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ORIGINAL ARTICLE

The RGD motif in VP31 of white spot syndrome virus is involved in cell adhesion

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Abstract Two WSSV envelope proteins, VP31 and VP33, contain a conserved Arg-Gly-Asp (RGD) sequence. In order to investigate the role of the RGD motif, wild-type and RGD-mutated VP31 and VP33 were recombinantly expressed in *E. coli*. The cell adhesion ability of the proteins was investigated in crayfish haemocytes using a fluorescence assay. The results showed that recombinant wild-type VP31 and VP33 had cell adhesion activity, and the RGD motif in VP31 was required for cell adhesion, which could be inhibited by an RGDT peptide. In contrast, the interaction of VP33 with cells did not require the RGD motif. These data indicate that the RGD motif plays an important role in the interaction between VP31 and host cells.

Introduction

White spot syndrome virus (WSSV), the sole member of the genus *Whispovirus*, family *Nimaviridae* (http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm), is an important pathogen of most species of crustaceans [3, 11, 17, 21], including crayfish [6]. WSSV is an enveloped virus with a large double-stranded circular DNA genome (~300 kb). With the development of proteomics, WSSV structural proteins have been identified [5, 15, 23, 26]. However, in-depth research on the interaction between the virus and its hosts is limited.

In order to enter host cells and initiate infection, viruses must first attach to the cell surface. An Arg-Gly-Asp (RGD) recognition motif has been found to be important for the binding of many virus proteins to host cells. Examples include the penton protein of adenovirus [7, 18] and the foot-and-mouth virus coat protein [10]. To date, at least 4 WSSV envelope proteins, VP31, VP110, VP187 and VP33 (VP36B/VP37/VP281), have been found to contain RGD motifs [23, 26]. VP110 and VP187 have been shown to interact with crayfish hemocytes, and this interaction could be blocked by synthetic RGD-containing peptides [12, 13]. The other two RGD-containing envelope proteins, VP31 and VP33, have been shown by neutralization experiment or virus overlay protein-binding assays to be involved in WSSV infection [14, 16]. However, the role of the RGD motif in these two proteins remains unknown. An exploration of the role of the RGD motif in virus envelope proteins will improve our understanding of the mechanism of virus infection. In this study, we investigate the interaction of two viral envelope proteins, VP31 and VP33, with host cells and explore the role of their RGD motif.

Materials and methods

Mutagenesis and expression of recombinant proteins

The vp31 and vp33 genes were amplified from WSSV genomic DNA by PCR. The primers were vp31f (AGAGGGGATCCATGTCTAATGGCGCAACTAT)/vp31r (AGAGGGAATTCCTCCTCCTTAAAAGCAGTGA) and vp33f (AGAGGGGATCCATGGCGGTAAACTTGGATAA)/

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vp33r (AGAGGGAATTCTGTCCAACAATTTAAAAAGA), respectively. The PCR products were digested with *BamH* I and *EcoR* I and inserted into the pMBP-P vector [2], in frame with the maltose-binding protein (MBP) and His tag. Site-directed mutagenesis was accomplished by PCR amplification [9]. Recombinant wild-type plasmids pMBP-vp31 and pMBP-vp33 were used as templates, and site-directed mutagenesis of the RGD motif of VP31 and VP33 was performed by PCR with the primer pairs vp31r/vp31m (AGAGGTCTAGATATAAACACACCCATATACTAAACGGAGAATCTGCAACATACGCTGTTAAACGTATAAAAAGAGATGGCACAAGGGACGATATATTG) and vp33r/vp33m (AGAGGGAGCTCATACAAGAAAGAGATGGTGAAATTACACCTTTGACT), respectively. Once the PCR was completed, the RGD sequence was mutated to RDG. Finally, the PCR fragments were digested with Xba I/EcoR I and Sac I/EcoR I and inserted into the corresponding sites of the expression vector. The constructs were verified by sequencing.

