Multiple proteins of *White spot syndrome virus* involved in recognition of β-integrin

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The recognition and attachment of virus to its host cell surface is a critical step for viral infection. Recent research revealed that β-integrin was involved in *White spot syndrome virus* (WSSV) infection. In this study, the interaction of β-integrin with structure proteins of WSSV and motifs involved in WSSV infection was examined. The results showed that envelope proteins VP26, VP31, VP37, VP90 and nucleocapsid protein VP136 interacted with LvInt. RGD-, YGL- and LDV-related peptide functioned as motifs of WSSV proteins binding with β-integrin. The β-integrin ligand of RGDT had better blocking effect compared with that of YGL- and LDV-related peptides. *In vivo* assay indicated that RGD-, LDV- and YGL-related peptides could partially block WSSV infection. These data collectively indicate that multiple proteins were involved in recognition of β-integrin. Identification of proteins in WSSV that are associated with β-integrin will assist development of new agents for effective control of the white spot syndrome.

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1. Introduction

White spot syndrome (WSS) is one of the most serious diseases of shrimp that is spreading widely (Lightner 1996). It affects most of the cultured shrimp species and has a wide host range and tissue tropism among crustaceans. WSSV is an enveloped, ellipsoid, large, double-stranded DNA virus. The complete genome sequences of the three WSSV isolates have been reported (Van Hulten *et al.* 2001a; Yang *et al.* 2001; Chen *et al.* 2002). Compared to significant progress on the WSSV's epidemiology, pathology and characterization, information concerning the mechanism of infection is lacking.

The recognition and attachment of the virus to its host cell surface is a critical step for viral infection. Many data indicated that multiple molecules are involved in binding and entry of WSSV (Chen *et al.* 2007; Li *et al.* 2007b; Liang *et al.* 2010; Huang *et al.* 2012). Integrins, a family of specific cell surface receptors, are transmembrane heterodimers composed of noncovalently associated α and β subunits. Certain viruses and bacteria, which contain canonical integrin-binding motifs in their surface, take advantage of this family of proteins to gain access into the permissive cells (Triantafilou *et al.* 2001). Recent research indicated that integrin plays a role in WSSV infection (Li *et al.* 2007a; Tang *et al.* 2012). The integrin receptor recognition site includes a highly conserved Arg-Gly-Asp (RGD) triplet. To date, only VP187 was reported to interact with integrin (Li *et al.* 2007a). Sequence assay showed that multi-protein of WSSV contains the integrin ligand sequence. Moreover, previous research indicated that virion attachment proteins (VAPs) do not completely contain Arg-Gly-Asp (RGD) motif on which integrins depend to bind with specific ligands (Xie and Yang 2005; Liu *et al.* 2011). Therefore, the WSSV proteins responsible for binding to integrin have not been completely identified.

In this study, we examined the interaction of β-integrin with structural proteins of WSSV and motifs involved. Multiple proteins of WSSV involved in recognition of β-integrin were found. Identification of proteins in WSSV associated with β-integrin will assist development of new agents for the disease control.

Keywords. Binding motif; β-integrin; proteins; recognition; WSSV

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2. Materials and methods

2.1 *Expression of recombinant β-integrin of L. vannamei (LvInt)*

Total RNA was extracted from the hepatopancreas of live *L.vannamei* (Lv) with Trizol reagent (Life Technologies, USA) and treated with RNAse-free DNase to remove contaminating DNA. First-strand cDNA synthesis was performed using M-MLV reverse transcriptase with oligo-$d(T)_{18}$ as the primers according to the instructions (Takara, Japan). LvInt (GeneBank No: ACY82398.1) was amplified using specific primer (table 1). Two microliters of cDNA was used as template for PCR amplification. The PCR products were assayed on a 1% agarose gel and gel fragments were cut out and cleaned using gel recovery kit (Tiangen, China). The PCR products and plasmid DNA of pBAD/gIII A (Life Technologies, USA) were separately digested with both *Nco*I and *Xba*I at 37°C for 2 h. After purification, the digested target gene fragments were ligated into the pBAD/gIII A vector. The ligated products were transformed into Top10 cells (Tiangen, China). Single colonies were picked and cultured overnight at 37°C. The recombinant plasmid DNA was extracted and digested with both *Sac*I and *Pst*I, and then applied on a 1% agarose gel to screen for positive clones. The sequences of the positive clones were confirmed by DNA sequencing analysis. The corrected sequence clone was induced in 0.02% L-arabinose Top10 *Escherichia coli* cells at 37°C for 5 h. The recombinant LvInt was purified using a column of TALON Metal Affinity Resins under denatured conditions and renatured by successive 12 h incubations with 6, 4, 2 and 0 M Guanidine-HCL in buffer (20 mM Tris-HCL,150 mM NaCl, 1 mM EDTA, 25 mM dethiothreitol, 0.1% Tween-20,10% glycerol, pH 7.5). Purity of LvInt was assayed by SDS-PAGE.

