JOURNAL OF VIROLOGY, Sept. 2006, p. 8830–8833 Vol. 80, No. 17
0022-538X/06/$08.00+0 doi:10.1128/JVI.00880-06

Functional Entry of Baculovirus into Insect and Mammalian Cells Is Dependent on Clathrin-Mediated Endocytosis

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Received 1 May 2006/Accepted 22 May 2006

Entry of the budded virus form of baculoviruses into insect and mammalian cells is generally thought to occur through a low-pH-dependent endocytosis pathway, possibly through clathrin-coated pits. This insight is primarily based on (immuno)electron microscopy studies but requires biochemical support to exclude the use of other pathways. Here, we demonstrate using various inhibitors that functional entry of baculoviruses into insect and mammalian cells is primarily dependent on clathrin-mediated endocytosis. Our results further suggest that caveolae are somehow involved in baculovirus entry in mammalian cells. A caveolar endocytosis inhibitor, genistein, enhances baculovirus transduction in these cells considerably.

The *Baculoviridae* are a large family of enveloped DNA viruses exclusively pathogenic to arthropods. Baculoviruses are divided taxonomically into two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (35). Baculoviruses produce two distinct virion phenotypes, occlusion-derived virus and budded virus (BV) (37), which are responsible for infection of insects and insect cells, respectively. Phylogenetic studies indicate that the NPV genus can be further subdivided into two subgroups, I and II (8, 12, 14). Members of the two NPV subgroups encode two different major BV envelope glycoproteins, GP64 for group I and F for group II, which mediate membrane fusion during viral entry (27).

The initial step for successful virus entry into target cells in general requires virion binding to cell surface-specific molecules, followed by internalization for viral infection or virus-based gene delivery to proceed. Viruses from various families utilize different internalization and trafficking pathways to enter target cells, including clathrin-mediated endocytosis, caveola-mediated endocytosis, macropinocytosis, and phagocytosis (9, 11, 23, 25, 28, 34). BVs of the baculovirus type species and group I NPV *Autographa californica* nucleopolyhedrovirus (AcMNPV) (3) are thought to enter insect cells via adsorptive endocytosis (13, 38), as evidenced by immunological and (electron) microscopy observations. However, no direct biochemical evidence is available that shows that this is indeed the case for all baculoviruses including group II NPVs, whether alternative routes such as through caveolae (29) or macropinocytosis (34) are used, whether clathrin-coated pits are involved, and whether this holds for the entry of baculovirus into insect as well as mammalian cells.

In order to study and dissect the entry process of baculoviruses, the effects of the inhibitors chlorpromazine (Sigma) (39) and genistein (Sigma) (10, 26) on baculovirus entry in insect and mammalian cells were studied. Chlorpromazine is a cationic, amphiphilic molecule that acts by shifting clathrin and the AP-2 complex to the late endosomal compartment, thus inhibiting clathrin-mediated endocytosis. Genistein interferes with caveola-mediated endocytosis by inhibiting viral internalization through caveolae; biochemically it blocks the phosphorylation of tyrosine kinase, which is involved in the formation of caveosomes. Bafilomycin A (Sigma) (1, 7), a specific inhibitor of endosome proton ATPase, was included to confirm that baculovirus BVs ultimately enter host cells in a low-pH-dependent manner.

After a 30-min treatment with the respective drugs, Sf21 cells were incubated for 1 h with an AcMNPV carrying a green fluorescent protein (GFP) gene under the control of a p10 promoter (21), at a multiplicity of infection (MOI) of 5 50% tissue culture infective dose ($TCID_{50}$) units per cell. Infected cells were incubated in drug-free Grace's medium supplemented with 10% fetal bovine serum (FBS). GFP expression was examined 24 h postinfection by fluorescence microscopy. The infectivity was estimated as percentage of GFP-expressing cells for each treatment. Bafilomycin A and chlorpromazine inhibited AcMNPV infection in Sf21 cells in a dose-dependent manner (Fig. 1A and C). In contrast, AcMNPV infection was not inhibited by genistein, even at a very high concentration, and this supports the view that AcMNPV entry into insect cells is primarily clathrin mediated. It has been demonstrated previously that lipid rafts are not involved in AcMNPV entry (40).

