Published in final edited form as:

Virus Res. 2014 March 6; 181: 81–91. doi:10.1016/j.virusres.2014.01.006.

Visualization of Intracellular Pathways of Engineered Baculovirus in Mammalian Cells

Yarong Liu[†], Kye-Il Joo[†], Yuning Lei[†], and Pin Wang^{†,‡,§,*}

[†]Mork Family Department of Chemical Engineering and Materials Science, University of Southern California, Los Angeles, CA 90089

[‡]Department of Biomedical Engineering, University of Southern California, Los Angeles, CA 90089, USA

[§]Department of Pharmacology and Pharmaceutical Sciences, University of Southern California, Los Angeles, CA 90089, USA

Abstract

Baculoviruses are a promising gene delivery vector. They have the ability to express large transgenes in mammalian cells without displaying pathogenicity in humans; however, little is known about their transduction mechanisms in target cells. In this study, we use colocalization and live-cell imaging studies to elucidate the internalization and intracellular trafficking pathways of baculoviruses through direct visualization of VP39-GFP-labeled viral particles and various endocytic structures within target cells. Drug inhibition and confocal microscopy results suggested that baculoviruses enter the cells via clathrin-mediated endocytosis in a dynamin-dependent manner. Viral particles were shown to traffic through early endosomes, triggering a low-pH-dependent endosomal fusion process of viruses. Suppressed autophagy activity enhanced viral transduction and overexpression of autophagosomes reduced viral transduction, suggesting that autophagy is involved in degradation process of viral particles. Actin filaments were involved in the viral transduction, while microtubules negatively regulated viral transduction by facilitating the fusion of autophagosomes with lysosomes to form autolysosomes, where degradation of viral particles occurs. These results shed some light on the essential cellular factors limiting viral transduction, which can be used to improve the use of baculoviral vectors in cell and gene therapy.

Keywords

Baculovirus; intracellular trafficking; endosomal fusion; autophagosome; microtubule

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*Address correspondence to Mork Family Department of Chemical Engineering and Materials Science University of Southern California 3710 McClintock Ave., RTH509 Los Angeles, CA 90089 Phone: (213)-740-0780 Fax: (213)-740-8053 pinwang@usc.edu.

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1.Introduction

Recently, the insect baculovirus *Autographa californica multiple* nucleopolyhedrovirus (AcMNPV) has emerged as a promising vector for gene therapy. This DNA virus is able to enter mammalian cells and express transgenes under the control of mammalian promoters (Boyce and Bucher, 1996; Condreay et al., 1999; Hofmann et al., 1995; Shoji et al., 1997; Tani et al., 2003), without any replication inside mammalian cells, thereby reducing the risk of side-effects (Kost and Condreay, 2002; Sandig et al., 1996; Shoji et al., 1997). The virus has a 130-kb double-stranded DNA (dsDNA) genome, which can accommodate foreign DNA fragments up to 38kb (Cheshenko et al., 2001), allowing for fast construction of recombinant viruses with high titer (Davies, 1994). Studying baculovirus-target cell interactions will further the development and optimization of more efficient gene therapy vector system.

Baculovirus AcMNPV has been shown to enter both insect and mammalian cells via adsorptive endocytosis (Blissard and Rohrmann, 1990; van Loo et al., 2001; Volkman and Goldsmith, 1985; Wang et al., 1997); however, how cell-surface molecules interact with the baculovirus during uptake is unclear. Previous reports suggest that baculoviruses can enter cells through either clathrin-dependent (Long et al., 2006; Matilainen et al., 2005) or clathrin-independent (Laakkonen et al., 2009) endocytosis pathways. Recently, other pathways, such as macropinocytosis, have also been associated with the internalization of baculoviruses (Kataoka et al., 2012). The major envelope glycoprotein of baculovirus AcMNPV, GP64, (Tani et al., 2003; Tani et al., 2001) has been shown to attach to the cells, which triggers receptor-mediated endocytosis (Hefferon et al., 1999). GP64 can also contribute to acid-induced endosomal escape through a conformational change that occurs in low-PH environments, like endosomes. This conformational change transforms GP64 to a fusion-competent protein, but the direct mechanism of the protein in endosomal fusion and escape of baculoviruses to the cytoplasm remain elusive (Blissard and Wenz, 1992; Markovic et al., 1998; van Loo et al., 2001). Understanding the entry mechanism and intracellular fate of baculoviruses will help establish methods to improve the efficacy of baculoviruses as gene delivery vehicles for gene therapy.

In this study, we investigated the intracellular trafficking routes of AcMNPV in mammalian cells using confocal microscopy to directly visualize the trafficking dynamics of individual fluorescent-tagged baculoviral particles. We also investigated the functional involvement of endocytic structures in the entry and endosomal fusion of baculoviruses in living cells. Our results suggest that baculoviruses enter HeLa cells through clathrin-mediated endocytosis in a dynamin-dependent manner, and continue to move along cytoskeleton networks inside the cells. Fusion of baculovirus envelopes occurs in early endosomes and is pH-dependent, as demonstrated by drug inhibition, dominant-negative mutation, and live-cell imaging of the fusion process. Autophagy plays an essential role in viral transduction, while microtubules negatively regulate viral transduction by transporting viral particles from autophagosomes to lysosomes for viral degradation. Here, we have attempted to unravel the process of baculovirus intracellular trafficking in mammalian cells to provide a better understanding of the rate-limiting steps required for successful transduction of baculoviruses and potentially expand the applicability of these vectors in gene therapy.