2.2 *SDS-PAGE and Western blot assay*

Expression cultures were subjected to 12% SDS-PAGE analysis according to the method of Laemmli (1970). For Western blotting, the separated proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were incubated in blocking buffer (1% bovine serum albumin, 5% skim milk, 50 mM Tris, 200 mM NaCl, pH 7.5) at 4°C overnight, followed by incubation with polyclonal mouse anti-(His)-HRP (1:10000) (Life Technologies, USA) for 2 h, respectively. Then the membrane was washed twice with 20 mL of PBST for 5 min with gentle agitation. Subsequently, detection was performed with a DAB (4-chloro-1-naphthol, Sigma-Aldrich, China) solution.

2.3 *Expression of VP28, VP26, VP31, VP37, VP90 and VP136*

Recombinant C-terminal VP28 (VP28C, 121-204 aa), VP26, VP31, VP37, VP90 (86-334 aa, containing RGD) and VP136 (846-1218 aa, containing RGD) were cloned using the specific forward primer and the reverse primer illustrated in table 1. The PCR products were digested with digestive enzyme respectively and cloned into pBAD/gIIIA vector. The recombinants were expressed in *E.coli* Top10 and confirmed by SDS-PAGE and Western blot assay. His-tag VP28C, VP26, VP31, VP37, VP90 and VP136 were purified using a column of TALON Metal Affinity Resins as described above.

Table 1. Primer sequence

Primer	Sequence (5′-3′)	Sequence information
LvInt(F)	TTCCATGGAGATGAGGCTGAGGCTGAGAGTC	Recombination
LvInt(R)	CCTCTAGATTGGCGACTTGATTAATCTG	Recombination
VP28(F)	CTACTCGAGATGGATCTTTCTTTCACTC	Recombination
VP28(R)	TATAAGCTTTCGGTCTCAGTGCCA	Recombination
VP26(F)	ACACCATGGATACACGTGTTGGAAG	Recombination
VP26(R)	GCGTCTAGAGTCTTCTTCTTGATTTCGT	Recombination
VP31(F)	GACTCGAGATGTCTAATGGCGCAACTA	Recombination
VP31(R)	GTGAATTCGCTCCTCCTTAAAAGCAG	Recombination
VP37(F)	GGTCGAATTCATGGCGGTAAACTTG	Recombination
VP37(R)	CGGCTCTAGAGGTGTCCAACAATTTAAAAA	Recombination
VP90(F)	CCCTCGAGATGAGGAGTGTCTACAGAGA	Recombination
VP90(R)	AAAAGCTTTCCTGCAGATGGGGATT	Recombination
VP136(F)	GAGAGCTCATGTCCATACTACTCTT	Recombination
VP136(R)	ACTCTAGACGAGTTCTGAAGCTAGA	Recombination

2.4 *Far-Western blot assay*

To confirm LvInt interaction with proteins of WSSV, a modified procedure was performed (Liu *et al.* 2006). Briefly, VP28C, VP26, VP31, VP37, VP90 and VP136 were separated by 12% SDS-PAGE, and transferred to a PVDF membrane. The membrane was renatured gradually at 4°C overnight in HEPES buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% Tween 20,10% glycerol, pH 7.5) containing 2% non-fat milk. The blot was washed with TBS, and incubated with 20 μg Digoxigenin (DIG)-labelled purified recombinant LvInt in 2 mL of HEPES buffer containing 1% non-fat milk for 4 h at 4°C respectively. After washing, the integrin-bound protein band was incubated with AP-conjugated anti-DIG antibody (1:2000 dilutions, Roche) for 1 h, and then the signals were visualized using substrate NBT kit (TopBio, China).

