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White spot syndrome virus enters crayfish hematopoietic tissue cells via clathrin-mediated endocytosis

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ABSTRACT

White spot syndrome virus (WSSV) is a major pathogen of aquacultured shrimp. However, the mechanism of its entry remains poorly understood. In this study, by analyzing the internalization of WSSV using crayfish hematopoietic tissue (HPT) cells, we showed that WSSV virions were engulfed by cell membrane invaginations sharing the features of clathrin-coated pits and then internalized into coated cytoplasmic vesicles. Further investigation indicated that WSSV internalization was significantly inhibited by chlorpromazine (CPZ) but not genistein. The internalized virions were colocalized with endogenous clathrin as well as transferrin which undergoes clathrin-dependent uptake. Preventing endosome acidification by ammonium chloride (NH_4Cl) or chloroquine (CQ) dramatically reduced WSSV entry as well. Moreover, disturbance of dynamin activity or depletion of membrane cholesterol also blocked WSSV uptake. These data indicate that WSSV enters crayfish HPT cells via clathrin-mediated endocytosis in a pH-dependent manner, and membrane cholesterol as well as dynamin is critical for efficient viral entry.

Introduction

Cell entry is the initial step of viral infection, which normally includes cell binding, penetration and viral genome delivery. Although some enveloped viruses can directly penetrate into the cytosol by fusing with the plasma membrane of host cells, most viruses enter cells via endocytosis which assists them to cross membrane barriers and deliver their genomes to the replication sites in the cytosol or nucleus (Mercer et al., 2010). Viruses have been reported to utilize various host endocytosis pathways including clathrin-mediated endocytosis (Hernaez and Alonso, 2010; Mendez et al., 2014), caveolea-dependent endocytosis (Engel et al., 2011; Smith et al., 2007), macropinocytosis (Mercer and Helenius, 2008; Amstutz et al., 2008) and less well-characterized clathrin- and caveolea-independent endocytosis (Quirin et al., 2008; Schelhaas et al., 2012).

Clathrin-mediated endocytosis is evolutionarily conserved among eukaryotes, including animals, plants and yeast (Chen et al., 2011; Weinberg and Drubin, 2012; McMahon and Boucrot, 2011). It is often hijacked by viruses to gain entry into host cells. Binding of viral particles to specific receptors on cell surface can trigger accumulation of key components of clathrin-mediated endocytosis including clathrin, AP2 and dymamin, leading to clathrin coat assembly, membrane curvature and cargo internalization (Mercer et al., 2010; McMahon and Boucrot, 2011). The examples of viruses that take advantage of clathrin-mediated endocytosis include astrovirus (Mendez et al., 2014), African swine fever virus (Hernaez and Alonso, 2010), vesicular stomatitis virus (Sun et al., 2005), etc. Viruses taken up via this method are subsequently delivered into early endosome within minutes and undergo endosome maturation. The acidification during this maturation process is essential for uncoating/penetration of a number of viruses (Hernaez and Alonso, 2010; Mendez et al., 2014).

Another well-characterized pathway is caveolae-dependent endocytosis, in which caveolin, dynamin and tyrosine kinases are involved (Lajoie and Nabi, 2010). Under physiological conditions, caveolaes bud from plasma membrane transport their viral cargos to early endosomes and eventually to endoplasmic reticulum (Mercer et al., 2010; Engel et al., 2011). Caveolae-dependent endocytosis was first observed in SV40 (Kartenbeck et al., 1989; Pelkmans et al., 2001). Since then, some viruses that belong to the polyomavirus family have been shown to enter cells via caveolae-dependent endocytic pathway (Gilbert and Benjamin, 2004; Richterova et al., 2001).

White spot syndrome virus (WSSV), the only member of the *Whispovirus* genus of the Nimaviridae family, is an enveloped, rod-shaped virus with a double-stranded circular DNA of approximately

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300 kb (Yang et al., 2001; van Hulten et al., 2001). As a causative agent, WSSV leads to high mortality in shrimp aquaculture. Although major progresses have been made in the past two decades to understand the genome, proteome of WSSV and the function of viral genes (Leu et al., 2009), the mechanism of WSSV entry is not precisely illustrated. There are two recent reports suggested that WSSV enters hemocytes of pacific white shrimp *Litopenaeus vannamei* and redclaw crayfish *Cherax quadricarinatus* via caveolae-mediated endocytosis based on the observations that viral internalization was inhibited by methyl-β-cyclodextrin (M-β-CD) (Duan et al., 2014; Huang et al., 2013). However, we've found that crayfish hemocytes do not support WSSV replication. Internalization of virions into hemocytes of red swamp crayfish *Procambarus clarkii* or *C. quadricarinatus* does not result in production of progeny virus (Wu et al., 2015). In addition, it is so far unclear whether WSSV uptake by penaeid shrimp hemocytes results in viral replication. Hence, how WSSV enters host cells to start a productive infection remains to be explored.

In our previous work, we found that internalization of WSSV into crayfish hematopoietic tissue (HPT) cells results in mass production of viral progeny in the nucleus (Wu et al., 2015). Mature virions are released at the final stage of infection by disrupting the host cells (Leu et al., 2009 and unpublished data). Therefore, in the present study, we used primary cultured HPT cells to investigate WSSV infectious entry by transmission electron microscopy (TEM) and endocytosis inhibition assay.

