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Entry of *Bombyx mori* nucleopolyhedrovirus into BmN cells by cholesterol-dependent macropinocytic endocytosis

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ABSTRACT

Bombyx mori nucleopolyhedrovirus (BmNPV) is a serious viral pathogen of silkworm, and no drug or specific protection against BmNPV infection is available at present time. Although functions of most BmNPV genes were depicted in recent years, knowledge on the mechanism of BmNPV entry into insect cells is still limited. Here BmNPV cell entry mechanism is investigated by different endocytic inhibitor application and subcellular analysis. Results indicated that BmNPV enters BmN cells by clathrin-independent macropinocytic endocytosis, which is mediated by cholesterol in a dose-dependent manner, and cholesterol replenishment rescued the BmNPV infection partially.

1. Introduction

BmNPV is an enveloped DNA virus that can produce virions of two phenotypes (BV and ODV) in the life cycle, ODV is responsible for the primary infection of the host insect while BV is responsible for the secondary infection [1]. BmNPV is a serious viral pathogen in sericulture industry, and no effective drug or specific prevention is available to date. Researchers gave major attention to inhibition of the virus replication by over-expression of antiviral exogenous gene [2] or RNA interference [3,4]. However, mechanisms of BmNPV BV entry into the host cell have not been addressed. Virus-cell interactions provide an area that is still incompletely explored and under-exploited for antiviral strategies, virus entry to the cells was a remarkable target for the development of viral protection methods. BmNPV entry mechanism needs to be obtained for future development of methods to block silkworm from BmNPV infection. In addition, BmNPV are increasingly applied as the tools to produce "foreign" protein using silkworm as a bioreactor [5], understanding on entry mechanism will facilitate its application in biomedical medicine research.

In the infection, viruses first bind to cell surface proteins, carbohydrates, and lipids, its interactions lead to the activation of cellular signaling pathways, then cells respond by internalizing the viruses using endocytic mechanisms [6]. Endocytosis is usually used by viruses to enter the host cell [7]. As a close relative of BmNPV, AcMNPV is the best-understood baculovirus, and it serves as a model for basic molecular research and the baculovirus infection cycle. AcMNPV exploits multiple endocytosis pathway according to the host range or cell type, it enters insect cells by clathrin-dependent endocytosis or direct fusion [8,9], whereas entry into mammalian cell is by clathrin-dependent endocytosis, lipid raft, or macropinocytosis pathways [8,10]. AcMNPV GP64 mediates virus absorption and fusion to insect cells [11–13], the CRAC of GP64 is efficiently bound to cholesterol of mammalian cells, which serves as an anchor in the transduction into mammalian cells, whereas this site is unnecessary for AcMNPV entry into Sf9 cells [14]. Although BmNPV genome is over 90% identical to that of AcMNPV [15], the host ranges of these two viruses have essentially no overlapping [16]. Furthermore, AcMNPV GP64 contains three CRAC sites [14], whereas four CRAC sites were found in BmNPV GP64, so we speculated that BmNPV may took a different entry mechanism with that of AcMNPV.

To address this hypothesis, the entry mechanism of BmNPV BV was explored with inhibitors of specific host cell signaling and cytoskeleton rearrangement, the infectivity of virus was checked by fluorescence expression or qPCR, with the depletion and replenishment of membrane cholesterol, we demonstrated that BmNPV BV entered BmN cells by macropinocytosis mechanism, which was mediated by cholesterol on the cell membrane.

Abbreviations: BmNPV, *Bombyx mori* nucleopolyhedrovirus; BV, budded virus; ODV, occlusion-derived virus; AcMNPV, *Autographa californica* MNPV; CRAC, cholesterol recognition amino acid consensus; h p.i, hour post infection; MOI, multiplicity of infection; DMA, N,N-dimethylacetamide; TCID50, 50% tissue culture infective dose; MβCD, methyl-β-cyclodextrin; RTKs, receptor tyrosine kinases.

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2. Materials and methods

2.1. Cells and virus

The BmN cell line was cultured at 27 °C in TC-100 insect medium (Applichem) supplemented with 10% fetal bovine serum (Gibco-BRL) using standard techniques.

BmNPV bacmid (BmBac-GFP) were constructed using pFastDual-hsp70-egfp according to the Bac-to-Bac system manual of Invitrogen [17], the recombinant bacmid was transfected into BmN cells for virus preparation, virus titer was determined by end point dilution assay [18].

2.2. Drugs treatments

Pharmacological inhibitors were purchased from Sigma Company and stock solutions were prepared either in water or in DMSO following the manufacturer's recommendations and used at the indicated concentration. BmN cells (10^5 cells)/dish were pre-seeded in tissue culture dishes for overnight, then the cells were incubated with drugs for the specific times as followed, chlorpromazine, nystatin and progesterone for 30 min; genistein, DMA, rottlerin, and Ehop-016 for 90 min; latrunculin A for 2 h. DMSO (no more than 2%) or PBS was added in the control group. After incubation, drugs were removed and the treated BmN cells were infected with BmBac-GFP at an MOI of 5 TCID50 unit per cell for 1 h without drug incubation. The viruses were then removed and the cells were washed twice with TC100 medium without FBS. The cells were cultured in TC100 medium with 10% FBS. Infectivity was recorded at 6 h p.i. by microscopy, and then the cells were applied to calculate the cells viability.