2.5 *Peptides synthesis*

Peptides (>90% pure by HPLC) contained in VP26 (Asn-Leu-Asp-Val-Ala, NLDVA), VP31 (Glu-Leu-Asp-Val-Arg, ELDVR; Lys-Arg-Gly-Asp-Thr, KRGDT), VP37 (Glu-Arg-Gly-Asp-Glu, ERGDE; Lys-Tyr-Gly-Leu-Arg, LYGLR), VP90 (Thr-Arg-Gly-Asp-Thr, TRGDT) and VP136 (Trp-Arg-Gly-Asp-Thr, WRGDT) were synthesized by Sangon company (Shanghai, China).

2.6 *Binding assay*

Ninety-six-well ELISA plates (Costar) were coated with VP26, VP31, VP37, VP90 and VP136 in coating buffer (1 μg/well) overnight at 4°C and then blocked with 2% non-fat milk in PBS buffer for 2 h at 37°C. The plates were washed three times with PBS buffer containing 0.05% Tween 20, following which DIG labelled LvInt (1 μg/well) was added for 1 h at 37°C. After 1 h incubation at 37°C, and three washes, anti-DIG-AP antibody (1:2000, Roche) was added. Finally the reaction was visualized using the AP substrate PNPP (Thermo), and stopped by the addition of 2 M NaOH. The absorbance was immediately read at 492 nm using a TECAN SAFIRE (Fluorescence, Absorbance and Luminescence) Reader. The LvInt incubated with 1 μg BSA/PBS was used as a control. The absorbance value was expressed as the value of sample divided by value of control (Liu *et al.* 2006).

To confirm binding motif presented in virus protein, competitive ELISA assay was performed. Briefly, 96-well ELISA plates (Costar) were coated with VP26, VP31, VP37, VP90 and VP136 in coating buffer (1 μg/well) overnight at 4°C and then blocked with 2% non-fat milk in PBS buffer for 2 h at 37°C. After washing, DIG-labelled LvInt (1 μg/well) were added and incubated with 0 (positive control) or 1 μg LDV-, RGD- and YGL-related peptides constituting in VP26, VP31, VP37, VP90 and VP136 for 1 h at 37°C respectively. Subsequent procedures were followed as described above. The absorbance was read at 492 nm and statistical analysis was performed using SPSS software. Differences were considered significant at $p < 0.05$.

2.7 *WSSV virions prepared and DIG-labelled*

The infection of healthy crayfish *Procambarus clarkii* and the purification of virus were performed as described previously (Xie and Yang 2005). WSSV virion numbers were calculated according to the method described by Zhou (Zhou *et al.* 2007). DIG-labelled WSSV was performed as before (Liu *et al.* 2006).

2.8 *Peptide blocking assay*

Ninety-six-well plates (Costar) were coated with 1 μg of LvInt in coating buffer (1 mM $MgCl_2$ in PBS) overnight at 4°C. Wells were washed three times with coating buffer, blocked with binding buffer (1% BSA in PBS with 1 mM $MgCl_2$) for 1.5 h at room temperature (RT), washed and pre-incubated with 50 μg of different peptides for 1 h at RT. The peptide solution was then removed before DIG-labelled WSSV (30 μL/well) was added. After 1 h incubation at room temperature, the unabsorbed virus was removed through washing, followed by incubation with AP-DIG antibody for 1 h. The washing step was repeated, and AP-substrate (PNPP) was added and absorbance was measured at the OD_{492} using a TECAN SAFIRE Reader. The wells coated with LvInt without peptides were used as control. Statistical analysis was done as described above.

