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Autographa californica Multicapsid Nucleopolyhedrovirus Efficiently Infects Sf9 Cells and Transduces Mammalian Cells via Direct Fusion with the Plasma Membrane at Low pH▽

Sicong Dong,[1,3] Manli Wang,[1] Zhijuan Qiu,[1] Fei Deng,[1] Just M. Vlak,[2] Zhihong Hu,[1] and Hualin Wang[1]*

State Key Laboratory of Virology and Joint Laboratory of Invertebrate Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, People's Republic of China[1]; Laboratory of Virology, Wageningen University, 6708 PB Wageningen, The Netherlands[2]; and Graduate School of the Chinese Academy of Sciences, Beijing 100039, People's Republic of China[3]

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The budded virus (BV) of the *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) infects insect cells and transduces mammalian cells mainly through the endocytosis pathway. However, this study revealed that the treatment of the virus bound to Sf9 cells at low pH could efficiently rescue the infectivity of AcMNPV in the presence of endocytosis pathway inhibitors. A colocalization assay of the major capsid protein VP39 with the early endosome marker EEA1 showed that at low pH, AcMNPV entered Sf9 cells via an endosome-independent pathway. Using a fluorescent probe (R18), we showed that at low pH, the viral nucleocapsid entered Sf9 cells via direct fusion at the cell surface. By using the myosin-specific inhibitor 2,3-butanedione monoxime (BDM) and the microtubule inhibitor nocodazole, the low pH-triggered direct fusion was demonstrated to be dependent on myosin-like proteins and independent of microtubules. The reverse transcription-PCR of the IE1 gene as a marker for viral entry showed that the kinetics of AcMNPV in cells triggered by low pH was similar to that of the normal entry via endocytosis. The low pH-mediated infection assay and VP39 and EEA1 colocalization assay also demonstrated that AcMNPV could efficiently transduce mammalian cells via direct membrane fusion at the cell surface. More importantly, we found that a low-pH trigger could significantly improve the transduction efficiency of AcMNPV in mammalian cells, leading to the potential application of this method when using baculovirus as a vector for heterologous gene expression and for gene therapy.

Enveloped viruses infect host cells by attaching to the cell surface and delivering their genetic material into the cytoplasm. One of the most crucial steps in this process is the fusion event between the viral envelope and a cellular membrane, which allows the passage of nucleocapsids through the cellular membrane into the cytoplasm. The fusion event could occur at the cell surface or with particular cellular vesicles, such as endosomes. Human immunodeficiency virus type 1 (HIV-1) and rotaviruses fuse at the cell surface (18, 26, 31) while other viruses, including influenza virus, Ebola virus, and vesicular stomatitis virus, take the endocytosis entry pathway and fuse with the endosome membrane (22, 40–41). Some viruses can utilize different ways to enter different cells. For example, Semliki Forest virus (SFV) is internalized into the endosomes when infecting CHO cells, while the virus infects BHK-21 cells by fusing with the cell membrane (12, 27, 51). Influenza virus normally infects cells via the endocytosis pathway (22, 29, 39), yet some researchers have demonstrated that it infects HeLa cells in the absence of clathrin-mediated endocytosis (37). Herpes simplex virus (HSV) infects Vero cells by fusion with the cell surface, while it enters HeLa and CHO-K1 cells through the endocytosis pathway (32).

Baculoviruses belong to the *Baculoviridae*, a family of rod-shaped enveloped viruses. There are two distinct virion phenotypes: occlusion-derived virus (ODV) and budded virus (BV). ODV infects the midgut cells of the insects and initiates primary infection, while BV infects the other tissues of the insect and is responsible for secondary or systemic infection (46). ODV enters the cell by direct membrane fusion between the ODV envelope and brush border membrane vesicles (BBMV) or host cell membrane (9, 15). It generally is believed that the clathrin-dependent endocytosis pathway is taken as the major pathway to infect the cells by BV, and it is a pH-dependent pathway (2, 24, 44). This conclusion was confirmed by the observation of virus particles in coated pits under the electron microscope (42). Entry by a clathrin-mediated endocytosis also was confirmed by the inhibition effects of chlorpromazine (CPZ) (25), which interfered with the infection of the virus by preventing the formation of the clathrin-coated vesicles (47), and ammonium chloride (NH_4Cl), which prevented the infection of the virus by elevating the pH of the late endosomes (11, 44). However, researchers have revealed that the BV of *Trichoplusia ni* NPV (TnNPV) penetrated the cytoplasm directly from the cell surface (21, 50). For AcMNPV in the genus *Alphabaculovirus*, immunoelectron microscopy studies also revealed that some viruses entered the cells by fusing with the cell membranes under certain conditions, but this alternate entry pathway was not considered efficient because it was believed that this pathway would lead to genomic degradation (45).

Baculoviruses have been used extensively for the expression

* Corresponding author. Mailing address: Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, P.R. China. Phone and fax: 86-27-87199353. E-mail: h.wang@wh.iov.cn.

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of recombinant proteins both in insect and mammalian cells (16, 20, 33). AcMNPV also has been successfully transduced into mammalian cells for gene expression and shows great potential for becoming a gene therapy vector (4, 14, 19, 36, 43). It has been well documented that baculoviruses utilize the clathrin-mediated endocytosis pathway to enter mammalian cells as it does in insect cells (25, 28, 42). One of the most important early events of a successful transduction is the binding of the virus to the receptor on the mammalian cells. Although the receptor on mammalian cells has not been identified, it is possible that electrostatic interactions between heparin sulfate and the major envelope protein GP64 are involved in this process (2, 5, 11). AcMNPV is absorbed into the endosomes, and the virus envelope is proposed to fuse with the endosome membrane with the help of GP64. After being released from the endosome, the nucleocapsids presumably travel through the nuclear pores to complete the transduction process (42).

Some envelope viruses, which enter cells mainly through the pH-dependent pathway, can be forced to fuse with the cell membranes by culturing the cell-bound viruses under low pH (27). The direct fusion between the virus envelope and the cell membrane also was observed after AcMNPV-bound cells were triggered under acidic conditions, even in the presence of sodium azide, which inhibited endocytosis (49). However, the consequence of direct fusion and whether it results in efficient infection is unknown. In this study, we explored whether low pH can cause the direct fusion of AcMNPV to host cell membranes for successful infection/transduction, especially in the presence of endocytosis inhibitors. We determined that a short-term exposure to low pH allowed AcMNPV to bypass the endocytosis pathway to gain entry into insect cells. We also investigated the involvement of cytoskeleton molecules and the entry kinetics of AcMNPV infection at low pH. Interestingly, in mammalian cells, the low-pH treatment not only allowed the virus to bypass endocytosis-mediated entry but also dramatically improved the AcMNPV transduction rate. This suggests that low-pH treatment is a very useful method when using baculovirus as a vector for gene expression in mammalian cells and for gene therapy.

MATERIALS AND METHODS

Cells and viruses. *Spodoptera frugiperda* Sf9 cells were propagated at 28°C in Grace's insect medium (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL), pH 6.0. The mammalian HepG2 and HeLa cell lines were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco-BRL) supplemented with 10% fetal bovine serum (FBS), pH 7.0. The AcMNPV bacmid was the commercially available bacmid bMON14272 (Invitrogen) and was propagated in *Escherichia coli* strain DH10B.