2. Materials and Methods

2.1. Cell lines, antibodies and reagents

HeLa cells were maintained in a 5% CO₂ environment with Dulbecco's modified Eagle's medium (DMEM, Mediatech, Inc., Manassas, VA) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO) and 2 mM L-glutamine (Hyclone Laboratories, Inc., Omaha, NE). The mouse monoclonal antibodies against clathrin and caveolin-1 and the rabbit polyclonal antibody specific to Cation-Independent Mannose 6-Phosphate Receptor (CI-MPR) were purchased from Abcam (Cambridge, MA). Mouse monoclonal anti-EEA1 antibody, rabbit polyclonal anti-Rab11 antibody, lysosome-associated membrane protein 1 (Lamp-1), anti-LC3A/B, and Alexa 647-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal antibody to α -tubulin was obtained from Sigma-Aldrich. Rhodamine-conjugated phalloidin, Texas red-conjugated and Alexa488-conjugated goat anti-mouse immunoglobulin G (IgG) antibody were purchased from Invitrogen (Carlsbad, CA). Bafilomycin A1, chlorpromazine, filipin, nocodazole, cyto-D, M β CD, rapamycin, and 3-MA were obtained from Sigma-Aldrich.

2.2. Vector production

To produce VP39-GFP-labeled baculoviruses (AcMNPV), the VP39 genes were amplified and then fused to a GFP gene via PCR using previously described methods (Kukkonen et al., 2003). The amplified DNA was then cloned into the pFastBac Dual construct. Similarly, to produce baculoviruses encoding a GFP gene, the GFP gene was amplified from pcDNA3-GFP and cloned into the pFastBac Dual construct. Recombinant baculoviruses with the described expression cassettes were produced and propagated in SF9 cells according to the manual of the Bac-to-Bac Baculovirus Expression system from Invitrogen. Briefly, the plasmids were transfected into cells using Cellfectin®II Reagent. Budded viruses were filtered through a 0.45- μ m pore size filter (Nalgene, Rochester, NY) to remove any cell debris and concentrated via centrifugation at 8000 $\times$ g for 5 hours at 20 °C. Viral pellets were resuspended in HBSS (Invitrogen). To determine the titers of the recombinant BVs, a qPCR assay was adapted to quantify the physical number of viral particles (Hitchman et al., 2007; Lei et al., 2011). The BacPAK Baculovirus Rapid Titer Kit (Clontech, Mountain View, CA) was then used to determine the infectious units.

2.3. Viral transduction

HeLa cells (0.1×10^6 per well) were seeded in a 24-well culture dish and transduced by baculoviruses (MOI~50) encoding a reporter GFP gene at 1050 $\times$ g at 30°C for 90 min (Sorval Legend centrifuge). The cells were then washed and cultured for 3 days before FACS analysis of GFP⁺ cells. For drug treatments, cells were incubated with one of the following cyto-D (20 μ M), nocodazole (30 μ M), bafilomycin A1 (50 nM), chlorpromazine (30 nM), filipin (15 nM), amiloride (1 mM), M β CD (15 mM), 3MA (5 mM), and rapamycin (1 μ M) for 30 min at 37°C before transduction, as described earlier. Drug concentration was maintained (except M β CD) during transduction.

2.4. Dominant-negative mutant for dynamin and endosomal dependency

The dominant-negative form (Dyn- K44A) of DsRed-dynamin was provided by Dr. Okamoto (Department of Pharmacology and Pharmaceutical Sciences, University of Southern California). HeLa cells were transfected with the construct encoding the dominant-negative mutant form of dynamin, Rab 5 or Rab7. 24 h post-transfection, cells were incubated with VP39-GFP-labeled baculoviruses and dye-labeled transferrin for 1 h. Following incubation, the cells were fixed and imaged. For the transduction experiment, 24 h post-transfection, the cells were seeded at 0.1×10^6 cells per well in a 24-well culture dish and transduced with baculoviruses encoding a GFP gene. The cells were analyzed for GFP expression by FACS 72 h post-infection.

2.5. Confocal imaging

A Yokogawa spinning-disk confocal scanner system (Solamere Technology Group, Salt Lake City, UT) with a Nikon eclipse Ti-E microscope equipped with a 60x/1.49 Apo TIRF oil objective and a Cascade II: 512 EMCCD camera (Photometrics, Tucson, AZ, USA) was used to acquire the fluorescent images. An AOTF (acousto-optical tunable filter)-controlled laser-merge system (Solamere Technology Group Inc., Salt Lake City, UT) was used to provide power for each of the laser lines: 491 nm, 561 nm, and 640 nm solid state lasers (50 mW for each laser).

2.6. Clathrin-dependent imaging study

For the colocalization study with clathrin or caveolin-1 markers, HeLa cells were incubated with VP39-GFP-labeled baculoviruses for 30 min at 4 °C to synchronize cell attachment and then shifted to 37 °C for the indicated time periods. Afterwards, the cells were fixed, permeabilized and immunostained with antibodies specific to clathrin and caveolin-1 or counterstained with DAPI. For real-time imaging of dynamic interactions between viral particles and clathrin structures, HeLa cells were transiently transfected with mRFP-clathrin. After 4 h of incubation at 37 °C, the media was replaced with fresh D10 media. At 24 h post-transfection, the cells were incubated with VP39-GFP-labeled baculoviruses for 30 min at 4 °C and then warmed to 37 °C to induce viral internalization. Confocal time-lapse images were acquired every 10 s over a time period of 10 min. Fluorescent intensity versus time within the regions of interest was measured and analyzed by using Nikon NIS-Elements software.