2.9 *Infection-blocking assays in vivo*

Health shrimp weighing about 7–10 cm were divided into six groups (20 shrimps for each group). Shrimp was injected as follows: group 1 with WSSV (10^6 virions/shrimp) (positive control), group 2 with TN buffer (20 mmol Tris–HCl, 400 mmol NaCl [pH 7.4]) (buffer negative control), group 3, with WSSV (10^6 virions/shrimp) plus integrin (20 μg/shrimp) as the recombinant protein test, and group 4–6 with WSSV plus different peptides (NLDVA, TRGDT, LYGLR) (final concentration 20 μg peptide/shrimp) and experiments were carried out twice. Cumulative mortality was recorded for 14 days after WSSV injection.

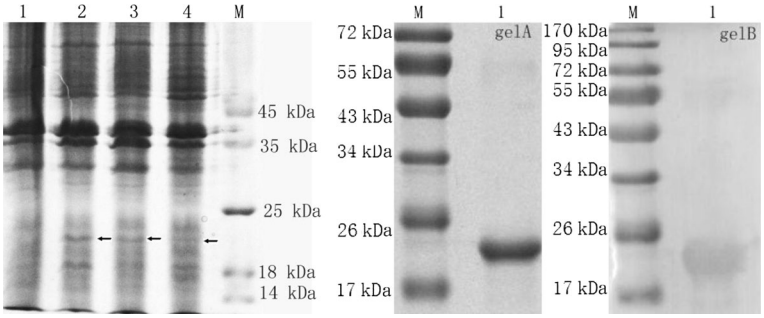

Figure 1. Analysis of recombinant expression of LvInt. Left gel: SDS-PAGE. 1, no-induced rLvAnt; 2–4, induced rLvInt; Gel A: SDS-PAGE profile of purified rLvInt. 1, rLvInt; Gel B: Western blot profile. 1, rLvInt.

3. Results

3.1 *Expression of LvInt*

The LvInt was cloned by RT-PCR and ligated with His-tag vector to constitute recombinant. The correct rLvInt was expressed in *E.coli* Top10, and SDS-PAGE (figure 1) assay revealed that the protein with estimated molecular weight around 22 kDa was expressed compared to non-transformant. The rLvInt was confirmed by Western blot analyses with mouse anti-(His) × 6-antibodies and purified LvInt was attained by affinity chromatography (figure 1).

3.2 *Multiple proteins involved in recognition LvInt*

To test whether LvInt binds to RGD-contained and other structural proteins, we produced the recombinant VP28C, VP26, VP31, VP37, VP90 and VP136 in *E. coli* expression system. The purified protein in each sample was tested by directly loading protein samples in an SDS-PAGE, then verified by Coomassie Blue staining of the gel (figure 2, left panel), and by Western blot with anti-His antibody (data not shown). DIG-labelled LvInt was used as a probe to interact with membrane blot of recombinant proteins. It was shown that LvInt was able to specifically interact with VP26, VP31, VP90, VP37 and VP136 (figure 2, right panel). Thus, the binding of WSSV to LvInt may be mediated via RGD-contained and other motifs for protein–protein interaction.

3.3 *Potential ligand sequences for LvInt*

In order to confirm the interaction of LvInt with VP26, VP31, VP37, VP90 and VP136, ELISA assay was performed. Ratio of absorbance of VP26, VP31, VP37, VP90 and VP136 to that of BSA was greater than 2. Results indicated that LvInt binding with VP26, VP31, VP37, VP90 and VP136 was specific. Higher binding activity was shown in that of VP26 (figure 3).

To identify LvInt ligand sequence, peptides containing putative LvInt ligand sequences present in VP26, VP31, VP37, VP90 and VP136 were used to further define the roles of these sequences in LvInt binding. Competitive ELISA results indicated that NLDVA peptide constituting of VP26 blocked the binding to LvInt. Peptides of ELDVR and KRGDT affected binding of VP31 to LvInt. Peptides of ERGDE and LYGLR reduced the binding of VP37 to LvInt. Peptides of TRGDT contained in VP90 also affect the binding of VP90 to LvInt. However, no significant inhibition