Recently a novel type of baculovirus BV envelope fusion protein, named F, was identified in NPV group II baculoviruses (16, 20, 27). In order to study the entry pathway of these viruses, the same experiment was performed as in Fig. 1A, but now by using BVs of a group II NPV, *Helicoverpa armigera* NPV (HearNPV), and *H. armigera* Am1 (HzAM1) cells. Results achieved were similar to those for AcMNPV (Fig. 1B and C; group I NPV). Chlorpromazine inhibition was almost complete at a concentration of 2 mM for both AcMNPV and HearNPV (Fig. 1C). The results obtained with these inhibitors provide independent, biochemical support for the view that BVs of both group I and group II baculoviruses enter insect cells primarily through clathrin-mediated endocytosis. The

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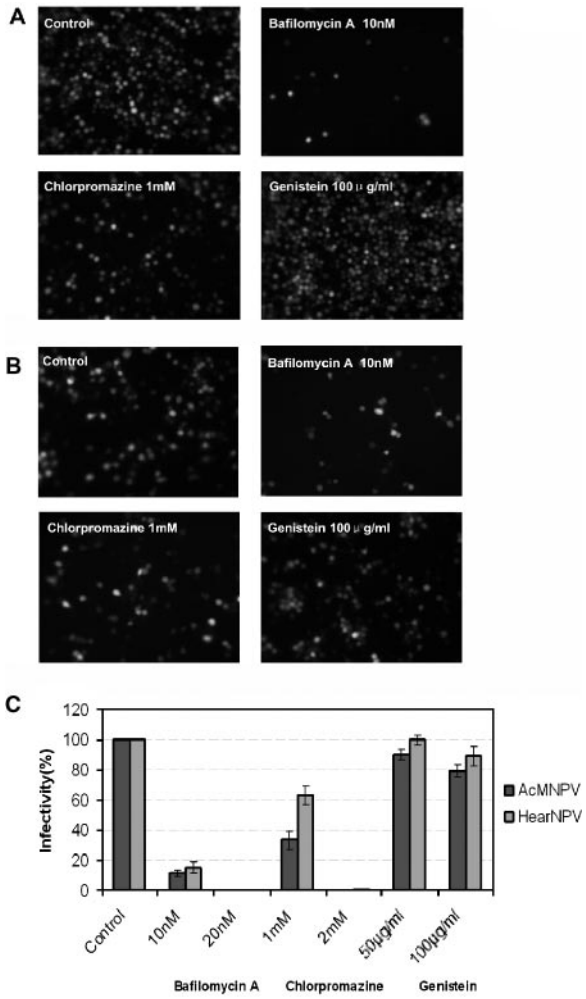

FIG. 1. Baculovirus NPVs infect insect cells through clathrin-mediated and low-pH-dependent endocytosis. (A and B) Insect cells were cultured in Grace's medium (GIBCO) supplemented with 10% FBS (GIBCO). Sf21 (A) and HzAM1 (B) cells were pretreated with 10 nM bafilomycin A, 1 mM chlorpromazine, and 100 μg genistein per ml and subsequently infected with AcMNPV (A) and HearNPV (B) at a multiplicity of infection of 5 $TCID_{50}$ units per cell for 1 h. Cells were rinsed twice with fresh medium and further cultured for 24 h. (C) Virus infectivity was quantified as the percentage of GFP-expressing cells relative to total cell number. The data shown are the means and standard deviations from three independent experiments.

caveola-mediated endocytosis pathway appeared to be not involved in baculovirus NPV BV infection of insect cells, as the virus uptake is unaffected by genistein (Fig. 1C).

Baculovirus BVs have been reported to effectively deliver genes into mammalian cells, and this has provided great impetus for the study and development of more effective baculovirus-based gene therapy vectors (6, 15, 18, 19, 30, 32). Understanding of the functional entry pathway of baculovirus into mammalian cells is thus pivotal for a successful entry process. Biochemical evidence suggested that baculovirus transduction into these cells is low pH dependent, and microscopy evidence suggested that baculovirus enters, e.g., human hepatoma cells through clathrin-mediated endocytosis and possibly through macropinocytosis (24, 36). But the functional

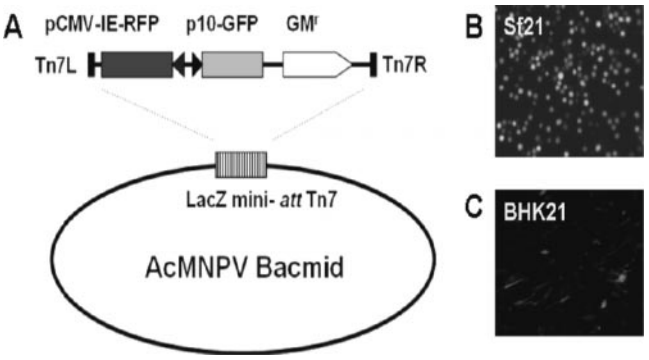

FIG. 2. Construction of AcMNPV-GR. (A) A p10 promoter-controlled enhanced GFP gene and a cytomegalovirus immediate-early promoter-controlled RFP gene were introduced into an AcMNPV bacmid, resulting in AcMNPV-GR. (B and C) GFP expression in Sf21 cells (B) and RFP expression in BHK21 (C) cells were examined.

entry pathway of baculovirus into mammalian cells, especially from the cell surface to the early endosome (23), requires further investigation. We therefore studied baculovirus entry into mammalian cells (Fig. 2) using the same set of inhibitor drugs as tested in insect cells (Fig. 1). By use of the bacmid system (22), a novel recombinant AcMNPV, AcMNPV-GR, carrying a GFP gene under the control of the AcMNPV p10 (late) promoter to evidence AcMNPV replication and a red fluorescent protein (RFP) gene under the control of the cytomegalovirus immediate-early (early) promoter was constructed to evidence entry, uncoating, and transport to the nucleus (Fig. 2A).