2.7. Imaging low-pH-dependent endosomal fusion of baculoviruses

For fusion studies, concentrated VP39-GFP-labeled baculoviruses were incubated with 100 μ M of DiD (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine), a lipophilic dye (Molecular Probes), for 1 h at room temperature. Unbound dye was removed by Microcon filter units with a 50 kDa cutoff (Millipore, Billerica, MA). To confirm the low-pH dependency of the viral fusion, the cells were treated with bafilomycin A1, which is known to elevate endosome pH, for 1h. Treated and untreated cells were incubated with the DiD/VP39-GFP-double-labeled baculoviruses for 30 min at 4°C to synchronize cell attachment and then shifted to 37 °C for 30 min. The cells were then fixed and imaged. For live-cell imaging of fusion, the cells were incubated with the DiD/VP39-GFP-double-labeled

2.11. Statistical Analysis

The results were expressed as means $\pm$ standard deviation. The significance of the difference in the means was determined by Student *t* test.

3. Results

3.1. Clathrin-dependent entry of recombinant baculoviruses

Consensus has held that clathrin- and caveolin-mediated pathways of endocytosis are the main routes of entry for many viruses (Mountain, 2000). Although it has been suggested that entry of baculoviruses is primarily dependent on clathrin-mediated endocytosis in both insect and mammalian cells (Long et al., 2006; Matilainen et al., 2005), clathrin-independent and macropinocytosis-mediated internalization were also reported (Kataoka et al., 2012; Laakkonen et al., 2009). To investigate the role of clathrin- or caveolin-dependent endocytosis on the entry of baculoviruses, we visualized the individual baculoviral particles and endocytic structures (clathrin or caveolin) in HeLa cells. To accomplish this, baculoviruses were labeled with green fluorescent protein (GFP) fused to the C-terminal end of the capsid protein, VP39. This construction did not affect natural infectivity (Kukkonen et al., 2003). As shown in Figure 1A and 1C, baculoviral particles were significantly colocalized with clathrin structures when VP39-GFP-labeled baculoviruses were incubated with HeLa cells for 15 min at 37°C. After 30 min of incubation, the colocalization of viral particles with clathrin was significantly decreased, suggesting that many viruses had already dissociated from the clathrin vesicles. Although some baculoviral particles were overlaid with caveolin structures, no significant colocalization was observed at either 15 or 30 min of incubation, suggesting that the caveolin-mediated pathways are not significantly involved in the entry of baculoviruses (Figure 1B and C).

To further confirm the importance of clathrin-dependent endocytosis over caveolin-dependent endocytosis, cells were treated with either chlorpromazine (CPZ), a drug known to prevent clathrin polymerization thus inhibiting internalization mediated by clathrin-coated vesicles (Rink et al., 2005), or filipin, a cholesterol-binding reagent that blocks caveolin-dependent internalization (Stoorvogel et al., 1991). As shown in Figure 1D, CPZ significantly inhibited baculovirus transduction of HeLa cells, while filipin exhibited no inhibitory effect, indicating a strong dependence on clathrin-dependent endocytosis. Also, cholesterol depletion with methyl-beta-cyclodextrin (M β CD) showed no inhibitory effect of viral transduction, indicating that cholesterol in the target cell membrane does not play a role in baculovirus internalization.

To monitor the interactions between baculoviruses and the clathrin structures in real time, VP39-GFP-labeled viruses were incubated with live HeLa cells expressing clathrin fused red fluorescent protein (mRFP-clathrin) and imaged using time-lapse spinning confocal microscopy. Figure 1E shows selected images obtained from the time series as well as a representative trajectory of VP39-GFP-labeled baculovirus. Colocalization of the viral particle (green) with the clathrin signal (red) started at 100s. This colocalization was maintained for 250 s, after which the clathrin signal rapidly disappeared, suggesting that the virus dissociated from the uncoated clathrin vesicle (Figure 1E and 1F). While the particle

was colocalized with the clathrin structure (100-250s), the instantaneous diffusion coefficients of the viral particle were significantly lower compared to those before association or after dissociation from the clathrin signal (Figure 1F), indicating that the viral particle entered through the confined region of the clathrin-coated pit on the cell's outer membrane.

Dynamin, a large GTPase, is associated with the formation of clathrin-coated vesicles, and may serve as another indicator of clathrin-dependent internalization (Van der Bliek et al., 1993). A dominant-negative (DN) mutant construct (Dyn-K44A) was used to disable dynamin function in order to investigate the functional involvement of dynamin on the internalization of baculoviruses (Joo et al., 2010). HeLa cells were transfected with either dynamin wild-type (WT) or DN mutant constructs 24 h prior to infection by baculoviruses. We used transferrin as a positive control for this experiment because it is known to enter cells through dynamin-dependent endocytosis. HeLa cells that expressed DsRed wild-type or Dyn-K44A mutants were incubated with VP39-GFP-labeled baculoviruses and dye-labeled transferrin for 60 min at 37 °C, followed by immunofluorescence microscopy. As shown in Figure 2A, the internalization of VP39-GFP-labeled baculoviruses and dye-labeled transferrin were significantly limited in the Dyn-K44A-expressing cells compared to the dynamin-wild type-expressing cells, indicating that the entry of baculoviruses into HeLa cells is dynamin-dependent. FACS analysis 3 days post-transduction showed that transduction by baculoviruses was significantly reduced in cells containing the DN construct compared to cells expressing wild-type dynamin (Figure 2B). Taken together, these results demonstrate that baculoviruses can be internalized by cells through clathrin-mediated endocytosis in a dynamin-dependent manner.