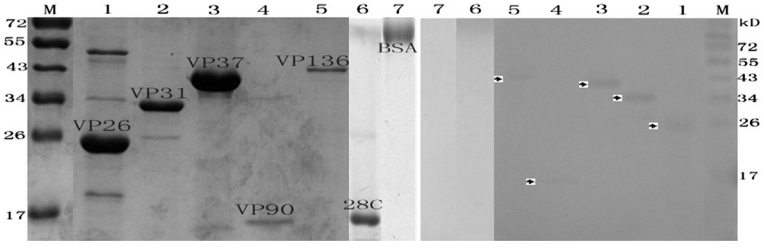

Figure 2. Far-Western blot assay of LvInt interact with VP26, VP31, VP37, VP90 and VP136. (Gel Left) Coomassie brilliant blue-stained 12% SDS/polyacrylamide gel profile. Gel right, blot on PVDF membranes overlayed with DIG-labelled intergrin. 1, rVP26; 2, rVP31; 3, rVP37; 4, rVP90; 5, rVP136; 6, VP28C; 7, BSA (arrow indicates positive band).

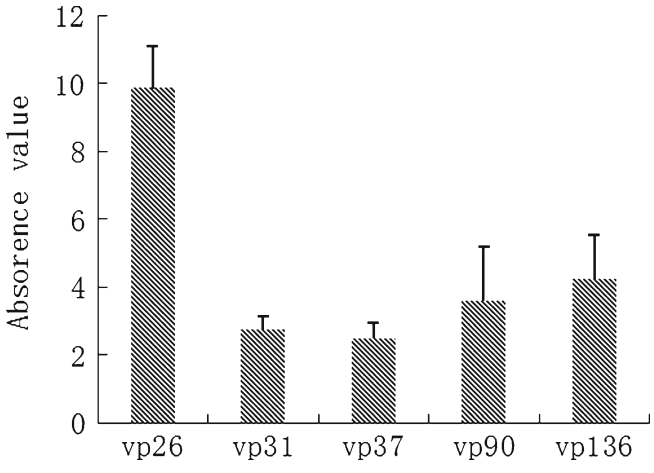

Figure 3. ELISA analysis of LvInt bind with VP26, VP31, VP37, VP90 and VP136. The 96-well plates were coated with 1 μg of VP26, VP31, VP37, VP90 and VP136. After incubation, DIG-labelled LvInt was added. Reaction was detected with substrate of DIG. BSA was used as control. Data are the average of three assays. Error bars indicate standard deviations.

effect of VP136 binding with LvInt was observed with the peptide WRGDT (figure 4).

3.4 *Peptide-blocking assay*

To study whether RGD-, LDV- and YGL-related peptides block direct virus–integrin interaction *in vitro*, the peptides were incubated with immobilized LvInt, followed by incubation with the DIG-labelled WSSV. The addition of different RGD-, LDV- and YGL-related peptides decreased virus binding to the LvInt compared to the addition of a negative-control peptide, thereby confirming that the RGD motif is not only one of the major WSSV binding sites for β-integrin. Other motif of LDV and YGL was involved in recognition of β-integrin. The peptides of TRGDT and KRGDT had better blocking effect compared with that of NLDVA, ELDVA, ERGDE and LYGLR peptides (figure 5).

3.5 *In vivo assay*

To investigate if LvInt and different motifs play a role in mediating the infection of WSSV, infection-blocking assays were carried out on shrimps *in vivo*. After WSSV challenge, shrimp mortality increased and reached to 100% at 11th day for the group injected with WSSV. In contrast, the group with WSSV pre-incubated with LvInt and different peptides showed lower mortality rate compared with WSSV group at 11th day (figure 6). All of the dead shrimp were WSSV-positive, which was confirmed by PCR test. These results showed that β-integrin and RGD, LDV and YGL-relative peptides could partially block WSSV infection.