Construction of vAc-EGFP and vAc-CMV-EGFP. The recombinant AcMNPV with the enhanced green fluorescent protein (*egfp*) reporter gene, vAc-EGFP and vAc-CMV-EGFP (Fig. 1), were generated according to the manufacturer's instructions for the Bac-to-Bac baculovirus expression system (Gibco-BRL). To generate the donor plasmid pFastBacDual-EGFP, the EGFP cassette was digested from the pEGFP-N1 plasmid (Clontech) as an NcoI-NotI fragment, blunted, and subcloned downstream of the p10 promoter of the transfer vector pFastBacDual (Gibco-BRL), which was blunted after being digested with SmaI and NcoI. To construct the donor plasmid pFastBacHTb-CMV-EGFP, the *Orgyia pseudotsugata* MNPV (OpMNPV) gp64 promoter (Op166) was cut from the plasmid pFB-Op166 (48) with BamHI and inserted into pEGFP-N1 to generate the plasmid pEGFP-N1-Op166. Subsequently, pEGFP-N1-Op166 was digested with NheI and SmaI to remove the residual multiple cloning sites and self ligated. The CMV-Op166-EGFP-SV40 poly(A) cassette then was digested from this plasmid by AseI and AflII, blunted, and ligated into the blunted EcoRI site of the vector pFastBacHTb (Gibco-BRL). Each of the donor plasmids (pFastBacDual-EGFP and pFastBacHTb-CMV-EGFP) was transformed into competent DH10Bac cells containing the AcMNPV bacmid and the helper plasmid expressing the transposase (Gibco-BRL). The transposition of inserts from donor plasmids to the AcMNPV bacmid was confirmed by diagnostic PCR. The identified bacmids were named Ac-EGFP and Ac-CMV-EGFP. Each purified bacmid DNA (1 μg) was transfected into Sf9 cells cultured in 35-mm-diameter tissue culture dishes at 1×10^6 cells per dish using 12 μl Lipofectin reagent according to the Bac-to-Bac expression system manual (Gibco-BRL). Supernatants were collected at 3 days posttransfection (p.t.) and used to infect Sf9 cells for viral amplification. The viruses were designated vAc-EGFP and vAc-CMV-EGFP, and the titers (50% tissue culture infectious doses [$TCID_{50}$], in units/milliliter) were determined by endpoint dilution assays (EPDA) on Sf9 cells.

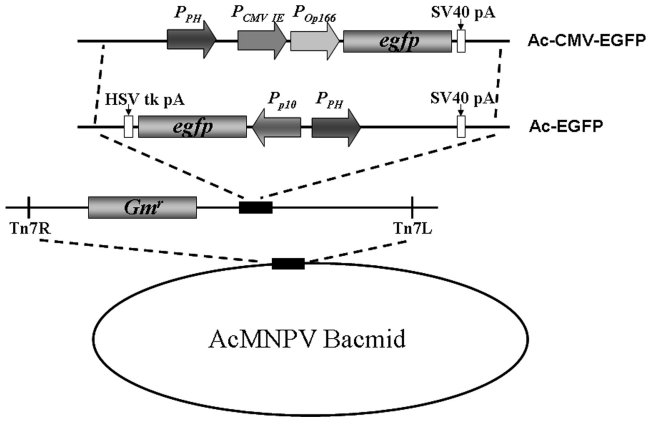

FIG. 1. Construction of recombinant baculoviruses. The reporter gene *egfp* is under the control of the P10 and CMV IE1/Op166 promoters in vAc-EGFP and vAc-CMV-EGFP, respectively.

Infection of Sf9 cells under low pH and with inhibitors of the endocytosis pathway. In this study, NH_4Cl or CPZ was used separately to inhibit the endocytosis pathway of Sf9 cells. The cells infected without chemical treatment under normal pH were set up as the control. The 24-well tissue culture plates were seeded prior to infection at a density of 2×10^5 cells per well and pretreated with Grace's medium containing either 25 mM NH_4Cl (Beijing YILI Fine Chemical Co., Ltd.) or 20 μg/ml CPZ (Sigma) at 28°C for 40 min and chilled to 4°C for an additional 20 min. Subsequently, the cells were incubated with vAc-EGFP at a multiplicity of infection (MOI) of 1 $TCID_{50}$ U/cell in the inhibitor-containing medium at 4°C for 1 h. After the incubation, supernatants containing viruses were removed, and the cells were incubated with acidic Grace's medium, pH 4.8, or normal Grace's medium, pH 6.0, in the presence of the inhibitor at 28°C for 5 min. Subsequently, the medium was removed and the cells were further incubated with fresh medium plus inhibitors at 28°C for 4 h. After being washed twice with fresh Grace's medium, the cells were cultured with normal Grace's insect medium supplemented with 10% FBS for 44 h before being harvested for flow-cytometric analysis. The experiments were repeated three times, and standard deviations were calculated by Microsoft Excel 2003.

Visualization of virus entry and membrane fusion. Fusion events of the virus envelopes with the Sf9 cell membranes were determined by using octadecyl rhodamine B chloride (R18)-labeled viruses under a fluorescence microscope. Purified AcMNPV was labeled with the R18 probe (Invitrogen) as described by Wang et al. (49), with slight modifications. Briefly, 100 μl of 10 mg/ml R18 was added to 50 ml of virus stock at a titer of 5×10^7 $TCID_{50}$/ml. The mixture was incubated for 60 min at room temperature, and the R18-labeled viruses were separated from free probes by centrifugation at 42,900 × *g* for 1 h at 4°C. Sf9 cells were incubated and infected with R18-labeled virus as described above, and the fluorescence was observed 60 min after infection.

Low pH-mediated infection of Sf9 cells with inhibitors of cytoskeleton molecules. Nocodazole and 2,3-butanedione monoxime (BDM) were used to inhibit the function of microtubules and myosin-like proteins, respectively, during infection triggered by low pH. The cells infected without chemical treatment under normal pH were set up as the control. The 24-well tissue culture plates were seeded in advance with a density of 2×10^5 cells per well and pretreated with Grace's medium containing either 20 μM nocodazole (Sigma) or 10 mM BDM

(Sigma) at 28°C for 40 min and chilled to 4°C for an additional 20 min. The rest of the experiments were the same as those described above for the NH_4Cl and CPZ inhibition experiments, except that in the control samples for BDM and nocodazole treatment, 1% methanol or dimethylsulfoxide (DMSO), instead of the chemicals, was added to the cells, respectively. Additionally, in the BDM inhibition assay, the BDM was maintained in Grace's medium until the flow-cytometric analysis. The experiments were repeated three times, and standard deviations were calculated. The inhibition rates of the nocodazole treatments with the control were analyzed with the Wilcoxon W test.

Comparison of viral entry kinetics of normal infection to those of low pH-mediated infection when endocytosis was inhibited. The kinetics of viral entry in Sf9 cells were determined by reverse transcription-PCR (RT-PCR) based on the transcription of the IE1 gene. Sf9 cells (1×10^6) cultured in 35-mm-diameter tissue culture dishes were preincubated at 28°C for 40 min in the presence or absence of 25 mM NH_4Cl. vAc-EGFP at an MOI of 1 was added in NH_4Cl-containing medium or normal medium and incubated at 4°C for 1 h. Subsequently, the supernatants were removed and marked as 0 min. The cells were treated with acidic (pH 4.8) or normal (pH 6.0) Grace's medium for 5 min at 28°C and then cultured with normal Grace's insect medium containing 10% FBS. In the low pH-triggered sample, NH_4Cl was present during the whole procedure. The total RNA was isolated with TRIzol (Invitrogen) according to the manufacturer's instructions at 0, 30, 60, 90, and 120 min after the binding procedure and then was treated with RQ1 RNase-Free DNase (Promega) to prevent DNA interference with the subsequent RT-PCRs. RT-PCR was performed using 1 μg of treated total RNA per time point as the template. The synthesis of the first-strand cDNA was performed using M-MLV Reverse Transcriptase (Promega) and oligo(dT) primers (5′-CTGATCTAGAGGTACCGGATCCTTTTTTTTTTTTTT-3′) according to the manufacturer's instructions. The PCR amplification of cDNA was performed using the IE1-specific primers IE1-F (5′-AAATACAGCAGCGTCGCTAATC-3′) and IE1-R (5′-CGTCGGACAACGGAACCAGA-3′).