3.2. Involvement of endosomal trafficking of baculoviruses

Endosomal transport of viral particles is considered an essential step of viral transduction. A low-pH endosomal environment is thought to induce the fusion of baculoviruses to the vessel membrane, mediated by the viral glycoprotein GP64 (Blissard and Wenz, 1992). The colocalization of baculoviruses with early endosomal compartments, but not late endosomes, was observed in several independent studies (Kukkonen et al., 2003; Matilainen et al., 2005). To explore the involvement of various endosomes in the intracellular routes of baculoviruses, we visualized HeLa cells incubated with VP39-GFP-labeled baculoviruses at different time points and antibody-stained for markers of early or late endosomes with early endosome antigen 1 (EEA1) (Lakadamyali et al., 2006; Sieczkarski and Whittaker, 2003; Vonderheit and Helenius, 2005) and cation-independent mannose-6-phosphate receptor (CI-MPR) (Lakadamyali et al., 2006; Urayama et al., 2004; Vonderheit and Helenius, 2005), respectively. After 15 min of incubation, the acquired images showed that ~33% of baculoviral particles were observed in endosomes positive for EEA1 (Figure 3A, left), while only ~20% of viral particles were colocalized with CI-MPR. At 30 min, over 60% of viral particles were seen in the early endosomes (EEA1, Figure 3A, middle) while only ~26% of particles were in late endosomes (CIMPR), indicating that early, not late, endosomes are involved in the intracellular trafficking of baculoviruses. After 60 min of incubation, a decreased level of colocalization between viral particles and early endosomes was observed, while no significant colocalization of viral particles with late endosomes was seen.

Quantitative colocalization data for these images is shown in Figure 3B, indicating that viral particles traffic from early endosomes to other compartments or directly escape from early endosomes to cytosol before nuclear transport.

To further validate the confocal imaging results, dominant-negative constructs of Rab proteins were used to perturb early (Rab5) or late (Rab7) endosome function. HeLa cells transfected with wild-type or dominant-mutant form of Rab5 or Rab7 were transduced with baculoviruses encoding a reporter GFP gene. As shown in Figure 3C, expression of dominant-negative Rab5, affecting early endosomes, significantly reduced viral transduction compared with that of wild-type Rab5-expressing cells ($p < 0.01$), confirming the functional involvement of early endosomes in viral transduction. In contrast, viral transduction in HeLa cells was not affected by expression of the dominant-negative form of Rab7, which affects late endosomes, suggesting that late endosomes are not required for the viral transduction pathway of baculoviruses, and confirming the confocal imaging results.

3.3. Endosomal fusion of baculoviruses

It is generally believed that enveloped viruses fuse their viral envelope with cell membrane or endosomal membranes to introduce their genetic materials into cells. Although the envelope glycoprotein GP64 of baculoviruses has traditionally been considered a viral attachment protein involved in host cell receptor binding (Hefferon et al., 1999; Zhou and Blissard, 2008), more and more evidence has shown that it undergoes a conformational change at low pH, which allows for a fusion-competent state that is necessary for the release of the nucleocapsid into cytosol before nuclear transport in insect cells (Blissard and Wenz, 1992; Kingsley et al., 1999; Markovic et al., 1998; Plonsky et al., 1999). To visualize the actual fusion event of baculoviruses within mammalian cells, a double-labeling method with a lipophilic dye, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine (DiD), and VP39-GFP was used to label the viral membrane and core of baculoviruses, respectively. The incorporation of DiD at high concentrations on viral membrane can lead to self-quenching of DiD fluorescence (Joo et al., 2008; Lakadamyali et al., 2006; Sakai et al., 2006). Viral fusion with endosomal membranes can be detected by DiD fluorescent dequenching that results from the dispersion of DiD dyes into endosomal membranes (Joo et al., 2010). The VP39-GFP fluorescent capsid enables us to track uncoated viral cores that have dissociated from the fused envelope and been released to the cytosol (Kukkonen et al., 2003).

To investigate the pH-dependency of baculovirus membrane fusion, HeLa cells were treated with bafilomycin A1, a specific inhibitor for vacuolar proton ATPases (Bowman et al., 1988), and incubated with DiD/VP39-GFP-double-labeled baculoviruses for 30 min. As shown in Figure 4A, after 30 min of incubation, many viral particles fused with endosomes, as demonstrated by the significant colocalization of baculoviral particles (VP39-GFP) and fusion signals (DiD) in untreated cells. However, no significant fusion signals (DiD) were observed in HeLa cells treated by bafilomycin A1, suggesting that a low-pH environment is required for the fusion event to occur. The functional involvement of low-pH endosomal environment in viral fusion was further confirmed by a significant inhibition of virus-mediated GFP expression in HeLa cells treated by bafilomycin A1 (Figure 4B).