2.3. Cholesterol depletion

BmN cells were treated with different concentrations of MβCD for 30 min, while treatment with PBS was used as the control. After drug treatment, cells were washed with TC100 medium without FBS twice, and then were infected with BmBac-GFP at an MOI of 5 for 1 h. Unattached viruses were removed and the cells were washed with TC100 medium without FBS twice. The cells were incubated in TC100 with 10% FBS at 27 °C. Infectivity and cells viability were recorded at 6 h p.i. by microscopy.

2.4. Cholesterol replenishment

BmN cells were treated with or without 10 mM MβCD for 30 min. Treatment with PBS was used as the control. The treated cells were washed with TC100 medium without FBS twice, and then an MβCD treatment was replenished with cholesterol (water-soluble cholesterol, Sigma product, final concentration 100 μg/ml). The cells were then infected with BmBac-GFP at an MOI of 5. Following infection, the virus was removed and the infected cells were washed with TC100 medium without FBS twice. The cells were incubated in TC100 with 10% FBS at 27 °C. Fluorescence was detected at 6 h p.i. by microscopy. Treated samples were collected for qPCR analysis.

2.5. qPCR

RNA of samples from cholesterol replenishment was extracted with Trizol (Invitrogen) according to the description. A total of 400 ng RNA was used in RT-PCR. Relative expressions of BmNPV IE1 in the different treatments were analyzed by qPCR with primers (IE1F:TGAGCAGTCTGTTGGTGTGA, IE1R:GCACAGCTTTGA ATTGTGCT). Actin expression set as internal control with primers (Actin F: GACCCTCAAATACCCCATCG, Actin R: CTTCCATACCCAA GAACGAGG).

2.6. Statistical analysis

The infectivity of different treatment was estimated as percentage of GFP-expressing cells for each treatment at 6 h p.i. as described in reference [8] and then the cells was applied to calculated viability by the trypan blue exclusion method according to the insect cell culture protocol of Invitrogen. The statistical analyses were performed by two tail *T* test in excel from three independent experiments.

2.7. TEM analysis

BmN cells in the dishes (60 mm) were incubated with/without 10 mM MβCD for 30 min, then the drug was removed and the cells were washed twice. The cells were then infected with BmBac-GFP using an MOI of 30 for 1 h at 4 °C. The virus-containing medium was removed and the cells were washed with PBS twice and fixed by glutaraldehyde for microscope observation and TEM analysis [19].

3. Results

3.1. BmNPV entry into BmN cells by clathrin-independent macropinocytosis

Since the closely related baculovirus AcMNPV take clathrin-mediated endocytosis enter host cell, effect of chlorpromazine, an inhibiter of clathrin-mediated endocytosis, was checked firstly. Result indicated chlorpromazine had no inhibitory function on BmNPV entry into BmN cells (Fig. 1A), even at a high concentration, cells, therefore, were infected efficiently, meanwhile, infectivity and viability of BmN cells incubated with 1 mM chlorpromazine decreased, which indicated chlorpromazine showed toxicity to cells. This result suggested that BmNPV took a clathrin-independent entry mechanism, so is it caveola/lipid raft endocytosis? Next, nystatin and progesterone were applied to verify whether caveola/lipid raft endocytosis was involved in. However, it had no effect on inhibition of BmNPV entry (Fig. 1B), even at a very high concentration (100 μg/ml), and BmN cell was more sensitive to the treatment of these drugs, cell viability decreased rapidly with the increasing drug concentration.

Viruses with large particles can enter cells via macropinocytosis, so BmNPV entry mechanism was supposed to be macropinocytic endocytosis. To validate this hypothesis, inhibitors of RTKs, Rac1 and PKC involving in macropinocytosis were used, including genistein, Ehop-016, and rottlerin. These chemicals blocked BmNPV entry efficiently without cell toxicity (Fig. 1C). Only 1.1% cells preincubated with 100 μg/ml genistein were infected; and about 3% cells showed fluorescence in treatment with 5 μM Ehop-016; while 1 μM rottlerin, inhibitor of PKC, blocked the virus entry activity completely. These results revealed that inhibition of kinase/GTPase resulted the failure of BmNPV into BmN cells efficiently. Other factors, including actin, Na+/K+ exchangers also involved in virus entry into BmN cells, which proved by the application of latrunculin A and DMA, the drugs decreased the infectivity significantly (Fig. 1C).

Taken together, these results indicated that BmNPV entry into BmN cells was clathrin- and caveolar/lipid raft-independent endocytosis, but was PKC-, Rac-1, and RTKs-dependent macropinocytic endocytosis.