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Identification of a protein binding to the phagocytosis activating protein (PAP) in immunized black tiger shrimp

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Abstract

The phagocytosis activating protein (PAP) gene was isolated from *Penaeus monodon* infected with the white spot syndrome virus (WSSV). The endocytosis pathway of GST–PAP (glutathione-*S*-transferase–PAP) that caused activation of phagocytosis of shrimp haemocytes was investigated. In this study, a yeast two-hybrid screening assay of a WSSV-infected *P. monodon* cDNA library, was performed using PAP as bait to test for the interaction between PAP and other proteins in WSSV-infected shrimp. An alpha-2-macroglobulin (α2M) was one of the proteins that interacted with PAP. A GST pull-down assay showed that GST–PAP was capable of co-precipitating α2M whereas GST itself was not. An intramuscular injection with inactivated WSSV caused a significant increase in the expression of both the PAP and α2M gene in the haemolymph. The highest expression of both genes was detected at 24 h post-injection and remained constant for 1 week. Moreover, the uptake of GST–PAP by shrimp haemocytes was higher in the presence of α2M than in its absence. The results indicated that α2M may facilitate the entry of GST–PAP into phagocytic cells and increase the survival rate of the shrimp after being infected with WSSV.

Keywords: Alpha-2-macroglobulin; Phagocytosis activating protein; *Penaeus monodon*; Real-time PCR; Ribosomal protein L26; White spot syndrome virus; Yeast two-hybrid

1. Introduction

Outbreaks of infectious diseases are causing significant economic losses for the shrimp farming industry, particularly that caused by white spot syndrome virus (WSSV) in *Penaeus monodon*. It is well known that crustaceans possess innate immune response systems including cellular and humoral mechanisms, that will rapidly and efficiently recognize and destroy non-self materials (Lee and SÖderhäll, 2002). Therefore, the development of basic knowledge of shrimp immunity is necessary to help establish strategies for prophylaxis and control of shrimp diseases in aquaculture (Bachere, 2000).

In our previous study, we isolated a phagocytosis activating protein (PAP) gene (GenBank accession no. AY680836) from the haemolymph of a WSSV-infected *P. monodon* (Deachamag et al., 2006). This gene is highly homologous to the ribosomal protein L26 (RPL26) from *Penaeus japonicus* (98.6%) (Watanabe, 1998) and similar to RPL26 from *Mus musculus* (63.1%). Although this gene is highly homologous to RPL26, it was named after its phagocytic activation activity in shrimp (Deachamag et al., 2006). The percentage of phagocytosis of haemocytes increased when the haemocytes were incubated with the glutathione-*S*-transferase–PAP (GST–PAP). Furthermore, an intramuscular injection of formalin-inactivated

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WSSV (white spot syndrome virus) produced a significantly increased expression (3-fold, $p<0.05$) of the PAP gene in *P. monodon* 1 week post-injection (Deachamag et al., 2006). In addition, the RPL26 gene was activated in a mouse macrophage cell line when treated with silica, LPS and IFNγ (Segade et al., 1996). Generally, RPL26 is located at the ribosomal subunit interface of the 60S subunit inside the cell (Villrreal and Lee, 1998) and our previous study found that the phagocytic activity of haemocytes increased after incubation with GST–PAP (glutathione-*S*-transferase–PAP) (Deachamag et al., 2006). However, the pathway of the GST–PAP that was used to activate phagocytic cells of the shrimp, has not been clearly characterized.

In present study, we have identified alpha-2-macroglobulin (α2M) as a PAP binding protein in the haemolymph of *P. monodon* by using the yeast two-hybrid screening assay. A correlation between the expression of the PAP and α2M in the WSSV-infected shrimp was determined by using the Quantitative Real-time Polymerase Chain reaction (Q-PCR). Furthermore, we have shown that PAP protects the shrimp from WSSV infection after intramuscular injection of the GST–PAP into the shrimp before being challenged with WSSV.