To visualize the multiple steps of endosomal fusion, DiD/VP39-GFP-double-labeled baculoviruses were incubated with HeLa cells at 37 °C for 30 min to induce virus–endosome fusion. Confocal time-lapse images were acquired every 10s over a time period of 10 min (Supplementary Movie 1). A typical viral trajectory, undergoing endosomal fusion, and the corresponding time trajectories of viral velocity and fluorescence intensity are presented in Figure 4C and 4D. From 0–200 s, only VP39-GFP viral capsid signals were observed, indicating that the fusion had not been initiated (Figure 4C). At 260 s, the viral particle shifts from green to yellow (a merging of the green and red dyes), suggesting that endosomal fusion had now taken place. The membrane fusion event was confirmed by the sudden increase in fluorescence ratio (DiD/VP39-GFP), shown in Figure 4D.

3.4. Baculovirus transport mediated by microtubule and actin

Cytoskeleton networks, such as microtubules or actin filaments, are known to play an essential role in cellular transport of many viruses to the nucleus. To elucidate the functional involvement of actin filaments and/or microtubules in the intracellular transport of baculoviruses, we conducted a colocalization experiment using α -tubulin-specific and actin-specific antibodies in HeLa cells. After 1 hour of incubation, a significant number of viral particles (VP39-GFP) were detected on both microtubules (Figure 5A) and actin filament (Figure 5B), implying that baculovirus might transport along both microtubule and actin networks within mammalian cells. The imaging results were further confirmed by a drug inhibition experiment using cytochalasin D (Cyto-D), which depolymerizes actin filaments, and nocodazole, which depolymerizes microtubules. FACS analysis of the GFP⁺ cells 3 days post-transduction (Figure 5C) revealed that actin blockage by Cyto-D significantly reduced the transduction level to about 39% of the control ($p < 0.01$), while microtubule depolymerization by nocodazole increased viral transduction ($p < 0.01$). This data suggests that both microtubules and actin filaments are involved in viral transduction, but that microtubules play a negative role in the viral transduction of baculoviruses. This has been reported by other groups (Salminen et al., 2005; van Loo et al., 2001), although the mechanisms by which microtubules inhibit transduction remain unknown.

3.5. Involvement of autophagy network in viral transduction

More and more evidence has suggested that autophagy networks play an essential role in the viral transduction process, aiding in the degradation of viruses or the replication or release of viruses from infected cells (Kirkegaard et al., 2004; Orvedahl and Levine, 2008; Shoji-Kawata and Levine, 2009; Wong et al., 2008). Moreover, it has been reported that some positive-strand RNA viruses, such as poliovirus, equine arterivirus or mouse hepatitis virus, could stimulate the accumulation of membranes bearing autophagic markers, which form autophagosomes (Kirkegaard et al., 2004; Snijder et al., 2001; Suhy et al., 2000). Investigation of the functional involvement of autophagy in the viral infection process of baculoviruses might provide a better understanding of the essential steps involved in the successful transduction of baculoviruses.

To investigate the involvement of autophagy in the intracellular trafficking pathway of baculoviruses, we visualized the individual VP39-GFP-labeled viral particles along with autophagosomes in HeLa cells expressing mRFP-LC3, which was used to monitor the

occurrence of autophagy (Kimura et al., 2007). As shown in Figure 6A, after incubation for 90 min, VP39-GFP was significantly colocalized with mRFP-LC3, suggesting that a good portion of baculoviruses tend to transport to autophagosomes. To verify the role of autophagy in viral transduction, HeLa cells were treated with rapamycin or 3-methyladenine (3-MA) before incubation with baculoviruses to enhance or reduce autophagy activity, respectively. As shown in Figure 6B, inhibition of autophagy activity by 3-MA significantly enhanced viral transduction efficiency compared to no drug treatment ($p < 0.01$), indicating that autophagy negatively affects the viral transduction of baculoviruses. On the other hand, the enhancement of autophagy by rapamycin treatment showed no effect on viral infectivity, suggesting that autophagy activity could not be further enhanced by drug treatment, probably because the viral infection itself had already enhanced autophagy activity to a significant extent. To test our hypothesis, mRFP-LC3-expressing HeLa cells were incubated with baculoviruses or rapamycin, and the maturation of autophagosome, defined as the colocalization of mRFP-LC3 and Lamp-1, was measured. As shown in Figure 6C and 6D, rapamycin treatment led to the colocalization of mRFP-LC3 and Lamp-1, while the signals remained distinct in untreated cells. Similarly, after 90 min of incubation with baculovirus, a significant colocalization of mRFP-LC3 with Lamp-1 was observed, indicating that viral infection could increase the activity of autophagy, which may lead to virus degradation in autolysosomes. The negative role of autophagy in viral transduction was confirmed by a significant decreased viral transduction in mRFP-LC3-expressing HeLa cells compared to that in mock-transfected cells (Figure 6E).