Low pH-mediated transduction of vAc-CMV-EGFP in mammalian cells. Cells transduced without chemical treatment under normal pH were set up as the control. HepG2 or HeLa cells were preincubated at 37°C for 30 min in the presence of 10 or 25 mM NH_4Cl, respectively. The cells were incubated for an additional 15 min at 4°C and subsequently transduced with vAc-CMV-EGFP at MOIs of 50 and 10. The cells were incubated at 4°C with NH_4Cl for 1 h to allow the viruses to bind the cells. After the binding period, the medium was replaced with phosphate-buffered saline (PBS) at pH 4.8 in the presence of NH_4Cl at room temperature for 5 min. The PBS was removed and fresh medium with NH_4Cl was added, and the cells were incubated at 37°C for an additional 2 h. The medium was removed, and the cells were washed twice. The fresh DMEM supplemented with 10% FCS was added, and the cells were incubated at 37°C for another 24 h before being harvested for flow-cytometric analysis. Standard deviations were calculated based on three replicates.

Immunofluorescence microscopy. HepG2 or Sf9 cells, grown in a 35-mm dish containing glass coverslips, were transduced or infected with vAc-CMV-EGFP (MOI = 50) or vAc-EGFP (MOI = 10) as described above. Thirty-five or 60 min after vAc-CMV-EGFP was added to the HepG2 cells, or 20 min after vAc-EGFP was added to Sf9 cells, the cells were washed twice with PBS and fixed with 96% ethanol for 15 min at room temperature. The fixed cells were washed three times with PBS and blocked with PBS containing 5% BSA for 60 min at 37°C. After that the cells were washed three times with PBS and incubated for 90 min in PBS containing anti-VP39 protein antiserum (1:500; kindly provided by Kai Yang, Sun-Yat Sen University, China) at 37°C. The cells were washed three times with PBS, followed by incubation for 90 min at 37°C in PBS containing rhodamine-conjugated goat anti-rabbit IgG (1:150; Millipore). Cells were washed three times with PBS and incubated with fluorescein isothiocyanate (FITC) mouse anti-human EEA1 (1:200; BD Transduction Laboratories) at 37°C for 90 min. After three washes with PBS, the cell nuclei were stained with Hoechst 33258 for 5 min at room temperature. Finally, the cells were mounted onto slides for capturing images with a fluorescence microscope.

RESULTS

Low pH efficiently triggers AcMNPV infection in Sf9 cells even when the endocytosis pathway is blocked. To test whether AcMNPV BV could infect Sf9 cells at low pH, we provided an acidic external pH environment in the presence or absence of the endocytosis inhibitors. Two chemicals were used as endocytosis inhibitors in our study: NH_4Cl, which blocks membrane fusion in the endosomes, and CPZ, which prevents the assembly of clathrin-coated pits. Sf9 cells were pretreated with NH_4Cl or CPZ and then incubated with vAc-EGFP in the presence of the chemicals for the duration of the infection. Cells were observed under a fluorescent microscope for the presence of green fluorescence at 48 h postinfection (p.i.), and the infection rate was analyzed by flow cytometry. The infection rate of the controls (normal infection without the treatments of the chemicals and low pH) were set as 100%. When virus-bound cells were treated with acidic medium in the absence of the chemicals at 28°C for 5 min, the proportions of infected cells were similar to those of the controls (Fig. 2). As predicted, treatment of cells with NH_4Cl and CPZ almost completely blocked the infection. The infection rate was only 8 and 18% for NH_4Cl- and CPZ-treated viruses, respectively, compared to results for the control (Fig. 2). When the virus-bound cells were exposed to the acidic medium in the presence of NH_4Cl or CPZ at 28°C for 5 min, vAc-EGFP could efficiently infect cells at a level of 114 and 85%, respectively, which is similar to that of normal infection (Fig. 2). Combined with the results of the NH_4Cl and CPZ inhibition assay, these results suggested that the acidic environment triggered the fusion process at the cell membrane that led to the effective infection of the virus-bound Sf9 cells, even in the presence of the endocytosis pathway inhibitors.

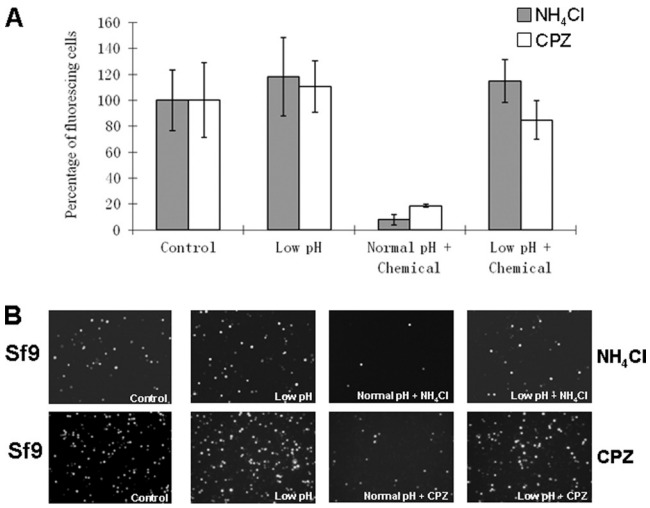

FIG. 2. Low pH-triggered infection assay in Sf9 cells with inhibitors of the endocytosis pathway. Normal infection without chemical treatment and low-pH treatment was set as the control. vAc-EGFP (MOI = 1) was allowed to bind to Sf9 cells at 4°C for 60 min, and then the unbound viruses were washed away and the pH was shifted to 4.8 for 5 min. The endocytosis pathway was blocked with NH_4Cl or CPZ. (A) Flow-cytometric analysis of the infection assay in Sf9 cells at 48 h p.i. The white or shaded control columns represent cells infected at normal pH from the CPZ or NH_4Cl series of experiments, respectively, but that were not treated with CPZ or NH_4Cl, respectively. The error bars represent standard deviations. (B) Fluorescence images of the infected cells at 48 h p.i. The upper images show the infected cells with NH_4Cl. The lower images show the infected cells with CPZ.

AcMNPV induced to enter Sf9 cells by low pH does not colocalize with the early endosome marker EEA1. The early endosome marker EEA1 (28) was used to further check whether viruses entered Sf9 cells through the endocytosis-

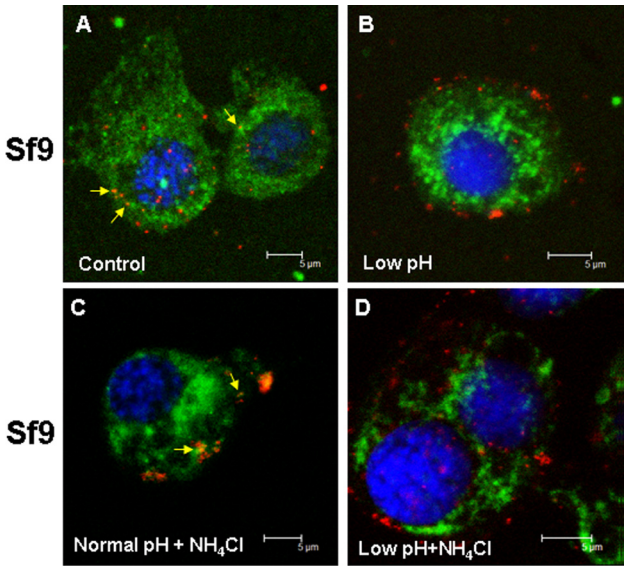

FIG. 3. Colocalization (yellow) of the vAc-EGFP (MOI = 10) capsid protein VP39 (red) with the early endosome marker EEA1 (green) at 20 min p.i. in Sf9 cells. EEA1 was conjugated with FITC while VP39 was detected with rhodamine-tagged goat anti-rabbit antibodies. Sf9 cells infected with neither NH_4Cl nor low-pH treatment were set up as the control sample. (A) In the control sample, VP39 colocalized with EEA1 (arrows). (B) In the low pH-treated sample, VP39 failed to colocalize with EEA1. (C) In the NH_4Cl-treated sample, VP39 colocalized with EEA1 (arrows). (D) In the low pH- and NH_4Cl-treated sample, VP39 also failed to colocalize with EEA1. Bars, 5 μm.