Sucrose-purified AcMNPV-GR BVs (20) were resuspended in phosphate-buffered saline and used to transduce BHK21 cells. GFP expression in AcMNPV-GR-infected Sf21 cells and RFP expression in AcMNPV-GR-transduced BHK21 cells were observed using fluorescence microscopy (Fig. 2B and 2C). Bafilomycin A, chlorpromazine, and genistein were applied to dissect the functional entry pathway of AcMNPV into BHK21 cells. After 30 min of drug treatment, BHK21 cells were transduced with AcMNPV-GR at an MOI of 100 $TCID_{50}$ units per cell for 1 h, and RFP expression was examined 24 h posttransduction. Transduction efficiency was calculated as the percentage of RFP-expressing cells. Similar to the results obtained from the baculovirus entry into insect cells (Fig. 1), bafilomycin A and chlorpromazine inhibited AcMNPV transduction into BHK cells (Fig. 3), suggesting that the clathrin-mediated and low-pH-dependent endocytic pathway is indeed involved in functional entry of AcMNPV into BHK21 cells.

Surprisingly, a high concentration of genistein (100 μg/ml), rather than being without effect on virus entry in insect cells, greatly increased AcMNPV transduction from 30% to more than 70% transduced cells (Fig. 3). This is an unexpected result, as entry of vertebrate enveloped virus either is not affected by inhibition of the caveolar pathway, e.g., influenza virus (33), or is reduced as in the case of murine leukemia virus (4). Our result may suggest that the caveola-mediated endocytic pathway is somehow involved in the functional entry of AcMNPV into BHK21 cells or is enhanced as a consequence of increased uptake of cholesterol (17). The enhanced transduction efficiency could also be explained by a lock-up of the

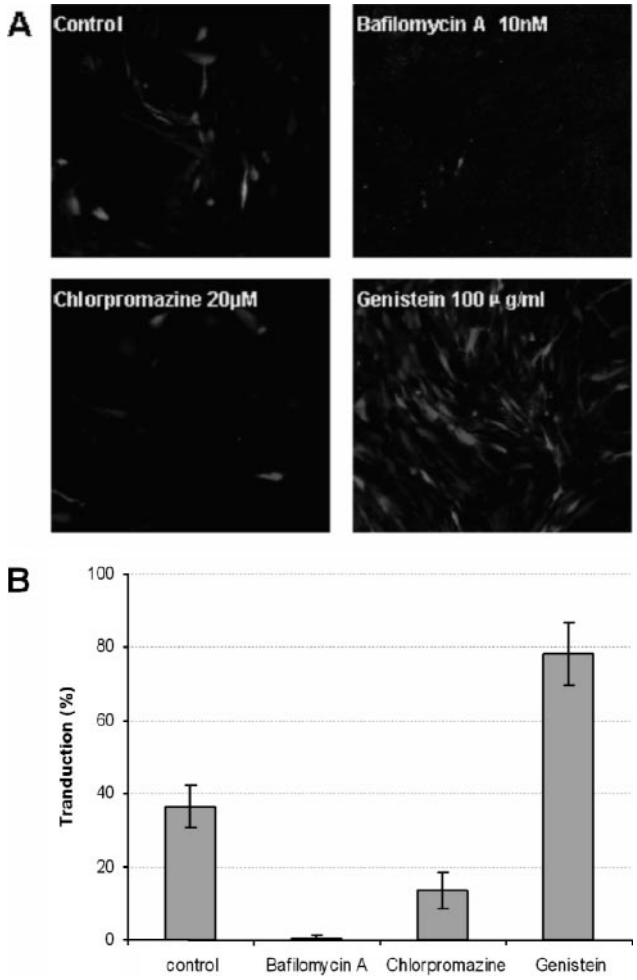

FIG. 3. Effects of drugs on AcMNPV-GR transduction into BHK21 cells. BHK21 cells were cultured in Dulbecco modified Eagle medium (GIBCO) supplemented with 10% FBS. (A) BHK21 cells were pretreated with 10 nM bafilomycin A, 20 μM chlorpromazine, and 100 μg genistein per ml and subsequently transduced with AcMNPV-GR at a multiplicity of infection of 100 $TCID_{50}$ units per cell for 1 h. (B) Transduction efficiency was evaluated as the percentage of RFP-expressing cells relative to the total cell number. The data shown are the means and standard deviations from three independent treatments.