It has been proposed that acetylated microtubules are required for the fusion of autophagosomes with lysosomes to form autolysosomes (Webb et al., 2004; Xie et al., 2010), suggesting that microtubules might be involved in both viral intracellular transport and the autophagosome-lysosome network. In order to verify the role of microtubules in virus interaction with the autophagosome-lysosome network, mRFP-LC3-expressing HeLa cells were treated with nocodazole to disrupt the microtubules and then incubated with baculoviruses. As shown in Figure 6C and 6D, nocodazole treatment significantly decreased the colocalization of mRFP-LC3 with Lamp-1 induced by viral infection, suggesting that microtubules were required for the formation of autolysosomes in which virus degradation may occur. This result was confirmed by the enhanced viral transduction of baculoviruses in the presence of nocodazole (Figure 5C).

4. Discussion

Understanding the interaction of baculovirus with target mammalian cells is essential for the development of baculovirus-based gene delivery systems. Baculoviruses are considered promising gene delivery vectors in various dividing and non-dividing mammalian cells based on their large capacity for foreign gene insertion and low cytotoxicity (Davies, 1994; Kost and Condreay, 2002; Sandig et al., 1996; Shoji et al., 1997). However, the mechanisms underlying cell entry and intracellular transport of baculoviruses in target mammalian cells have not been well characterized. In this study, we used a direct visualization technique to monitor the interaction of fluorescent-tagged baculoviral particles and cellular endocytic components in the target HeLa cells to characterize the internalization and intracellular transport of viral particles during the infection process.

Clathrin- and caveolin-mediated pathways are well characterized as the main internalization routes of many viruses in target cells (Mountain, 2000). Previous studies suggest that baculoviruses enter mammalian cells mainly through clathrin-mediated endocytosis (Long et al., 2006; Matilainen et al., 2005); however, more evidence has shown that other internalization mechanisms, such as macropinocytosis, also play an essential role in the endocytosis of baculoviruses (Kataoka et al., 2012). Although the envelope protein GP64 is thought to be involved in the binding or penetration of baculoviruses into mammalian cells, the natural receptor for the attachment of virions has not been identified (Hefferon et al., 1999). Here, using drug inhibition, dominant-negative mutant, colocalization, and live-cell imaging studies, we demonstrate that baculoviruses can be internalized into cells *via* clathrin-coated pits in a dynamin-dependent manner. The data from these experiments clearly indicate that baculoviruses use clathrin-mediated, not caveolin-dependent, endocytosis as a main entry pathway to mammalian cells; this is consistent with previous reports (Kataoka et al., 2012; Laakkonen et al., 2009; Long et al., 2006; Matilainen et al., 2005). Internalization of baculoviruses into insect cells is also dependent on clathrin-mediated endocytosis, suggesting that baculoviruses might utilize ubiquitously distributed molecules to enter both insects and mammals.

Clathrin-coated vesicles are known to deliver their contents to both early and late endosomes (Maxfield and McGraw, 2004). It has been shown that many viruses escape from early or late endosomes to release their viral capsid to the cytoplasm through conformational changes, possibly induced by the endosomal environment (Pelkmans and Helenius, 2003). Enveloped viruses are thought to respond to the pH drop in the acidic endosomes by undergoing conformational changes that lead to fusion envelope fusion with the vesicle membrane (Marsh and Helenius, 2006). Moreover, it has been proposed that baculoviruses fuse with early endosomes under the acidic condition with the help of the envelope protein GP64, followed by release of the capsid to the cytoplasm (van Loo et al., 2001). By tracking the fusion events of double-labeled baculoviruses in live-cell imaging and colocalization experiments, we demonstrated that most viruses undergo low-pH-dependent endosomal fusion. It was found that only early endosomes are functionally involved in the intracellular trafficking and transduction of baculoviruses indicating that trafficking through early endosomes may trigger the fusion process, as demonstrated by the colocalization and dominant-negative mutant studies. Compared to 30 min incubation, a decreased colocalization between baculoviruses and early endosomal marker was observed after 60 mins of incubation. It is noteworthy that a similar, relatively low, colocalization of viral particles with the late endosomal marker was observed at these two time points, indicating that baculoviruses can transport from early endosomes to other compartments or escape from early endosomes directly to cytosol.

In addition to the entry mechanism and endosomal fusion, the degradation pathway of viruses is another essential step in determining viral transduction efficacy. Autophagy plays a key role in viral degradation. It fuses with lysosomes and subsequently matures into autolysosomes, where viruses are degraded (Shoji-Kawata and Levine, 2009). Although it has been shown that autophagy aids in the degradation of other viruses in infected cells, such as vesicular stomatitis virus (VSV) (Shelly et al., 2009) and lentivector (Liu et al.,

2013), no evidence has shown that autophagy is involved in the degradation process of baculoviruses in mammalian cells. Through a colocalization study using autophagosomal and lysosomal markers and a viral transduction study using the autophagy inhibitor (3-MA), we demonstrated that the autophagosome-lysosome network is, indeed, involved in the degradation process of baculoviruses during transduction. It was also shown that viral infection significantly increased autophagy activity, which could explain why the autophagy inducer rapamycin could not further enhance autophagy activity.

