot obtained with crayfish nuclei was identified to be PC; its higher RF value may reflect the different fatty acid chains on the PC molecules.

Our data showed that the phospholipid profile of WSSV was identical to that of the host-cell nuclei. PC was the most abundant phospholipid and PE was also present in significant amounts, although the level of PC was double that of PE. By contrast, other types of phospholipid such as SM, PI and cardiolipin were present at very low levels and comprised less than 10% of the total phospholipids. This is in contrast to the phospholipid profile of crayfish membrane reported by Pruitt (1988), where the PE level was double that of PC and the gross amount of remaining phospholipids was approximately 40% of the total phospholipids, among which the SM content was greater than 10%. Thus, there are significant differences in the phospholipid profiles of crayfish nuclei and membranes, whereas WSSV and crayfish nuclei resembled each other in our study. Therefore, we considered that WSSV obtains its lipids from the host-cell nucleus.

There are several reports showing that the fatty acid compositions of different lipid classes are similar between viruses and their host-cell membranes (Laine et al., 1972; Voiland & Bardeletti, 1980). So far, no direct comparisons have been reported on the cholesterol and hydrocarbon compositions of viruses and their host-cell nuclei. The levels of these components are very low compared with the fatty acid levels; furthermore, these components are free within the membrane, so it is not necessary to perform a transesterified reaction before analysis. We attempted to detect small differences between WSSV and the host-cell nuclei by analysing the cholesterol and hydrocarbon levels. Interestingly, in spite of the fact that the GC-MS results showed that the six compositions that form the dominant amount of cholesterol and hydrocarbon in virus and host-cell nuclei were similar, the species of cholesterol and hydrocarbon in virus was increased compared with that in host-cell nuclei. This result indicates that the cholesterol and hydrocarbons of WSSV are more complicated than those of its host.

Based on the slight difference in the PC RF values and the species of cholesterol and hydrocarbon between WSSV virions and host nuclei, we suggest that there are two possible explanations. Firstly, a selective sequestration of lipids may occur during the assembly process, so that the cholesterol, hydrocarbon and PC of WSSV differ from those of the host nuclei. The same phenomenon was also reported by Blough & Lawson (1968), Blough (1971) and David (1971). Secondly, as the nuclei were isolated from uninfected crayfish, it is possible that infection by the virus changed the host lipid metabolism, resulting in lipid composition changes. Further research is required to obtain a more complete understanding of this process.

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