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Plasmolipin, *Pm*PLP1, from *Penaeus monodon* is a potential receptor for yellow head virus infection

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ABSTRACT

Plasmolipin has been characterized as a cell entry receptor for mouse endogenous retrovirus. In black tiger shrimp, two isoforms of plasmolipin genes, *Pm*PLP1 and *Pm*PLP2, have been identified from the *Penaeus monodon* EST database. The *PmPLP1* is highly up-regulated in yellow head virus (YHV)-infected shrimp. Herein, the function of *Pm*PLP1 is shown to be involved in YHV infection. The immunoblotting and immunolocalization showed that the *Pm*PLP1 protein was highly expressed and located at the plasma membrane of gills from YHV-infected shrimp. Moreover, the *Pm*PLP1 expressed in the Sf9 insect cells resided at the cell membrane rendering the cells more susceptible to YHV infection. Using the ELISA binding and mortality assays, the synthetic external loop of *Pm*PLP1 was shown to bind the purified YHV and neutralize the virus resulting in the decrease in YHV infection. Our results suggested that the *Pm*PLP1 was likely a receptor of YHV in shrimp.

1. Introduction

Yellow head virus (YHV) first emerged in the farmed black tiger shrimp (*P. monodon*) in Thailand in 1990 and become the major viral pathogen causing high and rapid mortality of both white shrimp (*Litopenaeus vanamei*) and black tiger shrimp (Chaivisuthangkura, 2014; Lightner and Redman, 1998). The entire crop is typically lost within a few days after the appearance of the gross signs of this disease (Chantanachookin et al., 1993). The YHV is a retrovirus containing a single-stranded RNA genome of about 27 kb in a rod-shaped envelope approximately 55 ± 5 nm in diameter and 195 ± 5 nm long (Sittidilokratna et al., 2009; Vatanavicharn et al., 2012). Not only black tiger shrimp can be infected by YHV, but other penaeid and palemonid shrimp species are also susceptible to YHV infection (Walker and Winton, 2010).

Basically, invasion of viruses into the host cell utilizes the membrane-bound proteins at the cell surface as receptors, e.g. integrin for white spot syndrome virus (WSSV) (Li et al., 2007), laminin receptor (Lamr) for WSSV (Liu et al., 2016), Taura syndrome virus (TSV) and YHV (Busayarat et al., 2011) and *Pm*YRP65 and *Pm*Rab11 in *Penaeus monodon* for YHV (Assavalapsakul et al., 2014; Kongprajug et al., 2017). In addition, the viral responsive genes in the shrimp have been shown to be involved in viral infection including the hemocyte homeostasis associated protein (*Pm*HHAP) (Prapavorarat et al., 2010), viral responsive gene (*Pm*VRP15) (Vatanavicharn et al., 2014), anti-lipopolysaccharide factor (ALF*Pm*) (Ponprateep et al., 2012) and plasmolipin (*Pm*PLP) (Vatanavicharn et al., 2012).

Plasmolipin (PLP) is a proteolipid protein and a member of the expanding group of tetraspan (4TM) myelin proteins. The plasmolipin functions as a voltage-dependent potassium ion channel that is mainly expressed in the nervous system of the brain and the apical surface of kidney tubular cells (Fischer and Sapirstein, 1994). Moreover, plasmolipin has been identified as the cell entry receptor for *Mus caroli* endogenous retrovirus (McERV) (Miller et al., 2008). In *Penaeus monodon*, two isoforms of plasmolipin (*Pm*PLP1 and *Pm*PLP2) are identified. The *Pm*PLP1 transcript levels is highly up-regulated in hemocytes after YHV infection. In contrast, *Pm*PLP2 was only slightly up-regulated with YHV (Vatanavicharn et al., 2012). The *Pm*PLP1 is

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expressed mainly in gills which is a primary target organ for YHV infection (Lu et al., 1995). Thus, the *Pm*PLP1 potentially can be a part of viral infection mechanism or defense response. In this research, we demonstrate the location of *Pm*PLP1 in the gill cell membrane, the neutralization of YHV with the synthetic extracellular loop of *Pm*PLP1 and the possible role of *Pm*PLP1 as a YHV receptor in black tiger shrimp.

2. Materials and methods

2.1. Shrimp and virus

The black tiger shrimp, *Penaeus monodon*, of 3–5 g in weight were obtained from the Shrimp Genetic Improvement Center, Thailand. The shrimp were acclimatized at an ambient water temperature of 28–30 °C and a salinity of 20 ppt for a few days before experiments. The shrimp were used for the detection of *Pm*PLP1 in shrimp gill, immunolocalization and viral infection experiments.

The yellow head virus (YHV) was isolated from infected black shrimp gills by using a method described by Xie et al. (2005) with modification. Briefly, gills were homogenized in TNE buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl and 0.1 mM EDTA) containing 1 mM phenylmethane sulfonyl fluoride (PMSF) and centrifuged at 3000 × g to collect the supernatant and then filtered through a 0.45 µm Millipore membrane (Merck Millipore, Germany). The filtrate was centrifuged at 16,000 × g at 4 °C for 30 min. The pellet was rinsed with TM buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$) and resuspended in TM buffer, while the supernatant was re-centrifuged at 16,000 × g at 4 °C. for 30 min. The pellet was resuspended in TM buffer, divided into aliquots and stored at −80 °C until use.

2.2. Antibody production

The extra cellular loop (EL) peptide of *Pm*PLP1, amino acid position of 127–140 residue, containing 14 amino acids of SSVTRPRPPSRLKP was synthesized and used for the production of rabbit polyclonal antibodies by commercial service (Bio-Synthesis, USA).