It has previously been reported that the cytoskeleton network, including the microtubule network and actin filaments, can facilitate the intracellular transport of baculoviruses (Salminen et al., 2005; van Loo et al., 2001). A colocalization study and transduction experiment were used to show that both microtubules and actin filaments are involved in the intracellular transport of baculoviruses. However, treatment of cells with nocodazole caused depolymerization of microtubules which significantly increased viral transduction, a finding previously reported (Salminen et al., 2005; van Loo et al., 2001), indicating that the inhibitory role of microtubules in baculovirus transduction is significant and requires further elucidation. It has been proposed that acetylated microtubules are required for the fusion of autophagosomes with lysosomes to form autolysosomes (Webb et al., 2004; Xie et al., 2010), suggesting that microtubules might be involved in viral degradation. By incubating mRFP-LC3-expressing HeLa cells with nocodazole followed by baculoviruses, we observed that disruption of microtubules by nocodazole significantly decreased the colocalization of autophagosomes with lysosomes, which should be induced by viral transduction, confirming that microtubules are involved in the fusion of autophagosome with lysosome to facilitate the degradation of viral particles.

In conclusion, this study has provided important clues that help to unravel the mechanism underlying cell entry and intracellular trafficking pathways of baculoviruses, thus improving our knowledge of the rate-limiting steps required for viral transduction, which might, in turn, facilitate the design of more efficient gene transfer strategies and expand the applicability of baculoviruses in gene therapy. For example, this study demonstrates that autophagy inhibitors could enhance the transduction efficiency of baculoviruses in target cells, suggesting that this type of inhibitors could be beneficial for virus-mediated gene delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Jennifer Rohrs for critical reading of the manuscript. This work was supported by grants from the National Institutes of Health (R01AI6897, R01CA170820 and P01CA132681) and a translational acceleration grant from the Joint Center for Translational Medicine.

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Figure 1.

Clathrin-dependent entry of baculoviruses. (A, B) HeLa cells were incubated with VP39-GFP-labeled baculoviruses (green) for 30 min at 4°C to synchronize infection, then shifted to 37 °C for either 15 or 30 min. After incubation, cells were fixed, permeabilized and immunostained with antibody against clathrin (A) or caveolin-1 (B). The boxed regions are enlarged in the bottom panels. Scale bar represents 5 μm. (C) Quantification of VP39-GFP-labeled baculoviral particles colocalized with clathrin and caveolin-1 signals after 15 or 30 min incubations. Overlap coefficients were calculated for over 50 cells using Mander's overlap coefficient with Nikon NIS-Elements software. A t-test was performed using Microsoft Excel software, and corresponding p-values are shown. (D) The effect of inhibitory drugs on baculovirus transduction. HeLa cells were preincubated with chlorpromazine (CPZ, 30 nM) or filipin (15 nM) for 30 min or MβCD (15 mM) for 15 min at 37 °C. The cells were spin-infected with baculovirus encoding a GFP gene for 90 min in the presence of drugs (CPZ and filipin) or in the absence of MβCD. After an additional 3 h of incubation, media were replaced, and the percentage of GFP-positive cells was analyzed by flow cytometry 72 h post-infection. All of the data was normalized by the mean percentage of GFP-positive cells in the no drug control group. All data is shown as the mean of triplicate experiments. A t-test was performed using Microsoft Excel software, and corresponding p-values are shown. (E) Real-time monitoring of virus internalization mediated by clathrin-coated pits. HeLa cells were transiently transfected with mRFP-clathrin (red), and incubated with VP39-GFP-labeled baculoviral particles (green) for 30 min at 4 °C. Cells were then warmed to 37°C to initiate virus internalization. Confocal time-lapse images were recorded and selected frames of real-time images are presented. Scale bar represents 2 μm. (F) Kinetics of the fluorescence intensity of mRFP-clathrin (red) and

diffusion coefficients (blue) of baculoviral particles, as indicated by the white circle, are shown.

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Figure 2.

Functional involvement of dynamin on the internalization of baculoviruses. (A) HeLa cells were transfected with DsRed-wild type-dynamin or DsRed-dynamin dominant mutant (Dyn-K44). 24 h post transfection, the cells were incubated with VP39-GFP-labeled baculoviral particles (green) and dye-labeled transferrin (blue) for 30 min at 4 °C. Cells were then warmed to 37°C for 60 min to initiate virus internalization, after which, the cells were fixed and imaged. Scale bar represents 5 μm. (B) HeLa cells were transfected with DsRed-wild type-dynamin or DsRed-dynamin dominant mutant (Dyn-K44). 24 h post transfection, cells were spin-infected with baculovirus encoding a GFP gene for 90min. After an additional 3 h of incubation, media was replaced. The percentage of GFP-positive cells was analyzed by flow cytometry 72 h post-infection. All of the data was normalized by the mean percentage of GFP-positive cells in the control group. Error bars represent the standard deviation from the mean for triplicate experiments. A t-test was performed using Microsoft Excel software, and corresponding p-values are shown.

Figure 3.

Functional involvement of various endosomes on viral transduction. (A) HeLa cells were incubated with VP39-GFP-labeled baculoviruses (green) for 30 min at 4 °C to synchronize infection. Cells were then shifted to 37 °C for different incubation periods (15, 30, 60 min), as indicated in the image. The cells were fixed, permeabilized and immunostained with antibodies against early endosomes (EEA1, red) and late endosomes (CI-MPR, blue). The boxed regions are enlarged in the bottom panels. Scale bar represents 5 μ m. (B) Quantification of VP39-GFP-labeled baculoviruses colocalized with EEA1⁺ or CI-MPR⁺ endosomes after 0, 15, 30 or 60 min of incubation. Overlap coefficients were calculated using Mander's overlap coefficient with the Nikon NIS-Elements software for more than 50 cells at each time point. (C) Functional involvement of early endosomes in baculovirus transduction. HeLa cells were transiently transfected with wild-type or dominant-negative mutant form of Rab 5 or Rab7. 24 h after transfection, cells were spin-infected with baculoviruses for 90 min. The percentage of GFP-positive cells was analyzed by flow cytometry at 72 h post-infection. All of the data was normalized by the mean percentage of GFP-positive cells in the control group. Error bars represent the standard deviation from the mean for triplicate experiments. A t-test was performed using Microsoft Excel software, and corresponding p-values are shown.