4. Discussion

Envelope proteins play vital roles in virus entry, assembly and recognition (Chazal and Gerlier 2003). Among WSSV proteins, VP26 is a major tegument protein loosely associated with both the envelope and the nucleocapsid (Tsai *et al.* 2006). VP26 was reported to be capable of binding to actin or hemocyte membrane proteins (Xie and Yang 2005; Liu *et al.* 2011; Weerayut *et al.* 2011). VP28, VP31, VP37 (or VP281) and VP90 were considered as envelope protein fractions (Tsai *et al.* 2004; Xie *et al.* 2006). VP37 with a cell attachment RGD motif was supposed to play an

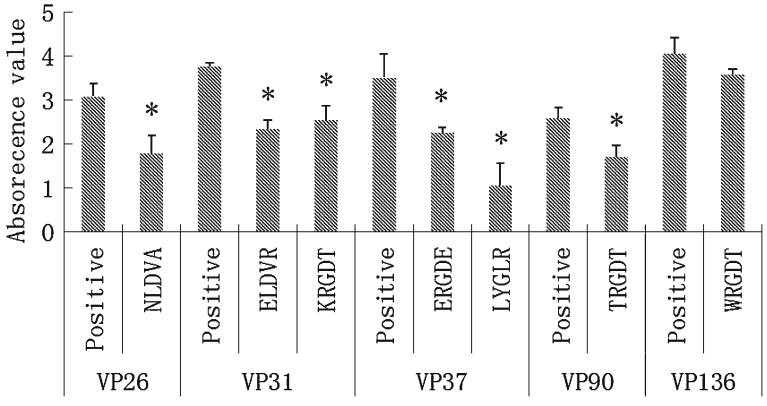

Figure 4. Binding of virus protein to rLvInt in the presence of synthetic peptides.VP26, VP31, VP37, VP90 and VP136 were coated in the 96-well plate (1 μg/well) and incubated with 50 μg of synthetic peptides then added DIG-labelled rLvInt. BSA was used as control. Data are the average of three assays. Error bars indicate standard deviations. Asterisk (*) means significantly different compared with positive control of virus protein (VP26, VP31, VP37, VP90, VP136).

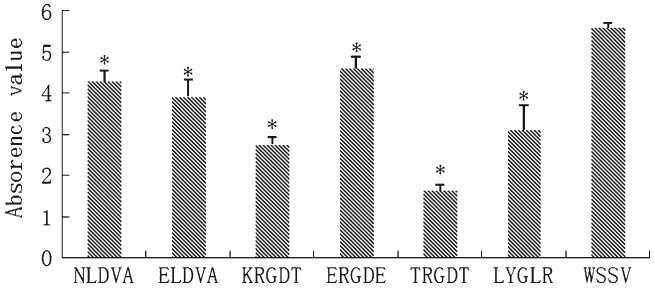

Figure 5. Peptide-blocking assay of WSSV binding with LvInt. The 96-well plates coated with 1 μg of integrin were incubated with 50 μg of different peptides before DIG-labelled WSSV was added. Data are the average of three assays. Error bars indicate standard deviations. Asterisk (*) means significantly different compared with the group of only WSSV without peptides.

important role in mediating WSSV infectivity and binding (Huang *et al.* 2002; Liu *et al.* 2006). VP31 have been reported to contain RGD motif (Tsai *et al.* 2004) and play an important role in WSSV infectivity (Li *et al.* 2006). VP90 was reported to be an envelope protein with RGD motif (Xie *et al.* 2006). VP28 was shown to be in the spike of the WSSV envelope that plays a role in the systemic infectivity of the virus in shrimp (Van Hulten *et al.* 2001b). However, only VP26, VP31, VP37 and VP90 interact with β-integrin. The possible role for VP26, VP31, VP37 and VP90 in cell attachment is also consistent with its interaction with β-integrin. This interaction with β-integrin may help explain the importance of VP31 and VP37 as a neutralizing antibody target (Li *et al.* 2005; Wu *et al.* 2005). It is likely that antibodies to VP31 and VP37 may neutralize WSSV by preventing their interaction with integrins.

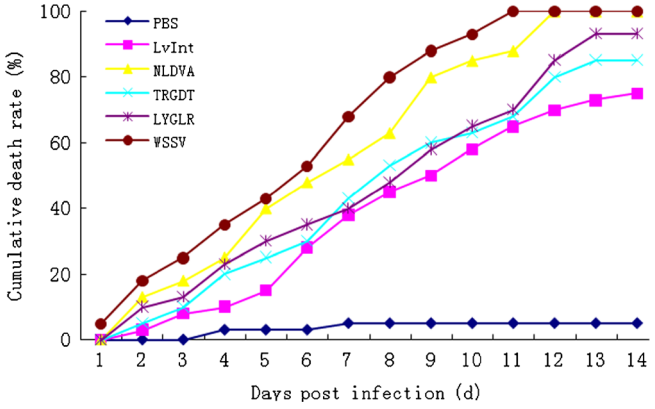

Figure 6. Infection-blocking assay *in vivo*. Shrimp were treated with WSSV, or WSSV pre-incubated with LvInt, NLDVA, TRGDT and LYGLR respectively. Shrimp treated with PBS was as a control.

