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RESEARCH ARTICLE

Improving Baculovirus Transduction of Mammalian Cells by Incorporation of Thogotovirus Glycoproteins

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Abstract

Baculovirus can transduce a wide range of mammalian cells and is considered a promising gene therapy vector. However, the low transduction efficiency of baculovirus into many mammalian cells limits its practical application. Co-expressing heterologous viral glycoproteins (GPs), such as vesicular stomatitis virus G protein (VSV G), with baculovirus native envelope protein GP64 is one of the feasible strategies for improving virus transduction. Tick-borne thogotoviruses infect mammals and their GPs share sequence/structure homology and common evolutionary origins with baculovirus GP64. Herein, we tested whether thogotovirus GPs could facilitate the entry of the prototype baculovirus Autographa californica multiple multiple nucleopolyhedrovirus (AcMNPV) into mammalian cells. The *gp* genes of two thogotoviruses, Thogoto virus and Dhori virus, were inserted into the AcMNPV genome. Both GPs were properly expressed and incorporated into the envelope of the recombinant AcMNPVs. The transduction rates of recombinant AcMNPVs expressing the two thogotovirus GPs increased for approximately 4–12 fold compared to the wild type AcMNPV in six of the 12 tested mammalian cell lines. It seemed that thogotovirus GPs provide the recombinant AcMNPVs with different cell tropisms and showed better performance in several mammalian cells compared to VSV G incorporated AcMNPV. Further studies showed that the improved transduction was a result of augmented virus-endosome fusion and endosome escaping, rather than increased cell binding or internalization. We found the AcMNPV envelope protein GP64-mediated fusion was enhanced by the thogotovirus GPs at relatively higher pH conditions. Therefore, the thogotovirus GPs represent novel candidates to improve baculovirus-based gene delivery vectors.

Keywords Autographa californica multiple nucleopolyhedrovirus (AcMNPV) · Baculovirus · Thogotovirus · Glycoprotein · Transduction · Mammalian cells

Introduction

Baculoviruses are a group of enveloped viruses with circular double stranded DNA genomes (80–180 kb), which exclusively infect insects in nature. These viruses are widely used as insecticides and have been applied as protein expression and vaccine production vectors extensively (Contreras-Gomez *et al*. 2014; Dai *et al*. 2018; van Beek and Davis 2016). Autographa californica multiple nucleopolyhedrovirus (AcMNPV) is the prototype member of family *Baculoviridae* and it is one of the best studied baculoviruses. AcMNPV is able to transduce a wide range of mammalian cells and express foreign genes under a mammalian promoter *in vitro* and *in vivo* (Mansouri and Berger 2018). Therefore, baculoviruses are considered promising gene delivery and gene therapy vector.

Compared to the traditional viral gene delivery vectors, such as retroviruses, lentiviruses, adeno-associated viruses and adenoviruses, baculovirus shows many advantages. There is no pre-existing antibody against baculovirus as it doesn't infect vertebrate in nature, and has no cytotoxic effects to mammalian cells (Ho *et al*. 2005; Strauss *et al*.

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2007). With the capacity of insertion of foreign DNA fragments as large as 38 kb, baculoviruses can transduce many kinds of human cells, including both dividing and non-dividing cells, such as hepatocyte, stem cells and cancer cells, without replicating and integrating into host chromosomes (Chen *et al*. 2011b; Cheshenko *et al*. 2001; Du *et al*. 2010; Makkonen *et al*. 2015; Merrihew *et al*. 2001). Additionally, the construction and propagation of recombinant baculovirus is bio-safe, scalable and cost efficient by infecting its natural host insect cells (Chen *et al*. 2011a; Felberbaum 2015).

Although great efforts have been made to advance the baculovirus technology from bench to bedside, there are still many roadblocks stand in the way. One of the most important problems is the low transduction efficiency of baculovirus in many kinds of cells. One common strategy is to display cell specific affinity proteins or peptides, *e.g.* the RGD containing peptides that recognized by cell-surface integrin, on the viral envelope by fusing with the native baculoviral glycoprotein (GP), thus baculovirus transduction was significantly improved in certain cells (Ernst *et al*. 2006; Makela *et al*. 2006). An alternative method is to incorporate heterologous GPs from viruses that infect mammalian cells, such as influenza virus neuraminidase, vesicular stomatitis virus G protein (VSV G) and the GP of rabies virus (Barsoum *et al*. 1997; Borg *et al*. 2004; Tani *et al*. 2003). Among these, displaying VSV G is the most widely adopted, which can enhance the transduction of AcMNPV up to 15 fold (Kolangath *et al*. 2014). However, VSV G exhibits significant cytotoxicity in the expressed cells and results in lower baculovirus production (Kaikkonen *et al*. 2006). Additionally, directed mutation and screening of baculovirus GP was also used to improve the transduction in human airway epithelia (Sinn *et al*. 2017).