E. coli BL21 (DE3) cells were transformed with the recombinant plasmids. Liquid cultures were grown in a shaking incubator (200 rpm) at 37°C until the OD_{600} reached 0.7, and the expression of target proteins was then induced by the addition of 0.2 mM IPTG for 5 h at 37°C. Cells were then harvested by centrifugation at 4000 × g for 5 min. The soluble forms of the MBP-His-tag fusion proteins wild-type MBP-VP31 (MBP-wtVP31), mutated MBP-VP31 (MBP-mtVP31), wild-type MBP-VP33 (MBP-wtVP33) and mutated MBP-VP33 (MBP-mtVP33) were purified by Ni-NTA affinity chromatography under native conditions using a QIAexpressionist Kit (QIAGEN).

SDS-PAGE and Western blot analysis

Samples were separated on a 12% sodium dodecylsulfate polyacrylamide gel (SDS-PAGE). For western blot analysis, the samples were transferred onto a PVDF membrane (GE Healthcare) by semi-dry blotting at a constant current density of 0.5 mA/cm^2 for 1.5 h at room temperature. The membrane was immersed in blocking buffer (1% BSA, 150 mM NaCl, 0.05% Tween 20, 20 mM Tris-HCl, pH 7.2) at room temperature for 1 h, followed by incubation with 6-his monoclonal antibody (GE Healthcare) for 0.5 h. Subsequently, alkaline-phosphatase-conjugated goat anti-mouse IgG (Promega) was added at a dilution of 1:7500, and signals were detected using a substrate solution containing 4-chloro-1- naphthol and X-phosphate (Promega).

Adhesion assay and competitive inhibition assay

The cell adhesion and competitive inhibition assay were performed as described previously [13]. In brief, haemocytes extracted from healthy crayfish (*Procambarus clarkii*) were seeded on 24-well poly-L-lysine-coated plates (10^5 cells per well). After incubation for 30 min at room temperature, the culture medium was removed and residual binding sites were blocked with CPBSB buffer (10 mM $CaCl_2$ and 3% BSA in PBS) for 45 min. Cells were then incubated with recombinant MBP fusion proteins (0.1 mg per well) or with the same amount of MBP alone for 45 min at room temperature. Subsequently, the wells were washed three times with PBS and incubated with anti-His-tag monoclonal antibody (diluted 1:2000 in CPBSB) for 30 min, followed by immunostaining with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (diluted 1:200 in CPBSB; Sino-American Biotechnology). Finally, stained cells were observed under a fluorescence microscope (Olympus IX70). For the competitive inhibition assay, cells were pre-incubated with synthetic RGDT or RDGT peptide (0.5 mg/ml, Shanghai Sangon) for 30 min before the addition of recombinant proteins.

Results

Mutagenesis, expression and purification of proteins

To test the role of the RGD motif in WSSV envelope proteins VP31 and VP33, the RGD motifs in VP31 (amino acids 244-246) and VP33 (amino acids 75-77) were mutated to RDG. Furthermore, MBP-wtVP31, MBP-mtVP31, MBP-wtVP33 and MBP-mtVP33 were expressed in *E. coli* (Fig. 1A). The soluble proteins MBP-wtVP31, MBP-mtVP31, MBP-wtVP33 and MBP-mtVP33 were purified and used in fluorescence analysis. Before cell adhesion and fluorescence assay, all recombinant proteins with His tags were validated by SDS-PAGE and Western blotting with 6-His monoclonal antibody (Fig. 1B).

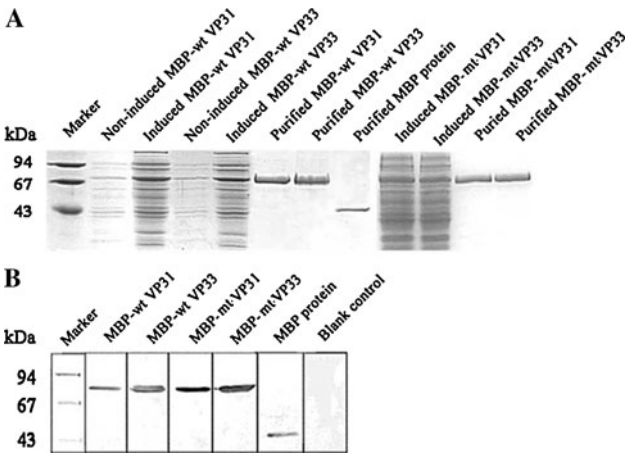

Fig. 1 (**A**) Expression of wild-type and mutant proteins in *E. coli* and (**B**) Western blot analysis with 6 His monoclonal antibody