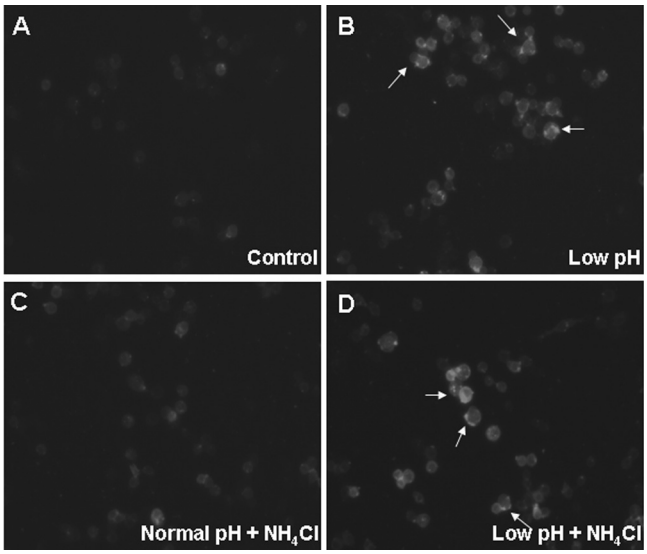

FIG. 4. Visualization of virus entry and membrane fusion. R18-labeled vAc-EGFP (MOI = 10) was allowed to bind to Sf9 cells at 4°C for 60 min. Unbound viruses then were washed away and the cells were treated at low pH (B, D) or left untreated (A, C). Membrane fusion between virus envelopes and cell membranes was observed by fluorescence microscopy at 60 min after infection. The endocytosis pathway was blocked with NH_4Cl (C, D).

dependent or -independent pathway induced by acid conditions. The viruses that entered the cells under normal conditions were used as the controls. Twenty minutes after vAc-EGFP was added, the cells were fixed for observation. In the control sample, VP39, as detected by anti-VP39 immunofluorescence, was shown to colocalize with EEA1 in the Sf9 cells at 20 min p.i. (Fig. 3A), which is consistent with the knowledge that baculoviruses normally enter the cells via endocytosis. The same colocalization was observed in the cells pretreated with NH_4Cl (Fig. 3C), indicating that when NH_4Cl blocked fusion at the endosome, the viruses remained in the endosomes. However, when the virus-bound cells were treated with the external acid environment, the capsid protein VP39 was detected in the cells but failed to colocalize with EEA1 (Fig. 3B). In the cells pretreated with NH_4Cl and treated with the acidic medium, the capsid protein VP39 also was found at the cell periphery but failed to colocalize with EEA1 (Fig. 3D). These observations revealed that the external acidic treatment of the virus-bound cells allowed the virus to enter the Sf9 cells through a nonendocytic pathway. Therefore, this modification of the entry pathway led to the recovery of virus infectivity in the NH_4Cl/CPZ inhibition assay.

Under low-pH conditions, AcMNPV entered the Sf9 cells via direct fusion of the virion envelope with the cell membrane. The experiments described above have shown that under low-pH conditions, AcMNPV entered the Sf9 cells via an endocytosis-independent pathway. In this experiment, the R18 probe was used to visualize whether the viruses entered the cells by direct membrane fusion at the cell surface. R18 is a fluorescent membrane probe inserted into the virus envelope that allows the location of the virus envelope to be tracked. AcMNPV were labeled with the R18 probe and used to infect Sf9 cells as described in Materials and Methods. Sf9 cells infected with viruses under the normal conditions were set as the control, and no obvious fluorescence was detected on the plasma membranes (Fig. 4A). In the NH_4Cl-treated cells, fluorescence could not be observed on the plasma membranes either (Fig. 4C). However, when the virus-bound cells were treated under low-pH conditions, fluorescence could be observed on the plasma membranes with or without NH_4Cl treatment (Fig. 4B, D). These results were consistent with a former report (49) and indicated that AcMNPV entered the cells via direct fusion at the cell surface induced by the acidic environment.

With low pH-induced viral entry, the transport of nucleocapsids to the nucleus is myosin-like protein dependent and microtubule independent. It has been demonstrated that the release of the BV nucleocapsids from endosomes into the cytoplasm induces the formation of the actin filaments, and the myosin-like proteins are involved in nucleocapsid transport to the nucleus (23). To investigate whether myosin-like proteins are involved in the infection process mediated by the direct fusion at the cell surface, Sf9 cells were infected in the presence of BDM for the low-pH treatment experiments as described in Materials and Methods, and the numbers of EGFP-positive cells in each condition were compared by flow-cytometric analysis. BDM inhibits myosin by blocking the release of phosphorylated protein after ATP hydrolysis (13). The infection rate of the control sample, which was not treated with BDM, was set as 100%. As shown in Fig. 5, in the presence of BDM, the infection rate of vAc-EGFP was greatly reduced in either low pH-treated cells (28%) or untreated cells (28%). These results implied that the myosin-like proteins play an important role in the transport of the nucleocapsids toward the

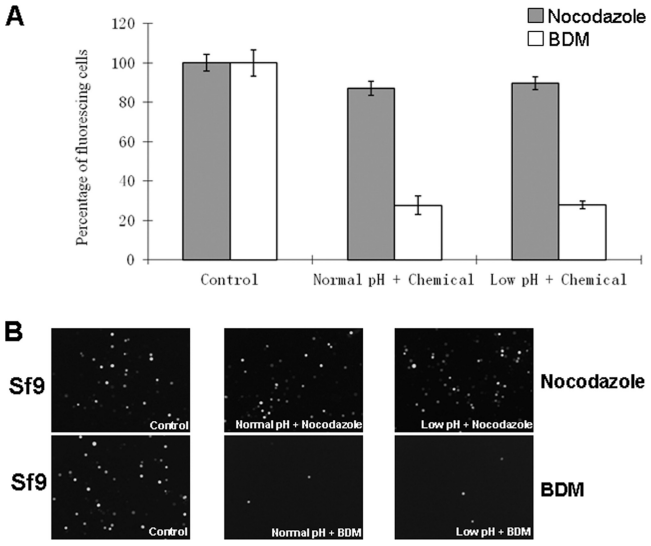

FIG. 5. Low pH-mediated infection of Sf9 cells with inhibitors of cytoskeleton molecules. Normal infection without the chemical treatment and low-pH treatment was set as the control. vAc-EGFP (MOI = 1) was allowed to bind to Sf9 cells at 4°C for 60 min. The unbound viruses then were washed away, and the pH was shifted to 4.8 for 5 min. The functions of microtubules and myosin-like proteins were blocked with nocodazole (gray bar) and BDM (white bar), respectively. (A) Flow-cytometric analysis of the infection rate of Sf9 cells at 48 h p.i. The white or shaded control columns represent cells infected at normal pH from the BDM or nocodazole series of experiments, respectively, but that were not treated with BDM or nocodazole, respectively. The error bars represent standard deviations. (B) Fluorescence images of the infected cells at 48 h p.i. The upper images show the infected cells with nocodazole. The lower images show the infected cells with BDM.

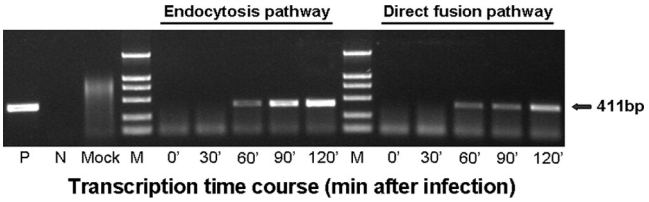

FIG. 6. Time course analysis of IE1 transcription in Sf9 cells infected by vAc-EGFP (MOI = 1) through the endocytosis or direct fusion pathway. The cell samples were collected at 0, 30, 60, 90, and 120 min p.i., and total RNA was extracted for the RT-PCR analysis of IE1 expression. P, positive control; N, template minus control; Mock, uninfected Sf9 cells.

nucleus following the direct fusion at the cell surface as well as in the classic endocytosis pathway.