Results

WSSV enters HPT cells via endocytosis

To investigate the earliest stages of WSSV entry, HPT cells were inoculated with purified WSSV virions for 30 min on ice. Attachment of virus particles along the cell surface was observed by TEM, but internalization did not occur at this moment (Fig. 1A). The cultures were quickly warmed up to 27 °C to allow internalization of the virions. At 20 min after temperature shift, virions were found to be engulfed by electron-dense membrane invagination on the cell surface (Fig. 1B–E) or internalized into tightly-fitting cytoplasmic vesicles with electron-dense membrane (Fig. 1F and G). These data suggest that the internalization process of WSSV in HPT cell might involve clathrin-dependent endocytosis.

Cytotoxicity tests of pharmaceuticals

To further identify the pathway(s) required for WSSV infection, endocytosis inhibitors were used in this study and their effects to viral entry were investigated. We first determined the cytotoxic effects of these drugs to primary cultured crayfish HPT cells. Cells were incubated with 50 μM CPZ, 60 μM CQ, 75 μM NH_4Cl, 20 μM dynasore, 50 μM genistein, 2 mM M-β-CD or 60 μg/ml nystain plus 20 μg/ml progesterone for 6 h. Cell viability was determined using CCK-8 after removal of the drugs. As shown in Fig. 2, the relative survival rates of the cells treated with all these drugs were above 80%, implying that the inhibitors at the indicated concentrations had little cytotoxicity effect on HPT cells within 6 h.

WSSV entry is clathrin-dependent

To explore the potential role of clathrin-mediated endocytosis and caveolae-dependent endocytosis in WSSV infection, we examined the ability of CPZ and genistein to block viral entry.

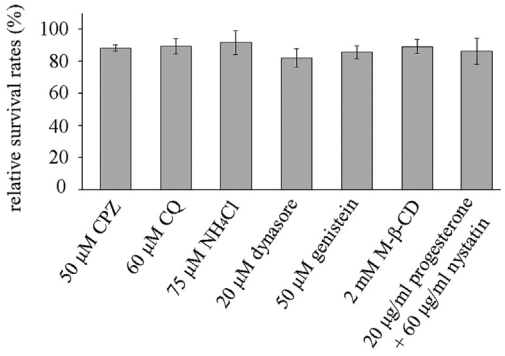

Fig. 2. Cytotoxic effects of endocytosis inhibitors.
HPT cells were treated with each of the inhibitors at indicated concentrations for 6 h at 27 °C. Untreated cells were used as the negative control. Cell viability was measured using CCK-8 after removal of the drugs. The relative cell viability was determined by comparing the absorbance at 450 nm for each treated sample with that of the control. The error bars represented the standard deviation ($n=3$).

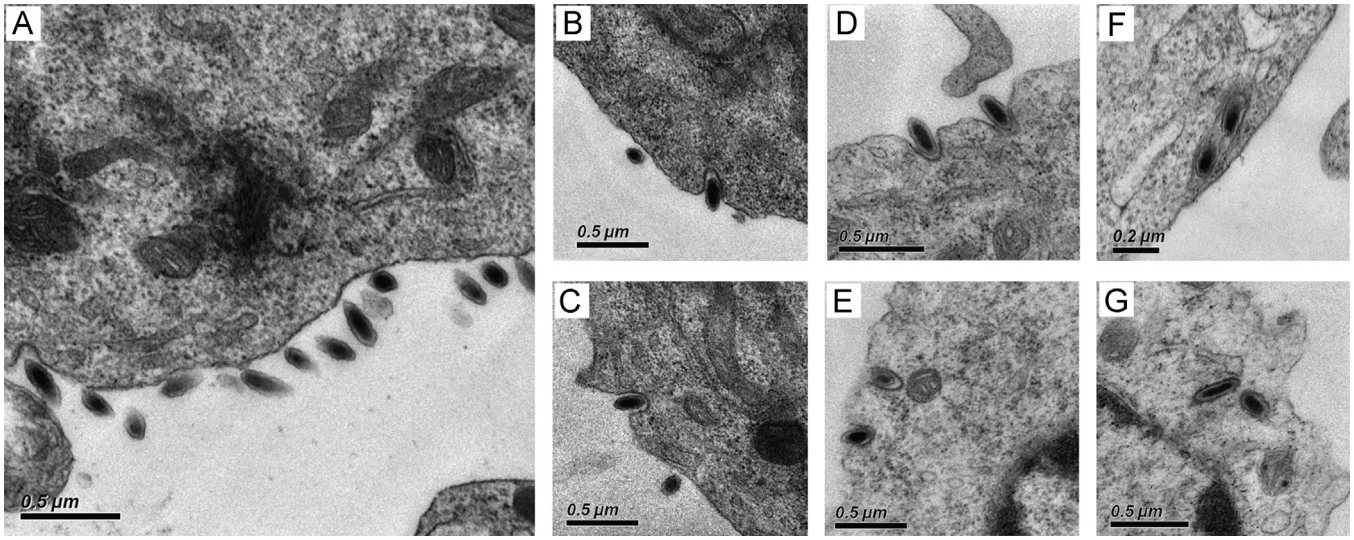

Fig. 1. Ultrastructural analysis of WSSV entry.
After incubation with WSSV at an MOI of 500 on ice, HPT cells were either fixed with 1% glutaraldehyde plus 2% paraformaldehyde (A), or were transferred to 27 °C to initiate viral internalization and fixed 20 min later (B–G). Samples were subjected to ultrathin sectioning. The slices were stained with 2% uranyl acetate plus 0.4% lead citrate, and visualized with a transmission electron microscope. Bars, 0.2 μm or 0.5 μm. The experiment was repeated more than three times.