Developmental and Comparative Immunology 44 (2014) 100–110

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

The essential role of clathrin-mediated endocytosis in yellow head virus propagation in the black tiger shrimp *Penaeus monodon*

Thapanan Jatuyosporn [a], Premruethai Supungul [a,b], Anchalee Tassanakajon [a], Kuakarun Krusong [a,*]

[a] Center of Excellence for Molecular Biology and Genomics of Shrimp, Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
[b] National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Pathumthani 12120, Thailand

ARTICLE INFO

Article history:
Received 19 August 2013
Revised 25 November 2013
Accepted 26 November 2013
Available online 9 December 2013

Keywords:
Clathrin coat assembly protein 17
Clathrin-mediated endocytosis
Penaeus monodon
Yellow head virus
YHV

ABSTRACT

Yellow head virus (YHV) is one of the most widespread viruses seriously affecting black tiger shrimp (*Penaeus monodon*) cultivation. A previous microarray study demonstrated that clathrin coat assembly protein 17 (AP17) was significantly up-regulated after YHV infection (Pongsomboon et al., 2011). Clathrin coat AP17 is a part of the assembly protein σ2 (AP-2) complex which is involved in clathrin-mediated endocytosis. Quantitative RT-PCR (qRT-PCR) revealed that the clathrin coat AP17 gene was up-regulated 3-fold at 12 h post YHV infection. In addition, immunofluorescence microscopy showed that clathrin coat AP17 was highly expressed in the cytoplasm of the YHV-infected hemocytes. Knockdown of the clathrin coat AP17 gene dramatically reduced YHV replicativity by 32-fold. Interestingly, shrimp pre-treated with chlorpromazine, a commercial drug that inhibits clathrin-dependent endocytosis, exhibited significantly low levels of YHV infection. Taken together, these results suggest that clathrin-mediated endocytosis is involved in YHV propagation in *P. monodon*.

1. Introduction

Yellow head disease was first observed in the black tiger shrimp *Penaeus monodon* in central Thailand in 1990 (Limsuwan, 1991) and since then, the disease has been commonly found throughout shrimp farming areas in Thailand (Flegel, 1997). Clinical signs of yellow head disease include a pale body appearance and a yellowish discoloration of the cephalothorax. The YHV type-1 reported from Thailand causes severe mortality of *P. monodon* while the second type (YHV-2), named as gill-associated virus (GAV), and other geographical types are considered to be less- or non-virulent (Wijegoonawardane et al., 2008). Age and shrimp species also affect the severity of disease symptoms (Spann et al., 2000, 2003). Juvenile to sub-adult shrimp are susceptible to YHV disease. Although *P. monodon* is the major natural host of YHV, the virus can also infect other shrimp species e.g. *Euphasia superba*, *Palaemon setiferus* (Flegel, 1997), *P. merguiensis*, *Metapenaeus ensis* (Chantanachookin et al., 1993), *P. vannamei*, *P. stylrostris* (Lightner et al., 1998; Lu et al., 1994, 1997), *P. setiferus*, *P. aztecus* and *P. duorarum* (Lightner et al., 1998).

YHV is a rod-shaped, single-stranded, positive-sense RNA virus with a spiked enveloped. The YHV genome is approximately 27 kb and the virus belongs to a new family *Roniviridae*, genus *Okavirus* (Walker et al., 2005; Wongteerasupaya et al., 1995). After YHV infection, a widespread necrosis is observed in the lymphoid organ, gills, connective tissues, hemocytes and hematopoietic organs of shrimp (Wang et al., 1996). Histopathological and YHV receptor studies suggest that the lymphoid organ is probably the primary target of YHV (Assavalapsakul et al., 2006; Lu et al., 1995). Three YHV structural proteins, including two-envelope glycoproteins gp116 and gp64 and a nucleocapsid protein p20, have been studied (Assavalapsakul et al., 2005; Jitrapakdee et al., 2003; Sittidilokratna et al., 2008).