Attachment of VP31 to crayfish haemocytes is mediated by the RGD motif

Haemocytes from healthy crayfish were seeded into 24-well plates, and the attachment of recombined VP31 and VP33 proteins to crayfish haemocytes was determined by immunofluorescence assay. As shown in Fig. 2, cells incubated with MBP-wtVP31, MBP-wtVP33 and MBP-mtVP33 showed a strong fluorescent signal (Fig. 2A, C, D), whereas no significant signal was observed in cells incubated with MBP-mtVP31 (Fig. 2B) or MBP alone (Fig. 2E). These results suggested that VP31 and VP33 could attach to crayfish haemocytes and that the RGD motif was required for cell adhesion of VP31 but not VP33.

To confirm the role of RGD motifs in these proteins, a competitive inhibition assay was performed using the synthetic peptides RGDT and RDGT. As shown in Fig.3, the binding of the MBP-wtVP31 protein to crayfish haemocytes was specifically abolished by the RGDT peptide (A) but not the RDGT peptide (B), indicating that the binding of VP31 to the cell membrane was mediated by its RGD motif. In contrast, neither the RGDT peptide nor the RDGT peptide affected the interaction between MBP-wtVP33 and the cell membrane (Fig. 3C, D), suggesting

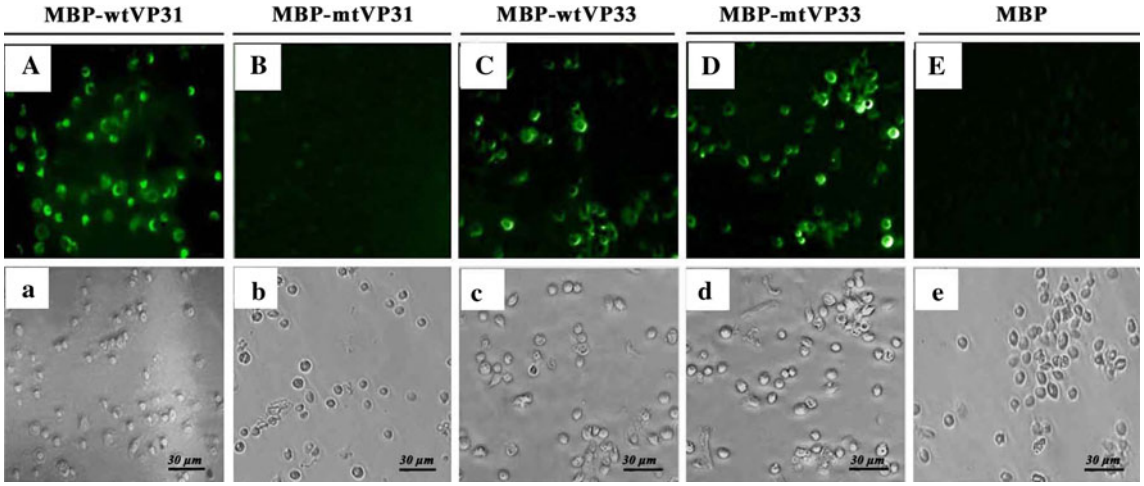

Fig. 2 Cell attachment assay. Crayfish haemocytes were incubated with wild-type proteins (MBP-wtVP31 and MBP-wtVP33), RGD-mutated proteins (MBP-mtVP31 and MBP-mtVP33) and MBP-His. Their attachment ability was evaluated by fluorescence microscopy using anti-His primary antibody and FITC-conjugated secondary antibody. The upper images (**A**-**E**) were made by fluorescence microscopy, and the corresponding lower images (**a**-**e**) show the same field by light microscopy. Bar = 30 μm