The characterization of nucleocapsid proteins in infection is also inadequate. Differing from those of many enveloped viruses, the replication and assembly of the WSSV envelope takes place in the nucleus (Lan *et al.* 2002), but little is known about the molecular events occurring during this process. The nucleocapsid protein VP136 is predicted to have TM domains as well as RGD motif (Xie *et al.* 2006), but it is less believed that they may have such a function as they are localized in the nucleocapsid (Li *et al.* 2007a, b). The interaction between VP136 and integrin was identified in this study. The process of nucleocapsid VP136 recognition integrin may be important for the naked nucleocapsid wrapped inside the envelope. The function of other proteins containing integrin motifs involved in recognition of β-integrin remains to be elucidated.

The integrin recognition motifs on several integrin ligands have been described, and it can often be reduced to small peptide sequences (Hynes 2002; Plow *et al.* 2000). Several viruses and bacteria, which contain canonical integrin-binding motifs in their surface, take advantage of this family of proteins to gain access into the cell (Triantafilou *et al.* 2001). In addition, some viruses have been found to interact with integrins through non-typical sequence motifs (Plow *et al.* 2000). To identify integrin ligand sequence, peptides containing the β-integrin ligand sequences present in WSSV were accessed for the roles of these sequences in WSSV binding. Competitive ELISA was performed to analyse the effects of peptides containing motif LDV, RGD and YGL on WSSV protein binding with integrin. The peptides of NLDVA, ELDVR, KRGDT, ERGDE, LYGLR and TRGDT competitively inhibit WSSV binding with LvInt significantly. Neutralization assay indicated that LDV, RGD and YGL-related peptides can partially neutralize WSSV infection. This suggested that WSSV bindes to the β-integrin mainly via ligand sequence RGD, LDV and YGL. Sequence analysis indicated that the RGD integrin ligand sequence was identified in envelope proteins such as VP31, VP37 and VP90. Potential ligand sequence LDV that is important for integrin recognition was also present in VP26 and VP31. VP37 domain also contains the sequence of YGL as well as RGD. This implies that VP26 binds integrin through LDV. VP31 binds integrin using LDV and RGD. VP37 binds integrin through RGD and YGL, and VP90 binds integrin by RGD motif.

Among RGD-, LDV- and YGL-related peptides, the differential effects of these peptides on WSSV binding were observed. A threonine at the fourth position of the RGD motif (TGDT), which was considered to be critical in interacting with integrin (Plow *et al.* 2000), gives better inhibition effect. This was probably related to sequence differences of the peptides within the binding regions in WSSV.

In previous studies, RGD structure has been reported widely to mediate the virus–host interaction (Tan *et al.* 2001; Wang *et al.* 2003). The role of the RGD sequence in cellular recognition has been shown through blocking assays with RGD-containing peptides, mutation of the sequence and function-blocking antibodies to integrin (Li *et al.* 2007b). These results strongly suggested that integrins play a central role in WSSV infection. However, not all RGD motifs mediate cell attachment (Israelsson *et al.* 2010). In this study, we found that the binding of integrin involves not only RGD loop but also LDV and YGL motifs. Integrin binding mediated by ligand is critical for the activation of intracellular signals, and more work is needed to explore whether integrin signalling contributes to WSSV-induced cell death.

Recent research indicated that the WSSV interaction with shrimp may be mediated through more than one cell receptors (Chang *et al.* 2010). These results further support the idea of multi-envelope acting as an 'infectome' for cell recognition, attaching and penetration into the cell. Taken together, our research will be helpful to expand the understanding of WSSV pathogenesis and to determine strategies to interfere with white spot syndrome disease.

Acknowledgements

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