A previous microarray study demonstrated that clathrin coat assembly protein 17 (AP17) was up-regulated (>4-fold) after YHV infection (Pongsomboon et al., 2011). Clathrin coat AP17 is a σ2 subunit of the assembly protein 2 (AP-2) complex, which is involved in clathrin-mediated endocytosis. In general, clathrin-mediated endocytosis usually occurs at specialized sites, where viruses bind to specific receptors, resulting in the assembly of clathrin and the formation of coated pits on the plasma membrane. The coated pits then invaginate and pinch off to form intracellular clathrin-coated vesicles. Depolymerization of these vesicles results in formation of early endosomes, which later form late endosomes that further fuse with lysosomes where the contents are ultimately degraded. Viruses somehow escape from the endosome to the cytosol via either membrane fusion or membrane disruption mechanisms (Mousavi et al., 2004). Several DNA viruses (e.g. African swine fever

Abbreviations: AP17, assembly protein 17; *Pm*, *Penaeus monodon*; YHV, yellow head virus.

* Corresponding author. Tel.: +66 (0)2 218 5413.
E-mail address: Kuakarun.K@chula.ac.th (K. Krusong).

0145-305X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.dci.2013.11.017

virus and Vaccinia virus) and RNA viruses (e.g. Ebolavirus, Hepatitis C virus and Influenza A virus) use clathrin-mediated endocytosis as an entry pathway (Bartosch and Cosset, 2006; Bernardes et al., 1998; Chou, 2007; Dimitrov, 2004; Husain and Moss, 2005; Simmons et al., 2003a,b; Suzuki et al., 2005).

Assembly of clathrin to form coated pits requires the heterotetrameric protein AP-2. AP-2 contains four subunits α, β2, μ2 and σ2. The α subunit is involved in targeting AP-2 to the plasma membrane while the β2 subunit is important for the interaction of AP-2 with clathrin. The β2 subunit may also function in the selection of specific cargo (Rapoport et al., 1998). The μ2 subunit contains a phosphoinositide-binding site and is involved in the recognition and sorting of protein cargo (Aguilar et al., 1997). The precise functions of the small σ2 subunit are not clear.

In this study, we aim to investigate the role of *Pm*-clathrin coat AP17, a σ2 subunit of AP2, in YHV infection in *P. monodon*. Quantitative Real-time RT-PCR was performed to confirm the previous microarray result. The *Pm*-clathrin coat AP17 gene was cloned, expressed and purified in order to produce an anti-Pm-clathrin coat AP17 antibody. Immunofluorescence confocal microscopy was used to examine the localization of *Pm*-clathrin coat AP17 in unchallenged- and YHV-challenged shrimp hemocytes. RNAi-mediated gene knockdown was carried out in order to investigate the role of clathrin coat AP17 in YHV infection. In addition, chlorpromazine, a commercial drug that inhibits clathrin-dependent endocytosis, was used in this study.

2. Materials and methods

2.1. Shrimp

Healthy black tiger shrimp, *P. monodon*, of approximately 10–15 g body weight were used in tissue distribution of *Pm*-clathrin coat AP17 transcripts, analysis of *Pm*-clathrin coat AP17 expression levels and localization of *Pm*-clathrin coat AP17 experiments. RNAi-mediated gene knockdown and chlorpromazine pre-treatment experiments were performed using shrimp of about 3 g in weight. Shrimp were acclimatized in artificially aerated water (20 ppt salinity) for at least 7 days before experimental use.

2.2. Preparation of YHV stock

Gills from YHV-infected moribund shrimp were collected and homogenized in cool TNE buffer (50 mM Tris–HCl, pH 7.4, 100 mM NaCl and 0.1 mM EDTA). The pellet was removed by centrifugation at 5,000x*g* for 5 min at 4 °C. The clear solution was filtrated with MILLEX®-HP Fillter Unit 0.45 μm and centrifuged at 30,000x*g* for 30 min at 4 °C. The pellet was washed twice with 1 mL of TM buffer (50 mM Tris–HCl, pH 7.5 and 10 mM $MgSO_4$) and centrifuged at 5,000x*g* for 5 min at 4 °C. The pellet was resuspended in TM buffer, aliquoted and kept at −80 °C until use.