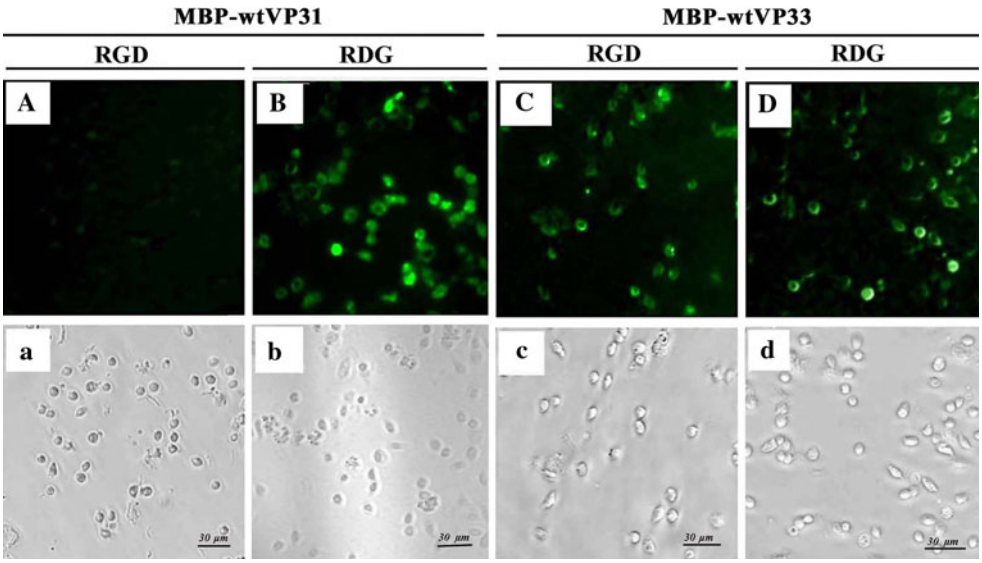

Fig. 3 RGD competitive inhibition experiments. Crayfish haemocytes were pretreated with synthetic RGDT or RDGT peptide prior to the addition of recombinant proteins. The other experimental conditions were the same as in the cell attachment assay. Adhesion of MBP-wtVP31 to cells was inhibited in the presence of RGDT. The upper images (**A**-**D**) were made by fluorescence microscopy, and the corresponding lower images (**a**-**d**) show the same field by light microscopy. Bar = 30 μm

that the RGD motif is not necessary for the attachment of VP33 to crayfish haemocytes.

Discussion

The RGD motif is known to mediate many cell-cell and virus-host interactions. For example, the RGD motif of bluetongue virus VP7 protein is responsible for core attachment to culicoides cells [22]. The RGD motif of foot-and-mouth disease virus VP1 contributes to the cell attachment site of the virus [4, 24]. The infection of human herpes virus 8 (HHV-8) is mediated by the RGD motif-integrin interaction [25], and its infectivity can be inhibited by RGD peptides [1]. Therefore, research on the interaction between host cells and envelope proteins, especially proteins possessing RGD motifs, will be helpful for a better understanding of the mechanisms of virus infection.

VP31 and VP33 are two WSSV envelope proteins that possess an RGD motif, and VP31 has a threonine at the fourth position after the RGD motif (RGDT), which has been reported to be important for binding to integrins via the RGD motif [19]. Previous studies have showed that these proteins are involved in WSSV infection. Antibody neutralization experiments showed that WSSV infection could be delayed or neutralized by antibodies against VP31 [14]. VP33 (VP37) has been shown, using a virus overlay protein binding assay, to bind to the shrimp cell membrane [16]. However, not all RGD motifs mediate cell attachment [8, 20]. In this study, we wanted to know whether the RGD motifs in VP31 and VP33 play a role in cell adhesion. First, we obtained soluble MBP-wtVP31 and MBP-wtVP33, as well as MBP-mtVP31 and MBP-mtVP33, by introducing point mutations in VP31 and VP33, converting RGD to RDG. A cell attachment assay showed that MBP-wtVP31 and MBP-wtVP33 can adhere to haemocytes, indicating that the VP31 and VP33 can interact with as yet unknown cell-membrane proteins. However, the RGD-mutated proteins MBP-mtVP31 could not bind to cells, suggesting that the RGD motif in VP31 may be involved in cell adhesion.

To confirm the role of the RGD motif, we carried out blocking experiments with synthetic RGDT and RDGT peptides in a competitive inhibition assay. We found that the interaction between MBP-wtVP31 and haemocytes was inhibited by the synthetic RGDT peptide but not by the RDGT peptide, whereas RGDT peptide did not affect the interaction between MBP-wtVP33 and haemocytes. Therefore, we conclude that the RGD motif is important for cell adhesion in VP31 but not in VP33.

These findings will contribute to the understanding of WSSV entry into host cells. Furthermore, VP31 may function as a potential target for the design of antiviral drugs.

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