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Expression kinetics of β-integrin in Chinese shrimp (*Fenneropenaeus chinensis*) hemocytes following infection with white spot syndrome virus

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ABSTRACT

Our previous study has demonstrated that an integrin β subunit of Chinese shrimp (*Fenneropenaeus chinensis*) (FcβInt) involved in WSSV infection. In order to further elucidate the potential role of the FcβInt in the WSSV infection, expression response of FcβInt to WSSV infection in shrimp hemocytes was investigated after intra-muscular injection with the virus. Following time-course hemocytes sampling, the expression variation of FcβInt in hemocytes was examined by flow cytometric immunofluorescence assay (FCIFA) and enzyme linked immunosorbent assay (ELISA) using the monoclonal antibody (Mab) 2C5 against FcβInt, which was successfully produced with recombinant partial FcβInt and exhibited binding to a 120 kDa hemocyte protein. Meanwhile, the dynamic state of FcβInt mRNA level and WSSV copies in hemocytes were determined by quantitative real-time PCR. The result of FCIFA showed that FcβInt was mainly expressed on the semi-granular and granular cells, which was down-regulated at 6 h post infection (p.i.), and significantly increased to the peak level at 12 h p.i., then decreased to the control level by 24 h. However, FcβInt on the hyaline cells was lowly expressed and didn't show active response to the viral infection. The variation of FcβInt concentrations in total hemocytes determined by ELISA was roughly in accordance with the changing tendency of FcβInt expressed on the semi-granular and granular cells. FcβInt mRNA level in total hemocytes was significantly up-regulated to the peak level at 12 h p.i. Moreover, the number of WSSV copies in hemocytes began to exhibit a significant increase at 24 h p.i. The present study demonstrated that WSSV infection would induce a differential regulation of FcβInt expression in different type hemocytes, which provided valuable evidences for the close correlation between FcβInt and WSSV infection.

1. Introduction

White spot syndrome virus (WSSV) is the sole member of the novel Nimaviridae family, and emerged globally as one of the most prevalent, widespread and lethal viral pathogens for shrimp populations [1–3]. In recent years, significant progresses have been made on the epidemiology, pathology and characterization of WSSV, but no effective treatment was available to control the occurrence and spread of the disease [4]. In order to gain insights into the WSSV infection mechanism and develop effective prophylactic measures, considerable efforts were focused toward virus–host interaction and obtained substantial information on the host cellular and viral molecules involved in WSSV infection [5–7]. Among them, the molecules involved in viral attachment and entry attracted the most attention due to their important roles in pathogenesis.

Integrins are heterodimeric cell surface receptors that consist of α and β subunits and function in mediating cell-matrix, cell–cell interactions as well as bidirectional signal transduction [8]. Due to their prominent and ubiquitous distribution on cell surface, integrins have emerged as receptors or co-receptors for a large number of viruses, such as adenovirus, ebola virus, echoviruses, hantavirus and papillomavirus [9–13]. Likewise, more recent studies also showed that integrin β subunits (β-integrin) in shrimps involved in WSSV infection. A β-integrin of *Marsupenaeus japonicus* exhibited specific binding activity to the WSSV envelope protein VP187, and the integrin, integrin-specific antibody as well as an RGD-containing peptide could block the WSSV infection *in vivo* and *in vitro* [14]. The previous work of this study also found that a β-integrin of *Fenneropenaeus chinensis* (FcβInt) could interact with WSSV, and the recombinant extracellular region of FcβInt owned partial neutralization activities against WSSV infection [15]. So,

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β-integrins might serve as putative receptors for WSSV attachment and entry. However, little information was known about the regulation of β-integrins in response to WSSV infection in the host cells.

Therefore, in the present study, monoclonal antibody (Mab) against FcβInt was produced and employed as a probe to investigate the expression variation of FcβInt in hemocytes following WSSV infection. Meanwhile, the dynamic state of FcβInt mRNA level and WSSV copies in hemocytes were determined by quantitative real-time PCR. The resultant data would further facilitate the understanding of the potential role of β-integrins in pathogenesis of WSSV infection.

2. Materials and methods

2.1. Hemocytes collection

The apparently healthy Chinese shrimps (*F. chinensis*) were caught from Yellow Sea of China. The hemolymph was withdrawn from the pericardial cavity using a syringe containing modified Alsever solution (27 mM Na citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7.2) as anticoagulant. The hemocytes in hemolymph were then pelleted by centrifuging at 200 *g* for 10 min at 4 °C and rinsed with prawn homoiosmotic phosphate buffered saline (PHPBS, 377 mM NaCl, 2.70 mM KCl, 8.09 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4, 780 mOsm/L), and pelleted again for later use.

2.2. Expression of partial FcβInt

The hemocyte total RNA was extracted with TRIzol® reagent (Life Technologies Corporation, USA) following the manufacturer's instruction and treated with RNase-free DNase (Promega RQ1 DNase I). The partial FcβInt cDNA located at the N-terminal portion of the extracellular region was refered to as von Willebrand factor type A domain (FcβInt-VWA), which was amplified by RT-PCR with the specific primer set InteF1 and InteR1 (Table 1) with recognition sequences for EcoRI and XhoI restriction enzyme (underlined). The purified PCR product was digested with the restriction enzymes and inserted into pET32a plasmid to generate pET32a-FcβInt-VWA recombinant. The recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3). The positive clones were screened by PCR and confirmed by sequencing, then incubated in LB medium and induced with 0.80 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The recombinant FcβInt-VWA (rFcβInt-VWA) with Trx/His/S-tag was purified with Ni^{2+}-affinity column (HiTrap HP column, GE) as described by the manufacturer's instruction. The samples, including uninduced *E. coli* BL21, induced *E. coli* BL21 and purified rFcβInt-VWA, were analyzed by SDS-PAGE and stained with coomassie brilliant blue R250.