2.3. Sequence alignment and phylogenetic analysis of clathrin coat AP17

The clathrin coat AP17 cDNA sequence was obtained from the *P. monodon* EST database (http://pmonodon.biotec.or.th). The sequence was then analyzed to gain information on protein domain (PROSITE program, http://prosite.expasy.org), signal peptide (SignalP 4.1 sever, http://www.cbs.dtu.dk/services/SignalP), molecular weight and isoelectric point (Compute pI/Mw, http://web.expasy.org/compute_pi). In addition, the basic local alignment search tool (BlastX, http://blast.ncbi.nlm.nih.gov/), multiple alignment (ClustalW2, http://www.ebi.ac.uk/Tools/msa/clustalw2/), Phylip, SeqBoot and BootStrap tools were used to construct the phylogetic tree of clathrin coat AP17.

2.4. Analysis of clathrin coat AP17 gene expression in shrimp

Shrimp tissues, such as antennal gland, epipodite, eyestalk, gill, heart, hemocyte, hepatopancreas, intestine, lymphoid organ and stomach, were collected from 3 normal shrimp and subjected to total RNA extraction by TRI reagent® (Molecular Research Center). After DNase treatment, the total RNA was used to synthesize cDNA by the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). The clathrin coat AP17 gene expression level in each tissue was identified by RT-PCR using 1 μl of cDNA template with clathrin coat AP17 (RT-PCR) primers (Table 1). Elongation factor 1 α (EF1α) gene was used as an internal control and amplified by EF1α (RT-PCR) primers (Table 1). The PCR reaction was started with 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 10 min. The PCR products were then analyzed by 2% (w/v) agarose gel electrophoresis.

2.5. Clathrin coat AP17 mRNA expression in unchallenged and YHV challenged shrimp hemocytes

Shrimp, of about 15 g body weight, were challenged by YHV at a dose that gave 100% mortality within 3 days. Control shrimp were injected with 150 mM NaCl. Shrimp hemocytes were collected from challenged- and unchallenged-shrimp (3 shrimp per group) at 6, 12, 24 and 36 h post-injection using 100 μl of anticoagulant, 10% sodium citrate, per 1 mL of hemolymph. Hemocytes were pelleted by centrifugation at 800x*g* for 10 min at 4 °C and used for total RNA extraction by FavorPrep™ Tissue Total RNA Mini Kit (Favorgen). cDNA synthesis was performed as previously described. Real-time RT-PCR was carried out using an equal amount of cDNAs in iCycler iQTM Real-Time detection system and the SsoFast™ EvaGreen® Supermix (Bio-Rad). The qRT-PCR condition was 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 55 °C for 10 s. The experiment was carried out in triplicates. Clathrin coat AP17 and EF1α (real-time PCR) primers were used (Table 1). Comparative C_T method was used to compare the gene expression in two different samples, in this case challenged-(sample A) and unchallenged (sample B) shrimp (Livak and Schmittgen, 2001). The fold change of gene expression was calculated using following formula

$$\text{Fold change} = 2^{-\Delta\Delta CT} \quad (1)$$

$$\begin{aligned}\Delta\Delta C_T = [&(C_T \text{ gene of interest} \\ &- C_T \text{ internal control}) \text{ sample A} \\ &- (C_T \text{ gene of interest} \\ &- C_T \text{ internal control}) \text{ sample B}]\end{aligned} \quad (2)$$

Statistical analysis was done using the one-way ANOVA followed by post hoc test (Duncan's new multiple range test). The result differences were considered significant at $P < 0.05$.

2.6. Production of recombinant Pm-clathrin coat AP17 and anti-Pm-clathrin coat AP17 Polyclonal antibody

Pm-clathrin coat AP17 gene was amplified from EST clone library of normal *P. monodon*, using PCR condition as followed: 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 10 min. The specific primers for amplification of *Pm*-clathrin coat AP17 were designed to contain 5′ *Nco* I and 3′ *Xho* I restriction sites (Table 1). The amplified full-length *Pm*-clathrin coat AP17 gene fragment