Microtubules are utilized by many viruses for transporting the nucleocapsids in the cytoplasm; however, they are not considered to be involved in the transport of baculovirus nucleocapsids following endocytosis (35, 42). We examined the role of the microtubule cytoskeleton in nucleocapsid transport after fusion at the cell surface. Nocodazole, a specific agent that depolymerizes microtubules, was added to the cells. The infection assay was performed as described in Materials and Methods. The infection rate of vAc-EGFP in the absence of nocodazole was set as 100%. Compared to results for the control sample, nocodazole showed no obvious effects on the infection rate of the viruses (87%; $P > 0.05$) entering through the endocytosis pathway. In the sample treated under low pH, nocodazole also had no obvious inhibitory effect on the infection rate (90%; $P > 0.05$) (Fig. 5). These data suggest that intact microtubules are not involved in the process of nucleocapsid transport in either the endocytosis or the direct fusion entry pathways.

AcMNPV entered Sf9 cells through the endocytosis pathway and direct fusion pathways at similar rates. The speed of entry is an important characteristic of a particular infection pathway, and it may differ between different infection pathways. It has been demonstrated that severe acute respiratory syndrome coronavirus (SARS-CoV) entering via the cell surface replicates 1 h ahead of the virus entering through the endocytosis pathway (30). To further define the entry kinetics of AcMNPV into Sf9 cells through different entry pathways, the IE1 gene was chosen in our study as the marker gene, because it is an immediate-early gene with transcription that is initiated as soon as the virus enters the nucleus (10). The transcription of IE1 was detected by RT-PCR. IE1-specific primers were used to detect the IE1 gene expression after DNase I digestion to eliminate DNA contamination. In infections under both normal conditions and low pH with blocked endocytosis conditions, IE1 transcription was detected as early as 60 min after infection (Fig. 6). As the synthesis of IE1 mRNA was considered the indicator of virus entry into the nucleus, this observation suggested that the viruses utilizing these two distinct entry pathways penetrated the cytoplasm and were transported to the nucleus at nearly the same speed. The infectious BV production of AcMNPV entry via the direct fusion pathway or the endocytosis pathway was analyzed by an endpoint dilution assay (EPDA). Results show that AcMNPV infection in Sf9 cells via two entry pathways has similar dynamics of BV production (data not shown).

The external acidic environment significantly increased the rate of the AcMNPV transduction of mammalian cells. AcMNPV can transduce several mammalian cell types, including HepG2 and HeLa cells, and it is considered a potential gene therapy vector (14, 19). However, AcMNPV has some shortcomings as a gene transduction vector, including a relatively low transduction rate compared to that of other systems. Only about 30% of cells can be transduced with viruses at an MOI of up to 100 at 4°C (28). Although some researchers have increased the transduction rate in certain mammalian cells by several surface-modified baculoviruses (1, 7, 8, 17, 34), further improvements are required. Considering that the virus fused at the cell surface can successfully infect the insect cells as well as when entering through the endocytosis pathway, we wondered whether the modification of the infection pathway for mammalian cells could increase the transduction rate.

In this study, HepG2 and HeLa were transduced as described in Materials and Methods. For all four controls, the percentage of transduced cells was less than 10%. On HepG2 cells, when the virus-bound cells were treated with acidic PBS, the proportion of the transduced cells showed a significant increase of up to 21.6% (MOI = 10) and 58.0% (MOI = 50) compared to levels of the control transduced cells. The acidic PBS treatment of the virus-bound cells also increased the proportion of the transduced cells even in the presence of NH_4Cl. To our surprise, the combined use of NH_4Cl and acidic PBS

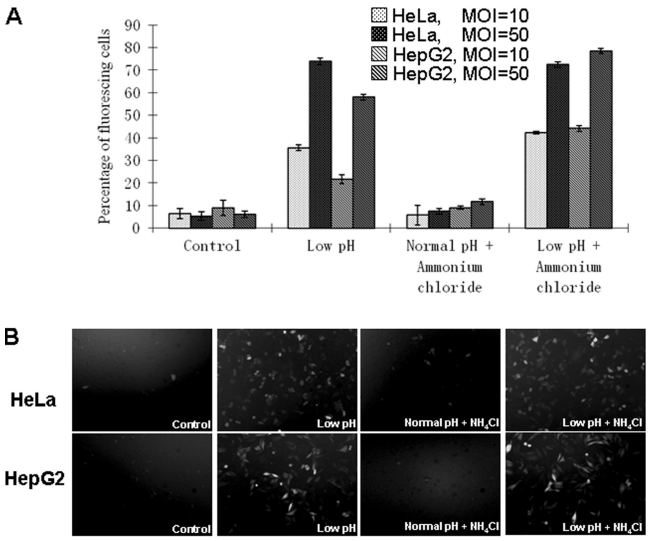

FIG. 7. Low pH-mediated transduction of mammalian cells by vAc-CMV-EGFP. Normal transduction without the chemical treatment and low-pH treatment was set as the control. vAc-CMV-EGFP (MOI = 10 or 50) was allowed to bind to HeLa and HepG2 cells at 4°C for 60 min. The unbound viruses then were washed away, and the pH was shifted to 4.8 for 5 min. The endocytosis pathway was blocked with NH_4Cl. (A) Flow-cytometric analysis of the transduction rate of mammalian cells at 24 h p.t. The error bars represent standard deviations. (B) Fluorescence images of the transduced cells with virus at MOI = 50 at 24 h p.t. The upper images show the transduced HeLa cells. The lower images show transduced HepG2 cells.

increased the proportion of the transduced cells to a much higher level than that observed with acidic PBS alone, up to 44.2% (MOI = 10) and 78.5% (MOI = 50). When the same experiment was carried out on HeLa cells, the acidic PBS treatment of the virus-bound cells also improved the transduction rate significantly, up to 35.6% (MOI = 10) and 73.9% (MOI = 50). After incubation with NH_4Cl, the acid treatment again increased the transduction rate. The proportions of transduced cells were 42.3% (MOI = 10) and 72.5% (MOI = 50) (Fig. 7). In these experiments, the acid treatment of the virus-bound mammalian cells showed an ability to dramatically increase the proportion of the transduced cells, and the combined use of the endocytosis inhibitor NH_4Cl with acidic treatment could increase the transduction rate more significantly than the use of the acid trigger alone, at least on HepG2 cells. These results indicated that the presumptive artificial shift of the entry pathway increased the transduction rate in certain mammalian cells.

AcMNPV entering HepG2 cells with a low-pH trigger did not colocalize with the early endosome marker EEA1. It is well established that AcMNPV enters mammalian cells mainly through the endocytosis pathway, and the major capsid protein VP39 colocalizes with the early endosome marker EEA1 (28). Therefore, in our study, we investigated the viral entry pathway after the acidic treatment of the virus-bound cells by observing the localization of VP39 and EEA1 by immunofluorescence microscopy. The virus entering the cells through the endocytosis pathway was set as the control. In the control sample, VP39 was shown to colocalize with EEA1 in HepG2 cells at 35 min and 60 min p.t. (Fig. 8A, A′). This observation was consistent with results shown in Fig. 3 and a previous report (28). The same colocalization was observed in the cells pretreated with NH_4Cl (Fig. 8C, C′). When the virus-bound cells were treated with an external acidic environment, VP39 could enter the cells but failed to colocalize with EEA1 (Fig. 8B, B′). In the cells pretreated with NH_4Cl, the acid trigger also showed that VP39 and EEA1 did not colocalize (Fig. 8D, D′). These observations suggested that the significant increase of the transduction rate on the acid treatment of the virus-bound mammalian cells was due to the virus entering via the cell surface instead of through the endocytosis pathway.