Table 1
Names and sequences of the primer sets used in this study.

Primer name	Sequences (5′ → 3′)
Integrin	
InteF1 (Forward)	GGAATTCGACCCGCTGAGTGATGTTTCG
InteR1 (Reverse)	CCTCGAGCAGGACGCCGAAAGATGAAGC
InteF2 (Forward)	GACCCGCTGAGTGATGTTTC
InteR2 (Reverse)	CTTGAACTGCGTCGTGAGG
β-actin	
AF (Forward)	GAA GTAGCCGCCCTGGTTG
AR (Reverse)	GGATACCTCGCTTGCTCTGG
WSSV	
VF (Forward)	AAGCATCGTGGAGACTCTTGC
VR (Reverse)	GAAGATTCGCCGCTCATACC

2.3. Production and characterization of Mabs against FcβInt

Mabs specific to rFcβInt-VWA were generated according to the protocol of Zhan et al. [16]. Briefly, spleen cells of BALB/c mice immunized with rFcβInt-VWA were applied to fuse with P3-X63-Ag8U1 myeloma cells using polyethylene glycol as fusogen. Hybridomas secreting specific antibodies against FcβInt were preliminarily screened by enzyme linked immunosorbent assay (ELISA) using rFcβInt-VWA as the target antigen, and further characterized by indirect immunofluorescence assay (IIFA) and western blotting, then the positive hybridomas were cloned by limited dilution.

For IIFA, the shrimp hemocytes collected above were suspended in PHPBS, settled onto glass sliders for 30 min subsiding, and then fixed with acetone for 15 min. The slides were overlaid with Mab 2C5 which showed a high binding activity to rFcβInt-VWA analyzed by ELISA. After incubation for 1 h at 37 °C in a moist chamber, the sliders were rinsed thrice with PHPBS for 5 min each time and incubated with goat-anti-mouse Ig-FITC (1:256, Sigma), contained 1 mg/ml Evan's blue dye (EBD, Fluka) as the counterstain, for 1 h at 37 °C in the dark. Finally, the slides were rinsed again and observed by fluorescence microscope. The Mab 2C5 was replaced by myeloma culture supernatant as control.

For western blotting, the total protein of induced *E. coli* BL21 harboring pET32a-FcβInt-VWA and collected hemocytes were separated by SDS-PAGE and electrophoretically transferred onto PVDF membrane (Millipore). After blocking with 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS, 0.01 M, pH 7.4) for 1 h at 37 °C, the membrane was incubated with Mab 2C5 for 1 h at 37 °C. After washing thrice with PBST (PBS containing 0.05% Tween 20), goat-anti-mouse Ig-alkaline phosphatase antibody (1:4000, Sigma) was added for 1 h incubation at 37 °C. Positive bands were developed with substrate solution (100 mM NaCl, 100 mM Tris and 5 mM $MgCl_2$, pH 9.5) containing 5-bromo-4-chloro-3-indolyphosphate (BCIP, Sigma) and nitroblue tetrazolium (NBT, Sigma) for 20 min, and stopped by washing with distilled water. The Mab 2C5 was replaced by myeloma culture supernatant as control.

2.4. WSSV infection and hemocytes sampling

Crude extract of WSSV (CE-WSSV) was prepared as described by Jiang et al. [17] for shrimp infection. Briefly, 1 g gill tissue of natural heavily infected *F. chinensis* was homogenized in 10 ml PBS and centrifuged at 600 *g* for 20 min at 4 °C. The supernatant was passed through a 450 nm membrane filter to obtain the CE-WSSV, which was diluted 1000 times with PBS for inoculation.

The healthy shrimps (body length, 15 ± 2 cm) caught from Yellow Sea were acclimatized for 7 days at 25 °C prior to WSSV infection, which were confirmed to be WSSV-free by PCR [18]. For WSSV infection, each shrimp was intra-muscularly injected with 50 μl CE-WSSV suspension. At 0, 6, 12, 18, 24, 36, 48, 60 and 72 h post infection (p.i.), hemocytes were sampled as previously described from randomly selected 12 shrimps. All the hemocytes were averagely divided into four groups, one for flow cytometric immunofluorescence assay (FCIFA) suspended with PHPBS, one for ELISA stored at −80 °C, one for RNA extraction stored in RNAlater (Sigma) at −80 °C, and the last for DNA extraction stored in 95% ethanol at −80 °C. An equal number of shrimps were intra-muscularly injected with 50 μl PBS as control and the hemocytes were sampled in parallel.

2.5. Detection of FcβInt by FCIFA

The concentrations of hemocyte samples collected at different time points were adjusted to 5×10^6 cells/ml in PHPBS, and 1 ml of cell suspension incubated with 100 μl of MAb 2C5 for 1 h at room