2.3. Protein expression level of PmPLP1 in normal and YHV-infected shrimp

The protein level of *Pm*PLP1 in the gills of YHV-infected shrimp was investigated using rabbit polyclonal antibody against the extra cellular loop peptide of *Pm*PLP1 (EL*Pm*PLP1). To control all shrimp in the same amount of YHV, intramuscular injection was performed. Two groups of five shrimp were injected with 100 µL of 0.85% NaCl (treatment 1) and YHV (treatment 2). The amount of YHV used (3×10^3 copies per shrimp) caused 100% shrimp death in 4 days. After 48 h post injection, shrimp gills were collected for the extraction of total protein. The samples were washed three times with 0.85% NaCl and homogenized in cold homogenization buffer (10 mM Tris-HCl pH 7.2, 250 mM sucrose and 1 mM EDTA). The homogenates were centrifuged at 500 × g at 4 °C for 10 min. The supernatant tissue lysates were collected and determined their protein contents using Bio-Rad protein assay (Bio-Rad, USA).

The protein level of *Pm*PLP1 was analyzed using Western blot analysis. The tissue lysate of 50 µg from each group was subjected to 12% SDS-PAGE and the protein bands were transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with 5% skim milk in PBS buffer for 30 min and washed with washing buffer (PBS pH 7.4, 0.1% Tween20). The membrane was incubated with rabbit anti-EL*Pm*PLP1 or mouse anti-β-actin (Merck Millipore, Germany) in 1% skim milk in washing buffer at 4 °C for an overnight. The membrane was washed six times with washing buffer and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG monoclonal antibodies (Merck Millipore, Germany). The target protein was detected by chemiluminescence using Clarity Western ECL substrate (Bio-Rad, USA).

2.4. Immunolocalization of PmPLP1 in normal and YHV-infected gill

The shrimp gills from healthy shrimp and 24 h-YHV-infected shrimp were collected and fixed in 10% formaldehyde neutral buffer (10 mL of 37% formaldehyde solution, 0.8 g NaCl, 0.4 g NaH_2PO_4, 0.65 g Na_2HPO_4 and distilled water to 100 mL). The dehydrated gills were embedded in Paraplast, cut into 6 µm sections and mounted on poly-L-lysine coated slides. The slides were stored at 4 °C until use.

To detect the *Pm*PLP1, the slides were deparaffinized and rehydrated. Briefly, the slides were dipped into two consecutive jars of xylene for 2 min each. The slides were rehydrated with decreasing concentrations (100, 95, 70, 50%) of ethanol solutions for 5 min each and rinsed with cold tap water to remove traces of ethanol. The slides were blocked with blocking buffer (1% fetal bovine serum in PBS pH 7.4) at room temperature for 1 h and probed with the rabbit anti-EL*Pm*PLP1 at 4 °C for an overnight. The slides were washed three times with washing buffer (PBS pH 7.4, 0.1% Tween20) and, subsequently, incubated with the secondary goat anti-rabbit antibody conjugated with fluorescence dye, Alexa Fluor 488 (Invitrogen, USA) at room temperature for 1 h. After incubation, the slides were washed three times with washing buffer, incubated with PureBlu Hoechst 33342 nuclear staining dye (Bio-Rad, USA) to stain the nuclear DNA at room temperature for 15 min. The slides were washed with PBS and mounted in ProLong Gold antifade (Invitrogen, USA). The samples were examined under a confocal laser scanning microscope (Olympus, Japan). Bright field and fluorescence images were collected for the analyses.

2.5. Binding assay of ELPmPLP1 to YHV using ELISA technique

The Costar Clear 96-well plate (Corning) were coated with 100 µL PBS or 1 µg/mL purified YHV solution at 37 °C for 1 h and the solutions were discarded. The coated plates were blocked with 150 µL of 5% bovine serum albumin (BSA) in PBS at 4 °C for an overnight. The plates were incubated with EL*Pm*PLP1 or PBS at 37 °C for 1 h and washed three times with washing buffer (PBS buffer containing 0.05% Tween20), incubated with primary anti-EL*Pm*PLP1 antibody diluted 1:1000 in 1% BSA at 37 °C for 1 h and washed three times with washing buffer. The plates were incubated with secondary anti-rabbit conjugated AP antibody diluted 1:3000 in 1% BSA at 37 °C for 1 h and washed three times with washing buffer. The color was developed with *p*-nitrophenylphosphate (Bio-Rad, USA) and monitored A_{405} for 30 min by FLUOstar OPTIMA microtiter plate reader (BMG LABTECH, Germany).

2.6. Effect of ELPmPLP1 on the replication of YHV

To study the effect of EL*Pm*PLP1 on YHV infection using real-time RT-PCR, the shrimp were divided into three groups of 5 individuals each. The first control group was intramuscularly injected with 50 µL of 0.85% NaCl. The second and third groups were injected with 50 µL of YHV pre-incubated with 4.2 µg of EL*Pm*PLP1 or BSA, respectively. The amount of YHV used (3×10^3 copies per shrimp) caused 100% shrimp death in 4 days. The gills from individual shrimp were collected for total RNA extraction at 24 h post-infection using TRI Reagent (Molecular Research Center) following the manufacturer protocol followed by the first-strand cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA).

Quantitative real-time PCR using the fluorescent dye SYBR Green (Luna Universal qPCR Master Mix: NEB, USA) was performed to determine the replication of YHV viral gene in YHV-infected gill. The specific primers of *GP64* gene and *EF1-α* gene were used for the amplification (Table 1). The real-time PCR condition was 40 cycles of 95 °C 15 s, 60 °C for 30 s. Each sample group was from 5 individual shrimp-