DISCUSSION

In this report, we demonstrated that low-pH conditions can induce BV of AcMNPV to enter Sf9 cells by direct fusion with

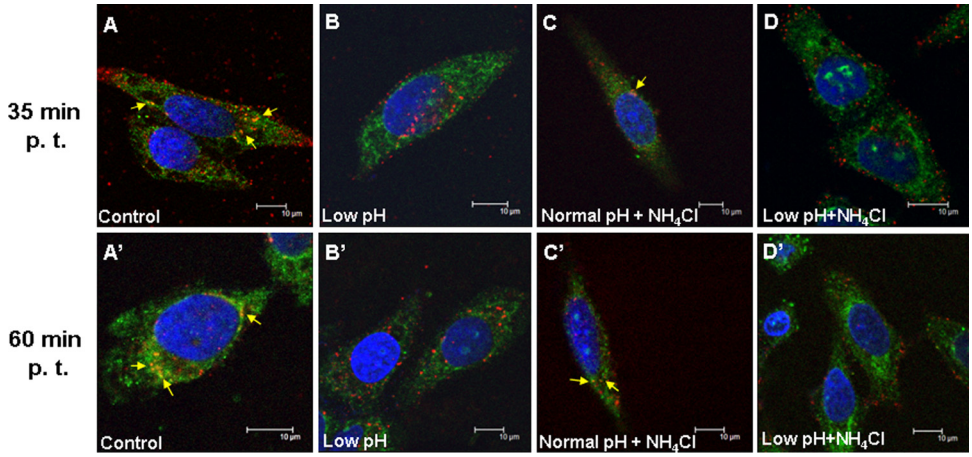

FIG. 8. Colocalization (yellow) of the vAc-CMV-EGFP (MOI = 50) capsid protein VP39 (red) with the early endosome marker EEA1 (green) at 35 or 60 min p.t. in HepG2 cells. EEA1 was conjugated with FITC, while VP39 was detected with rhodamine-tagged goat-anti rabbit antibodies. HepG2 cells transduced with neither NH_4Cl nor low-pH treatment was set up as the control sample. (A, A′) In the control sample, VP39 colocalized with EEA1 (arrows). (B, B′) In the low-pH sample, VP39 failed to colocalize with EEA1. (C, C′) In the NH_4Cl-treated sample, VP39 colocalized with EEA1 (arrows). (D, D′) In the low pH- and NH_4Cl-treated sample, VP39 also failed to colocalize with EEA1. Bar, 10 μm.

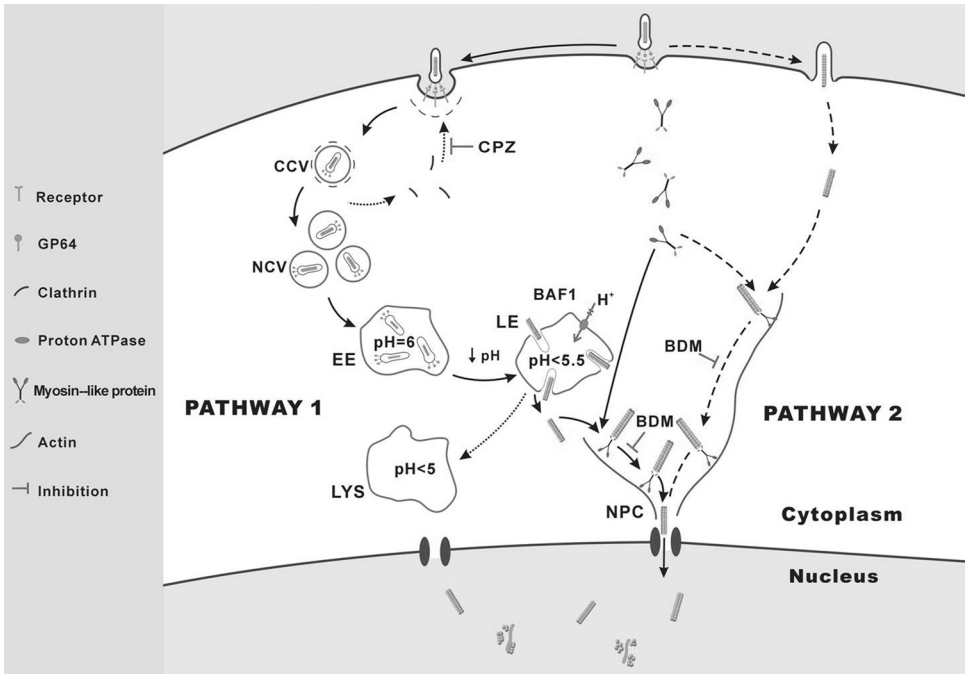

FIG. 9. Schematic of two different entry pathways of AcMNPV in Sf9 cells. Pathway 1 indicates the classic endocytosis pathway. BV enter the cells via clathrin-mediated endocytosis, and nucleocapsids are released from the endosomes and transported to the nucleus with the help of myosin-like proteins. Pathway 2 indicates the hypothetical direct fusion pathway. Virus envelopes fuse with the cell membrane and release the nucleocapsids into the cytoplasm when triggered by acidic conditions. The nucleocapsids then are transported to the nucleus with the help of myosin-like proteins. Both pathways lead to successful infection in Sf9 cells. CCV, clathrin-coated vesicle; NCV, noncoated vesicle; EE, early endosome; LE, late endosome; LYS, lysosome; NPC, nuclear pore complex; BAF1, bafilomycin A1.

the cell surface. This conclusion was supported by the following experiments. At normal pH, BV infection in Sf9 cells was sensitive to NH_4Cl and CPZ. This is consistent with the former reports that demonstrate that the clathrin-dependent endocytosis pathway is the major productive infection pathway (25, 28). However, the infectivity of the virus was recovered when the virus-bound cells were triggered by the acidic environment for a short time even in the presence of the endocytosis inhibitors (Fig. 2), suggesting that the acidic trigger forced AcMNPV to enter the Sf9 cells through an endocytosis-independent pathway. Immunofluorescent analysis showed that with low pH-induced entry, virus nucleocapsid remains at the cell periphery and did not colocalize with the early endosome marker EEA1, further confirming that virus did not enter via the endocytosis pathway. The R18-labeled membrane fusion assay further demonstrated that BV entered Sf9 cells via direct membrane fusion at the cell surface (Fig. 4). Taken together, these results illustrated well that the acidic environment could cause BV of AcMNPV to fuse with the cell surface and productively infect the cells. Furthermore, since ODV of AcMNPV (9) and *Lymantria dispar* NPV (LdNPV) (15) were demonstrated to enter insect cells via the direct fusion of ODV envelop with the host cell membrane, the direct membrane fusion pathway is suggested as being an important pathway for baculovirus nucleocapsid entry.

In addition, by using BDM and nocodazole, it was demonstrated that the infection via direct membrane fusion induced by low pH was myosin-like protein dependent and microtubule independent (Fig. 5). By integrating previous studies with our current observations, we generated a schematic representation of entry pathways of AcMNPV BV (Fig. 9). In this model, BV can enter and infect Sf9 cells through a clathrin-mediated endocytosis pathway during normal infection or a direct fusion pathway at the cell surface induced by low pH. In the clathrin-mediated endocytosis pathway, the virus is sorted into the clathrin-coated vesicles and then transported into the early endosomes and late endosomes. After fusion with the endosomes, the virions are released and transported into the nucleus with the help of actin and myosin-like proteins (23). In the direct fusion pathway, virus fused with the cell membrane under low pH and entered the cytoplasm. A most reasonable speculation of this process is that after viruses bind to the cell surface, external low pH induces the conformation change of GP64, which leads to the exposure and insertion of the fusion peptide into the plasma membrane and finally results in the fusion and release of the nucleocapsids. Considering the sensitivity to BDM, the inhibitor of the myosin-like proteins, which was consistent with the virus entering through the endocytosis pathway (Fig. 4), it was most likely that the virus utilized the same mechanism to transport their nucleocapsids into the nucleus (Fig. 9).