Figure 4.

Low-pH-mediated endosomal fusion of baculoviruses. (A) Low pH is required for endosomal fusion of baculoviruses. DiD/VP39-GFP-double-labeled baculoviruses were added to bafilomycin A1 (BAF)-treated or untreated HeLa cells for 30 min at 4 °C to synchronize infection. Cells were then shifted to 37 °C for 30 min. The cells were fixed and imaged. Yellow signals indicate viral particles fused with endosomes. Scale bar represents 5 μm. (B) The effect of BAF on baculovirus transduction. HeLa cells were preincubated with BAF for 30 min at 37 °C. The cells were then spin-infected with baculoviruses for 90 min in the presence of BAF. After an additional 3 h of incubation, media was replaced. The percentage of GFP-positive cells was analyzed by flow cytometry 72 h post-infection. All of the data was normalized by the mean percentage of GFP-positive cells in the control group. Error bars represent the standard deviation from the mean for experiments performed in triplicate. A t-test was performed using Microsoft Excel software, and corresponding p-values are shown. (C) Real-time monitoring of the endosomal fusion of baculoviruses. DiD/VP39-GFP-double-labeled baculoviruses were incubated with HeLa cells for 30 min at 4 °C to synchronize infection. Cells were then shifted to 37 °C for 30 min to induce virus–endosome fusion. Confocal time-lapse images were acquired every 10 s over a time period of 10 min. Yellow signals indicate viral particles fused with endosomes. Scale bar represents 2 μm. (D) Time trajectories of the velocity (black) and the fluorescent ratio of DiD and VP39-GFP fluorescent emission (red) of the virus inside the white circle shown in Figure 4(C).

Figure 5.

Cytoskeleton-mediated intracellular transport of baculoviruses. (A, B) VP39-GFP-labeled baculoviruses (green) were incubated with HeLa cells for 30 min at 37 °C. The cells were then fixed, permeabilized, and immunostained for microtubules, with the monoclonal antibody to α -tubulin (red, A), or actin filaments, with rhodamine-conjugated phalloidin (red, B). The boxed regions are enlarged in the right panels. Scale bar represents 5 μ m. (C) The effect of microtubule and actin filament inhibitors on virus transduction. HeLa cells were preincubated with nocodazole (30 μ M) or cyto-D (20 μ M) for 30 min, after which the cells were spin-infected with baculoviruses for 90 min. The percentage of GFP-positive cells was analyzed by flow cytometry 72 h post-infection. All of the data was normalized by the mean percentage of GFP-positive cells of the control group. Error bars represent the standard deviation from the mean for experiments performed in triplicate. A t-test was performed using Microsoft Excel software, and corresponding p-values are shown.

Figure 6.

Functional involvement of the autophagy and lysosome network in baculovirus transduction. (A) The involvement of autophagy in the intracellular trafficking pathway of baculoviruses. HeLa cells were transfected with mRFP-LC3 to label autophagosomes. 24 h post transfection, the cells were incubated with VP39-GFP-labeled baculoviruses for 90 min. The cells were then fixed, permeabilized and immunostained with an antibody against lysosomes (Lamp-1, green). Scale bar represents 5 μ m. (B) The effect of autophagy activity on baculovirus transduction. HeLa cells were preincubated with 3-MA (5 mM) or rapamycin (1 μ M) for 30 min at 37 $^{\circ}$ C. The cells were then infected with baculoviruses carrying a reporter GFP gene for 90 min in the presence of drugs. After an additional 3 h of incubation, the media was replaced with fresh media. The percentage of GFP-positive cells was analyzed by flow cytometry 72 h after infection. (C) Imaging the autolysosome formation in cells after treatment with rapamycin alone, baculovirus alone, or nocodazole followed by baculovirus. mRFP-LC3-transfected HeLa cells were incubated with rapamycin, baculoviruses, or nocodazole followed by baculoviruses for 90 min at 37 $^{\circ}$ C. The cells were then fixed, permeabilized and immunostained with antibody against lysosome (Lamp-1, green). Scale bar represents 5 μ m. (D) Quantification of autophagosome and lysosome colocalization data in cells that are untreated or treated as described in Figure 6(C). Overlap coefficients were calculated for more than 40 cells at each time point using Mander's overlap coefficient with Nikon NIS-Elements software. (E) The effect of overexpression of autophagosomes on virus transduction. HeLa cells were transfected with mRFP-LC3. 24 h post-mRFP-LC3 transfection, cells were infected for 90 min, in the presence of drugs, with baculoviruses carrying a reporter GFP gene. After an additional 3 h of incubation, the media was replaced with fresh media. After 72 h, the percentage of GFP-positive cells was analyzed by flow cytometry. All the data was normalized by the mean percentage of GFP-positive cells in the

control group. All data are shown as the mean of triplicate experiments. A t-test was performed using Microsoft Excel software, and corresponding p-values are shown.