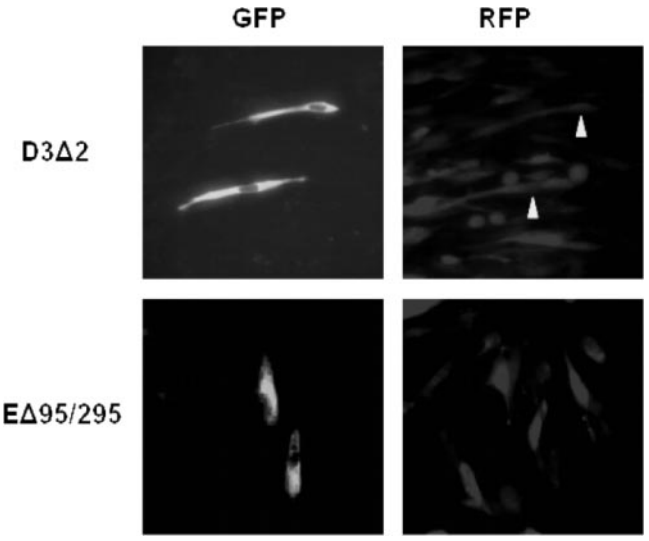

FIG. 4. Transduction is inhibited in cells expressing EΔ95/295. BHK21 cells were transfected with plasmids encoding either GFP-D3Δ2 (upper panels) or GFP-EΔ95/295 (lower panels). Forty-eight hours after transfection, cells were transduced with AcMNPV-GR BVs. Twenty-four hours after transduction, GFP (left panels) and RFP (right panels) expression was examined.

caveola-mediated endocytic pathway by genistein, possibly driving more virus particles to enter mammalian cells through clathrin-mediated endocytosis. Alternatively, the response of insect cells to genistein showing no effect on virus uptake may be different although the primary biochemical response, i.e., the inhibition of tyrosine kinase-mediated phosphorylation, is the same (2, 31).

BHK21 cells are sensitive to high concentrations (50 μg/ml) of chlorpromazine; thus, complete inhibition of transduction into BHK21 cells by chlorpromazine does not occur. To confirm that the clathrin-mediated endocytosis is part of the functional entry of AcMNPV into mammalian cells, we used EΔ95/295, a dominant-negative form of Eps15 (*e*pidermal growth factor receptor *p*athway *s*ubstrate clone 15), which specifically interferes with clathrin-coated vesicle formation at the plasma membrane and thus inhibits virus entry (5). As a control, we used epidermal growth factor receptor mutant D3Δ2, another form of Eps15 with no dominant-negative effect on clathrin-mediated endocytosis. EΔ95/295 or D3Δ2 with an N-terminally fused enhanced GFP gene to allow detection was transiently expressed in BHK21 cells by using Lipofectin (Invitrogen). Forty-eight hours after transfection BHK21 cells were transduced with AcMNPV-GR at an MOI of 100 $TCID_{50}$ units per cell. Twenty-four hours after transduction, RFP and GFP expression was determined using fluorescence microscopy. As expected, D3Δ2 expression had no effect on AcMNPV transduction, whereas AcMNPV transduction was strongly inhibited in cells transfected with EΔ95/295 (Fig. 4). This result is consistent with the result from chlorpromazine treatment (Fig. 3) and proved that clathrin-mediated endocytosis is the major functional pathway for AcMNPV entry into BHK21 cells.

In conclusion, our results using various inhibitor drugs and reporter constructs support the view that baculovirus NPVs, regardless of whether they belong to group I (GP64) or group II (F) NPVs, primarily enter insect and mammalian cells through a clathrin-mediated, low-pH-dependent endocytic pathway. Baculovirus may enter mammalian cells through multiple pathways, but the caveola-dependent entry is somehow involved as genistein enhances transduction. These data imply that baculoviruses may be tailored by genetic engineering to enter mammalian cells more efficiently through clathrin-mediated endocytosis and promote the idea that tyrosine kinase inhibitors may be used as novel agents to enhance baculovirus-based gene delivery in these cells.

We are grateful to A. Benmerah (Cochin Institute, Paris, France) for kindly providing EΔ95/295 and D3Δ2 constructs.

This work was funded by grants from the National Science Foundation of China (NSFC 30300012 to Gang Long) and the Royal Academy of Sciences of The Netherlands (KNAW) (project number 04-PSA-

BD-02) and by a Ph.D. sandwich grant to G.L. from Wageningen University.

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