Previously, by analyzing the residual infectivity of AcV1 (a monoclonal antibody against GP64)-treated BV and immunoelectron microscopy data, it was reported that direct fusion with the cell surface is an alternative infection pathway of BV in insect cells, although it is considered an inefficient infection because it is thought to lead to the degradation of the virus genome (45). In our experiments, we also showed that at normal pH even if there is any direct fusion, it would be unde-

tectable, since NH_4Cl and CPZ could inhibit most of the infection (Fig. 2). However, when the AcMNPV infection was triggered with low pH, it resulted in a completely different outcome that is definitely considered the result of a successful infection in this particular circumstance.

For some viruses using the endocytosis pathway, the endosomal proteolysis is critical for viral infection. The endosomal proteolysis of the glycoprotein is necessary for infection by Ebola virus and SARS-CoV (3, 30, 38). Proteolysis by endosomal cathepsin L or B also is suggested to be a crucial mediator for reovirus disassembly that led to the release of the transcriptionally active virions in murine fibroblast cells (6). Although baculoviruses normally use endocytosis to enter insect and mammalian cells, it is suggested that the endocytic modification of the nucleocapsids is not necessary for the baculoviruses to travel to the nucleus in human hepatocytes. In these experiments, the nonenveloped nucleocapsids were microinjected into HepG2 cells, and the localization of nucleocapsids then was monitored by immunolabeling. At 6 h p.i., the stained nucleocapsids were observed in both the cytoplasm and nucleus (35). In our experiment, acid treatment forced the AcMNPV to fuse at the cell surface, releasing the nucleocapsids into the cytoplasm and finally enabling the productive infection in Sf9 cells. Since the virions were released at the cell surface and infected Sf9 cells through a nonendocytosis pathway, it is likely that the modifications of the nucleocapsids in the endosomes were not essential for the AcMNPV infection in insect cells.

BV of AcMNPV can transduce many mammalian cells and is considered a potential transduction vector for foreign gene expression and gene therapy (19, 20). Although the baculovirus-derived vector has emerged as an efficient gene transfer vector in mammalian cells, the large amount of virus needed for successful transduction imposed a restriction on the application of this system. Recently, several baculoviruses with a variety of different membrane attachment proteins were developed and showed an improvement of the transduction rate in certain mammalian cells (7, 8, 17, 34). However, most of these improvements require the modification of the baculoviruses, and a simpler method is required. In our experiment, once the entry pathway was modified with the acidic environment, a great improvement in the transduction rate was observed in both HepG2 and HeLa cells, even in the presence of NH_4Cl (Fig. 6). The great improvement of the transduction rate with the simple modification of the entry pathway made it much easier to transduce the mammalian cells, and a lower viral dose was needed to reach the same transduction rate without any gene modifications. However, whether this supplemental infection pathway also could improve the transduction rate in other mammalian cells or even expand the transduction range remains to be investigated.

In conclusion, this study revealed that with a low-pH trigger, BV of AcMNPV can infect insect cells by fusion at the cell surface as efficiently as the clathrin-mediated endocytosis pathway. This is the first demonstration of the existence of a second efficient infection pathway in BV of AcMNPV. We found that after fusion with the cell membrane, the myosin-like proteins aid the transport of the nucleocapsids to the nucleus while the intact microtubules are not necessary for this process. More importantly, we found that a low-pH trigger could significantly improve the transduction efficiency of AcMNPV in mammalian cells, which implies the potential application of this method when using baculovirus as a vector for heterologous gene expression and for gene therapy.

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REFERENCES

1. **Barsoum, J., R. Brown, M. McKee, and F. M. Boyce.** 1997. Efficient transduction of mammalian cells by a recombinant baculovirus having the vesicular stomatitis virus G glycoprotein. Hum. Gene Ther. **8:**2011–2018.
2. **Blissard, G. W., and J. R. Wenz.** 1992. Baculovirus gp64 envelope glycoprotein is sufficient to mediate pH-dependent membrane fusion. J. Virol. **66:**6829–6835.
3. **Chandran, K., N. J. Sullivan, U. Felbor, S. P. Whelan, and J. M. Cunningham.** 2005. Endosomal proteolysis of the Ebola virus glycoprotein is necessary for infection. Science **308:**1643–1645.
4. **Condreay, J. P., S. M. Witherspoon, W. C. Clay, and T. A. Kost.** 1999. Transient and stable gene expression in mammalian cells transduced with a recombinant baculovirus vector. Proc. Natl. Acad. Sci. USA **96:**127–132.
5. **Duisit, G., S. Saleun, S. Douthe, J. Barsoum, G. Chadeuf, and P. Moullier.** 1999. Baculovirus vector requires electrostatic interactions including heparan sulfate for efficient gene transfer in mammalian cells. J. Gene Med. **1:**93–102.
6. **Ebert, D. H., J. Deussing, C. Peters, and T. S. Dermody.** 2002. Cathepsin L and cathepsin B mediate reovirus disassembly in murine fibroblast cells. J. Biol. Chem. **277:**24609–24617.
7. **Ernst, W., T. Schinko, A. Spenger, C. Oker-Blom, and R. Grabherr.** 2006. Improving baculovirus transduction of mammalian cells by surface display of a RGD-motif. J. Biotechnol. **126:**237–240.
8. **Ge, J., Y. Huang, X. Hu, and J. Zhong.** 2007. A surface-modified baculovirus vector with improved gene delivery to B-lymphocytic cells. J. Biotechnol. **129:**367–372.
9. **Granados, R. R., and K. A. Lawler.** 1981. In vivo pathway of Autographa californica baculovirus invasion and infection. Virology **108:**297–308.
10. **Guarino, L. A., and M. D. Summers.** 1987. Nucleotide sequence and temporal expression of a baculovirus regulatory gene. J. Virol. **61:**2091–2099.
11. **Hefferon, K. L., A. G. Oomens, S. A. Monsma, C. M. Finnerty, and G. W. Blissard.** 1999. Host cell receptor binding by baculovirus GP64 and kinetics of virion entry. Virology **258:**455–468.
12. **Helenius, A., J. Kartenbeck, K. Simons, and E. Fries.** 1980. On the entry of Semliki forest virus into BHK-21 cells. J. Cell Biol. **84:**404–420.
13. **Herrmann, C., J. Wray, F. Travers, and T. Barman.** 1992. Effect of 2,3-butanedione monoxime on myosin and myofibrillar ATPases. An example of an uncompetitive inhibitor. Biochemistry **31:**12227–12232.
14. **Hofmann, C., V. Sandig, G. Jennings, M. Rudolph, P. Schlag, and M. Strauss.** 1995. Efficient gene transfer into human hepatocytes by baculovirus vectors. Proc. Natl. Acad. Sci. USA **92:**10099–10103.
15. **Horton, H. M., and J. P. Burand.** 1993. Saturable attachment sites for polyhedron-derived baculovirus on insect cells and evidence for entry via direct membrane fusion. J. Virol. **67:**1860–1868.
16. **Jones, I., and Y. Morikawa.** 1996. Baculovirus vectors for expression in insect cells. Curr. Opin. Biotechnol. **7:**512–516.
17. **Kaikkonen, M. U., J. K. Raty, K. J. Airenne, T. Wirth, T. Heikura, and S. Yla-Herttuala.** 2006. Truncated vesicular stomatitis virus G protein improves baculovirus transduction efficiency in vitro and in vivo. Gene Ther. **13:**304–312.
18. **Kaljot, K. T., R. D. Shaw, D. H. Rubin, and H. B. Greenberg.** 1988. Infectious rotavirus enters cells by direct cell membrane penetration, not by endocytosis. J. Virol. **62:**1136–1144.
19. **Kost, T. A., and J. P. Condreay.** 2002. Recombinant baculoviruses as mammalian cell gene-delivery vectors. Trends Biotechnol. **20:**173–180.
20. **Kost, T. A., J. P. Condreay, and D. L. Jarvis.** 2005. Baculovirus as versatile vectors for protein expression in insect and mammalian cells. Nat. Biotechnol. **23:**567–575.
21. **Kozuma, K., and T. Hukuhara.** 1994. Fusion characteristics of a nuclear polyhedrosis virus in cultured cells: time course and effect of a synergistic factor and pH. J. Invertebr. Pathol. **63:**63–67.
22. **Lakadamyali, M., M. J. Rust, and X. Zhuang.** 2004. Endocytosis of influenza viruses. Microbes Infect. **6:**929–936.
23. **Lanier, L. M., and L. E. Volkman.** 1998. Actin binding and nucleation by Autographa california M nucleopolyhedrovirus. Virology **243:**167–177.
24. **Leikina, E., H. O. Onaran, and J. Zimmerberg.** 1992. Acidic pH induces

