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Research article

Bombyx mori nucleopolyhedrovirus utilizes a clathrin and dynamin dependent endocytosis entry pathway into BmN cells

Min Feng, Jianjia Zhang, Weifan Xu, Haiping Wang, Xiangshuo Kong, Xiaofeng Wu*

College of Animal Sciences, Zhejiang University, Hangzhou, 310058, China

ABSTRACT

Bombyx mori nucleopolyhedrovirus (BmNPV) is a leading cause of silkworm mortality and economic loss to sericulture. The entry of BmNPV budded virus (BV) into host cells is a fundamental process required for the initiation of infection. However, our understanding of the mechanism of virus entry is limited and it is unclear whether BV enter BmN cells via clathrin-mediated endocytosis. In this study, we found that BV enter BmN cells through a low-pH-dependent endocytosis pathway. Inhibition assays, transmission electron microscopy (TEM) analysis, and small interfering RNAs (siRNAs) knockdown assays revealed that BV entry into BmN cells is mediated by clathrin-dependent endocytosis. Moreover, after treated with dynasore, an inhibitor of dynamin, BmNPV entry was markedly reduced, indicating that dynamin also participates in the efficient internalization of BmNPV. In addition, suppression of Rab5, Rab7 or Rab11 through siRNAs demonstrated that BV requires early and late endosomes for endocytosis in infection of BmN cells. Taken together, BmNPV uses a clathrin- and dynamin-mediated endocytic pathway into BmN cells that requires participation of Rab5 and Rab7 but not Rab11.

1. Introduction

Bombyx mori nucleopolyhedrovirus (BmNPV) belongs to the alphabaculovirus genus of the baculovirus family. It is a major pathogen in sericulture industry, and can cause losses as high as 70% of total sericultural production annually (Dong et al., 2015). BmNPV possesses a large double-stranded circular DNA genome (approximately 128 kb), and is predicted to encode about 136 proteins (Gomi et al., 1999). Two different virion phenotypes with the same genetic material are produced in the virus infection cycle; they include budded virus (BV) and occlusion-derived virus (ODV) (Rohrmann, 2013). These two virion phenotypes differ in their role in virus transmission. ODVs infect the host midgut epithelial cells after ingestion and initiate the primary infection, and are responsible for virus transmission between insect larvae. In contrast, BVs mediate the infection between cells and tissues within individual larvae and cause systemic infection throughout the host. The entry of BV into BmN cells is mediated by the envelope glycoprotein GP64 (Blissard, 1992).

Endocytosis is a common cellular process utilized by many viruses to enter their target cells, and can be divided into clathrin-mediated endocytosis, caveolae, macropinocytosis, and non-clathrin, non-caveolae routes (Sieczkarski and Whittaker, 2002). *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the type species of the alphabaculoviruses, and the mechanism of its entry has been intensively studied. AcMNPV entry into insect and mammalian cells is primarily dependent on clathrin-mediated endocytosis (Long et al., 2006; Volkman and Goldsmith, 1985), as well as direct fusion with the plasma membrane under low pH conditions (Dong et al., 2010). Moreover, endocytosis of AcMNPV into non-host mammalian cells relies on cholesterol in the plasma membrane, dynamin- and caveolae-dependent endocytosis, and micropinocytosis (Kataoka et al., 2012; Long et al., 2006). However, for BmNPV entry into BmN cells, it is still unclear whether it enters host cells via clathrin-mediated endocytosis. Previous studies reported that clathrin is indispensable for the entry of BmNPV into the silkworm cell line NIAS-Bm-oyanagi2 (Fujita et al., 2009). However, another study that utilized Chlorpromazine (CPZ), an inhibitor of clathrin-dependent endocytosis, indicated that BmNPV enters BmN cells through clathrin-independent micropinocytosis (Hu et al., 2017; Huang et al., 2014). Apart from clathrin, many other proteins, including dynamin and Rab proteins are involved in clathrin-mediated endocytosis (Doherty and Mcmahon, 2009).