2. Materials and methods

2.1. Yeast two-hybrid screening

2.1.1. Plasmid construction

The PAP gene encoding a 144 amino acid protein (GenBank accession no. AY680836) was amplified by PCR from a PAP-pGEX-4T-1 plasmid produced in our previous study (Deachamag et al., 2006). The forward primer was flanked by a *Bam*HI site (5′-GGG ATC CGG ATG AAG ATC A-3′) and the reverse primer flanked by a *Sal*I site (5′-CCG TCG ACT TAA GAT GAG GTG-3′). PCR was performed in a final volume of 50 μl containing 200 ng DNA templates, 0.4 μM each of primer, 0.2 mM each of dNTP, 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% TritonX-100 and 2.5 U Taq DNA polymerase. Thirty cycles of PCR were carried out with 2 min of denaturation at 94 °C, 1 min of annealing at 50 °C followed by 1 min of extension and terminated by 10 min of incubation at 72 °C. All PCR experiments were performed using a thermocycler (Hybaid Limited, USA). The PCR product of 452 bp was subcloned in frame at the *Bam*HI site and *Sal*I sites into the pGBKT7 (CLONTECH), a bait vector that encodes the Gal4 DNA binding domain and the plasmid was named as PAP-BD. Plasmid DNA was prepared using the QIAprep Spin Miniprep Kit (QIAGEN).

2.1.2. Protein interaction screening

The yeast two-hybrid screening was carried out with the MATCHMAKER Gal4 Two-Hybrid System3 (CLONTECH). The bait PAP-BD (PAP-GAL4 DNA binding domain) was used to screen 10^5 independent recombinant clones of a haemocyte cDNA library from black tiger shrimp, obtained from a previous work in *Saccharomyces cerevisiae* strain AH109 (Tonganunt et al., 2005). The cDNA is expressed as a fusion to the GAL4 activation domain (AD). When bait and library fusion proteins interact in *S. cerevisiae* (AH109), the PAP-BD and AD-PAP binding proteins are brought into proximity, thus activating transcription of four reporter genes [*ADE2*, *HIS3*, *MEL1* (encoding α-galactosidase) and *lacZ* (encoding α-galactosidase) while transformant markers for AH109 containing BD and AD plasmids are *trp1* and *leu2*, respectively. Positive blue clones were selected for growth on a synthetic droupout (SD) plate lacking adenine, histidine, leucine and tryptophan (SD/−ade/−his/−leu/−trp) containing 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-Gal). Plasmid DNA isolated from those clones that activated all four yeast reporter genes [*ADE2*, *HIS3*, *MEL1* (encoding α-galactosidase) and *lacZ* (encoding β-galactosidase) was transformed into *E. coli* Top10F′ to recover the plasmid and nucleic acid sequencing was carried out. Searching for gene database sequences was performed through the National Center for Biotechnology Information (NCBI) using BlastX. To confirm the screening result, β-galactosidase activity was activated when PAP-BD interacted with AD-PAP binding protein. Therefore both of the PAP-BD and AD-PAP binding protein plasmids were retransformed into a host strain and plated on SD/−ade/−his/−leu/−trp. The transformants were tested for β-galactosidase activity by filter lift assay according to the manufacturer's instructions. Briefly, a single colony of the transformant was streaked on (SD/−ade/−his/−leu/−trp) plate and incubated at 30 °C, overnight. A sterile dry nitrocellulose filter was placed over the surface of the colonies to be assayed. The filter was lift and frozen in liquid nitrogen for 10 s. After thawing, the filter with colonies side up was placed on a presoaked filter with X-Gal solution to allow for the appearance of blue colonies.

2.2. In vitro pull-down assay

2.2.1. Expression of recombinant 6xHis-α2M

As the clone identified by the yeast two-hybrid screening had homologies with α2M (GenBank accession no. AY826818) encoding 181 amino acid residues of the C-terminal. Therefore the α2M-pQE40 was