fusion of cells infected with baculovirus to form syncytia. FEBS Lett. **304:** 221–224.
25. **Long, G., X. Pan, R. Kormelink, and J. M. Vlak.** 2006. Functional entry of baculovirus into insect and mammalian cells is dependent on clathrin-mediated endocytosis. J. Virol. **80:**8830–8833.
26. **Maddon, P. J., J. S. McDougal, P. R. Clapham, A. G. Dalgleish, S. Jamal, R. A. Weiss, and R. Axel.** 1988. HIV infection does not require endocytosis of its receptor, CD4. Cell **54:**865–874.
27. **Marsh, M., and R. Bron.** 1997. SFV infection in CHO cells: cell-type specific restrictions to productive virus entry at the cell surface. J. Cell Sci. **110**(Pt 1)**:**95–103.
28. **Matilainen, H., J. Rinne, L. Gilbert, V. Marjomaki, H. Reunanen, and C. Oker-Blom.** 2005. Baculovirus entry into human hepatoma cells. J. Virol. **79:**15452–15459.
29. **Matlin, K. S., H. Reggio, A. Helenius, and K. Simons.** 1981. Infectious entry pathway of influenza virus in a canine kidney cell line. J. Cell Biol. **91:**601–613.
30. **Matsuyama, S., M. Ujike, S. Morikawa, M. Tashiro, and F. Taguchi.** 2005. Protease-mediated enhancement of severe acute respiratory syndrome coronavirus infection. Proc. Natl. Acad. Sci. USA **102:**12543–12547.
31. **McClure, M. O., M. Marsh, and R. A. Weiss.** 1988. Human immunodeficiency virus infection of CD4-bearing cells occurs by a pH-independent mechanism. EMBO J. **7:**513–518.
32. **Nicola, A. V., A. M. McEvoy, and S. E. Straus.** 2003. Roles for endocytosis and low pH in herpes simplex virus entry into HeLa and Chinese hamster ovary cells. J. Virol. **77:**5324–5332.
33. **Possee, R. D.** 1997. Baculoviruses as expression vectors. Curr. Opin. Biotechnol. **8:**569–572.
34. **Raty, J. K., K. J. Airenne, A. T. Marttila, V. Marjomaki, V. P. Hytonen, P. Lehtolainen, O. H. Laitinen, A. J. Mahonen, M. S. Kulomaa, and S. Yla-Herttuala.** 2004. Enhanced gene delivery by avidin-displaying baculovirus. Mol. Ther. **9:**282–291.
35. **Salminen, M., K. J. Airenne, R. Rinnankoski, J. Reimari, O. Valilehto, J. Rinne, S. Suikkanen, S. Kukkonen, S. Yla-Herttuala, M. S. Kulomaa, and M. Vihinen-Ranta.** 2005. Improvement in nuclear entry and transgene expression of baculoviruses by disintegration of microtubules in human hepatocytes. J. Virol. **79:**2720–2728.
36. **Shoji, I., H. Aizaki, H. Tani, K. Ishii, T. Chiba, I. Saito, T. Miyamura, and Y. Matsuura.** 1997. Efficient gene transfer into various mammalian cells, including non-hepatic cells, by baculovirus vectors. J. Gen. Virol. **78**(Pt 10)**:**2657–2664.
37. **Sieczkarski, S. B., and G. R. Whittaker.** 2002. Influenza virus can enter and infect cells in the absence of clathrin-mediated endocytosis. J. Virol. **76:** 10455–10464.
38. **Simmons, G., D. N. Gosalia, A. J. Rennekamp, J. D. Reeves, S. L. Diamond, and P. Bates.** 2005. Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. Proc. Natl. Acad. Sci. USA **102:**11876–11881.
39. **Skehel, J. J., P. M. Bayley, E. B. Brown, S. R. Martin, M. D. Waterfield, J. M. White, I. A. Wilson, and D. C. Wiley.** 1982. Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. Proc. Natl. Acad. Sci. USA **79:**968–972.
40. **Sun, X., V. K. Yau, B. J. Briggs, and G. R. Whittaker.** 2005. Role of clathrin-mediated endocytosis during vesicular stomatitis virus entry into host cells. Virology **338:**53–60.
41. **Takada, A., C. Robison, H. Goto, A. Sanchez, K. G. Murti, M. A. Whitt, and Y. Kawaoka.** 1997. A system for functional analysis of Ebola virus glycoprotein. Proc. Natl. Acad. Sci. USA **94:**14764–14769.
42. **van Loo, N. D., E. Fortunati, E. Ehlert, M. Rabelink, F. Grosveld, and B. J. Scholte.** 2001. Baculovirus infection of nondividing mammalian cells: mechanisms of entry and nuclear transport of capsids. J. Virol. **75:**961–970.
43. **Volkman, L. E., and P. A. Goldsmith.** 1983. In vitro survey of Autographa californica nuclear polyhedrosis virus interaction with nontarget vertebrate host cells. Appl. Environ. Microbiol. **45:**1085–1093.
44. **Volkman, L. E., and P. A. Goldsmith.** 1985. Mechanism of neutralization of budded Autographa californica nuclear polyhedrosis virus by a monoclonal antibody: inhibition of entry by adsorptive endocytosis. Virology **143:**185–195.
45. **Volkman, L. E., P. A. Goldsmith, and R. T. Hess.** 1986. Alternate pathway of entry of budded Autographa californica nuclear polyhedrosis virus: fusion at the plasma membrane. Virology **148:**288–297.
46. **Volkman, L. E., and M. D. Summers.** 1977. Autographa californica nuclear polyhedrosis virus: comparative infectivity of the occluded, alkali-liberated, and nonoccluded forms. J. Invertebr. Pathol. **30:**102–103.
47. **Wang, L. H., K. G. Rothberg, and R. G. Anderson.** 1993. Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. J. Cell Biol. **123:**1107–1117.
48. **Wang, M., Y. Tan, F. Yin, F. Deng, J. M. Vlak, Z. Hu, and H. Wang.** 2008. The F protein of Helicoverpa armigera single nucleopolyhedrovirus can be substituted functionally with its homologue from Spodoptera exigua multiple nucleopolyhedrovirus. J. Gen. Virol. **89:**791–798.
49. **Wang, P., D. A. Hammer, and R. R. Granados.** 1997. Binding and fusion of Autographa californica nucleopolyhedrovirus to cultured insect cells. J. Gen. Virol. **78:**3081–3089.
50. **Wang, X., and D. C. Kelly.** 1985. Baculovirus replication: uptake of Trichoplusia ni nuclear polyhedrosis virus particles by insect cells. J. Gen. Virol. **66:**541–550.
51. **White, J., J. Kartenbeck, and A. Helenius.** 1980. Fusion of Semliki forest virus with the plasma membrane can be induced by low pH. J. Cell Biol. **87:**264–272.