Rab proteins, which are small GTPases of the Ras superfamily,

Abbreviations: BmNPV, *Bombyx mori* nucleopolyhedrovirus; BV, budded virus; ODV, occlusion-derived virus; AcMNPV, *Autographa californica* multiple nucleopolyhedrovirus; CHC, clathrin heavy chain; hpi, hours post infection; siRNA, small interfering RNA; RFI, relative fluorescence intensity; TEM, transmission electron microscopy; MOI, multiplicity of infection; CPZ, Chlorpromazine

* Corresponding author.
E-mail address: wuxiaofeng@zju.edu.cn (X. Wu).

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regulate cellular transport and vesicle fusion activities in the endocytic pathway (Jordens et al., 2005). Among the Rab GTPase family, Rab5, Rab7 and Rab11 have been revealed as important regulators in virus endocytosis. Rab5 is involved in controlling an early endocytic fusion event (Bucci et al., 1992; Gorvel et al., 1991). Rab7 associates with early endosomes to mediate sorting and transport to late endosomes (Vonderheit and Helenius, 2005), and vesicle recycling to the plasma membrane depends on Rab11 (Sheff et al., 1999). Numerous reports have shown that Rab proteins participate in the life cycle of many viruses such as Japanese encephalitis virus, classical swine fever virus, and bovine ephemeral fever virus (Cheng et al., 2012; Liu et al., 2017; Shi et al., 2016). However, the roles of these three Rab proteins in BmNPV entry into host cells are unclear.

Viruses often use multiple strategies to invade the host cells, and whether alternative pathways exist in the BmNPV entry into BmN cells still needs to be elucidated. In this study, we investigated the infection process of BmNPV and found BmNPV endocytosis is low pH-dependent. In order to determine the functional role of the clathrin-related pathway in the entry process of BmNPV, inhibition assays, siRNA knockdown assays and transmission electron microscopy (TEM) analysis were performed, the results demonstrated that BVs enter BmN cells via clathrin-mediated endocytosis. In addition, a dynamin inhibitor significantly decreased BmNPV reproduction, indicating that dynamin also participates in the internalization of BmNPV. The experiments of siRNA interfering against Rab5 and Rab7 revealed that the early and late endosome compartments are involved in BV entry. Our findings demonstrated that the entry of BmNPV into BmN cells is a low pH dependent process involving both clathrin and dynamin, and regulats by Rab5 and Rab7, but not Rab11.

2. Results

2.1. BmNPV internalizes into BmN cells through an endosomal pathway

The acidic pH of endosomes is thought to play an essential role in triggering fusion events. To determine whether BmNPV internalizes into cells through endosomes, BmN cells were treated with various concentrations of ammonium chloride (NH_4Cl), which could inhibit endosome acidification by raising the pH of intracellular acidic vesicles. When treated with NH_4Cl at concentration up to 50 mM, no obvious effect was exhibited on cell viability by using the MTT assay (Fig. 1A). After NH_4Cl treatment, BmN cells were then infected with recombinant virus v-Bm$^{VP39\text{-}EGFP}$ and flow cytometry was used to examine the cellular fluorescence intensity at 48 hours post-infection (hpi). Compared with untreated cells, relative fluorescence intensity (RFI) in cells treated with NH_4Cl was reduced in a dose-dependent manner (Fig. 1A), indicating that treatment with NH_4Cl significantly inhibited virus reproduction.

In addition, NH_4Cl treatment was performed before BmNPV infection, and the virus yield also appeared to be significantly reduced (Fig. 1B). Accordingly, the expression of EGFP was almost completely blocked under the same drug treatment, with a greater than 90% reductions of fluorescence in the cells. Moreover, a lower infection efficiency, as indicated by fluorescence expression, was detected when NH_4Cl was added an hour after virus infection (Fig. 1B). RFI of EGFP revealed BmNPV infection was blocked by the preceding or following NH_4Cl treatment (Fig. 1C), which indicated pH changes in endosomes not only affected the early step of viral internalization, but also influenced the virus transmission. Thus, it could be concluded that BmNPV internalized into BmN cells via a low-pH-dependent endocytosis pathway.

2.2. BmNPV uses a clathrin-mediated endocytic pathway to enter BmN cells

Clathrin-mediated endocytosis is one of the most widely-used routes of viral entry into host cells. In order to investigate whether clathrin is required for BmNPV entry, we employed chemical inhibition assays using the inhibitors of clathrin-mediated endocytosis, Pitstop2 and Chlorpromazine (CPZ). The former acts via blocking ligand access to the clathrin terminal domain (Von et al., 2011), while CPZ prevents the assembly of clathrin-coated pits at the plasma membrane (Wang and Rothberg, 1993). RFI was significantly reduced by Pitstop 2 and CPZ pretreatment in a dose-dependent manner (Fig. 2**A** and **B**) and this impaired of virus infection was not due to the drug toxicity as nearly all cells (> 90%) were viable after treatment with these two inhibitors (Fig. 2**A** and **B**). Thus, these data suggested that clathrin-mediated endocytosis is involved in BmNPV entry into BmN cells.

As inhibitors of clathrin-mediated endocytosis, Pitstop 2 and CPZ have been applied extensively to mammalian cells, however, there is a lack of evidence for their inhibitory role in insect cells. To provide more specific support for clathrin-mediated endocytosis in BmNPV entry, we then examined the involvement of clathrin in BmNPV entry by siRNA-mediated knockdown. BmN cells were transfected prior to infection with virus v-Bm$^{VP39\text{-}EGFP}$, and siRNAs (siCHC-1, siCHC-2 and siCHC-3) and a mixture of all three (siCHC-Pool) were designed to target the clathrin heavy chain (CHC) coding gene. We found that two siRNAs (siCHC-2 and siCHC-Pool) caused a marked reduction (about 70%) in the expression of *CHC* as judged by qRT-PCR (Fig. 2C). After siRNA transfection, cells were infected with recombinant virus v-Bm$^{VP39\text{-}EGFP}$ for 48 hours, fluorescence microscopy indicated that virus infection was clearly inhibited in clathrin-knockdown cells compared with the control, suggesting that clathrin-mediated endocytosis is required for BmNPV entry (Fig. 2D). Flow cytometry assays also confirmed that BmNPV infection was significantly inhibited after siRNA treatment with over 80% reduction in fluorescence (Fig. 2E), proving that CHC plays a vital role in BmNPV entry.

Binding of BmNPV to BmN cells was further observed using TEM. BmN cells were inoculated with BmNPV (MOI of 200) and incubated at 4 °C for an hour to allow particle cell surface adsorption. Next, the cells were incubated at 27 °C for 0 min, 5 min, 10 min, and 20 min, and then fixed and embedded. Ultra-thin sections were cut and stained with uranyl acetate for observation by TEM. Non-infected cells were used as control (Fig. 3A). The virus particles initially attached to the surface of the cells at 0 min post infection (Fig. 3B), and then most viruses were found in close proximity to or at the base of cellular protrusions resembling membrane ruffles (Fig. 3**C and D**). At 20 min post infection, the electron-dense cell coat around viral particles were formed (Fig. 3E), and were reminiscent of clathrin-coated vesicles. Some viral particles were also observed in larger vesicles in the cytoplasm (Fig. 3F). Taken together, these data presented above demonstrated that BmNPV internalization into BmN cells is dependent on clathrin-mediated endocytosis.

2.3. Dynamin participates in the internalization of BmNPV

Dynamin, a crucial factor in endocytosis, is a member of the GTPase family that participates in membrane fission, and exerts mechanoenzymatic activity in endocytosis (Roux et al., 2006). To determine whether BmNPV entry is also dependent upon dynamin, dynasore, which is an inhibitor of dynamin that targets dynamin-1 and dynamin-2, was used to treat BmN cells. MTT assays showed that dynasore treatment had no toxicity to cells at the concentrations up to 80 μM (Fig. 4A). Cells were infected in the presence of the drug, then removed the supernatant and continued infection for 48 h, the RFI of EGFP showed that dynasore treatment significantly inhibited viral reproduction in a dose-dependent manner (Fig. 4A). Furthermore, fluorescence microscopy demonstrated that virus infection was obviously inhibited in dynasore-treated cells compared with the control (Fig. 4B), indicating that dynamin participates in the internalization of BmNPV.