The major envelope GP of AcMNPV, GP64, is responsible for cell attachment, viral fusion and budding in insect cells (Blissard and Theilmann 2018). GP64 also contributes to the virus entry into mammalian cells (Kataoka *et al*. 2012). Like in insect cells, AcMNPV enters mammalian cells mainly through receptor-mediated, clathrin-dependent endocytosis pathway (Dong *et al*. 2010; Kataoka *et al*. 2012; Long *et al*. 2006), although macropinocytosis and clathrin-independent pathway were also reported (Kataoka *et al*. 2012; Laakkonen *et al*. 2009). Once the virions are endocytosed, the acidic environments in the endosomes will trigger the conformation change of GP64 to mediate fusion of viral envelope and endosomal membrane. Thus, the nucleocapsids are released into the cytosol and further transported to the nucleus (Liu *et al*. 2014). Being a class III viral fusion protein, GP64 is structurally related to the other fusion proteins in this group, including the G proteins of rhabdoviruses, the gB proteins of herpesviruses and the GPs of thogotoviruses (Mas and Melero 2013).

Thogotoviruses, belong to the *Thogotovirus* genus of family *Orthomyxoviridae*, are tick-borne viruses that infect mammals including human (Ejiri *et al*. 2018). The GPs of two thogotoviruses, Thogoto virus (THOV) and Dhori virus (DHOV), show no obvious similarity to that of Influenza viruses and Isavirus, but in contrast, are quite structurally related to baculovirus GP64 protein, although with a low sequence homology ($\sim$ 28% amino acid identity) (Peng *et al*. 2017). Interestingly, only the Group I alphabaculoviruses and a betabaculovirus possess GP64, while other baculoviruses use a distinct F protein as their envelope fusion proteins (Ardisson-Araujo *et al*. 2016a, b; Wang *et al*. 2014). It has been proposed that an ancient Group I alphabaculovirus obtained the GP64 protein from a thogotovirus-like ancestor during evolution, which may render the virus the ability to transduce a wide range of mammalian cells (Pearson and Rohrmann 2002; Peng *et al*. 2017).

In this study, we tested whether thogotovirus GPs will facilitate the entry of AcMNPV into mammalian cells. By incorporating the GPs of THOV and DHOV into AcMNPV envelope, the baculovirus transduction of a variety of mammalian cells was found to be significantly improved and showed different cell tropisms to the virus expressing VSV G. Further studies showed that the improvement was a result of enhanced virus-endosome fusion by the incorporation of thogotovirus GPs. Thus, the GPs of thogotoviruses provide novel candidates to improve baculovirus based gene delivery vectors.

Materials and Methods

Cell Culture and Viruses

A549, HeLa, Hep2, HepG2, HMC3, HEK 293, HUVEC, RD, SW13, SH-SY5Y, U87MG and Vero cells were obtained from the American Type Culture Collection (ATCC). A549, HeLa, HMC3, HEK 293, HUVEC, RD, SW13 and U87MG cells were cultured at 37 °C in Eagle's Minimum Essential Medium (EMEM; Gibco-BRL) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL). Hep2, HepG2, SH-SY5Y and Vero cells were cultured at 37 °C in Dulbecco's modified Eagle medium (DMEM; Gibco-BRL) supplemented with 10% FBS. Sf9 cells were cultured at 27 °C in Grace's insect medium (Gibco-BRL) supplemented with 10% FBS. The *gp64*-null AcBacmid (AcBacΔ*gp64*) was previously constructed at our laboratory (Wang *et al*. 2008).

Construct of Recombinant Baculoviuses

The open reading frame (ORF) of THOV GP (ThGP, GenBank accession code: NC006506) and DHOV GP (DhGP, GenBank accession code: GU969310) were chemically synthesized with a C-terminal HA tag ($ThGP_{HA}$/$DhGP_{HA}$). The P_{Op166}-*gp64*-SV40 poly(A) cassette of pFB-P_{Op166}-*gp64* (Wang *et al*. 2008) was cut and inserted to pFastBactHTb (Invitrogen) under the *polyhedrin* promoter (P_{Polh}), and the P_{CMV}-P_{Op166}-*egfp* cassette of pFB-P_{CMV}-P_{op166}-*egfp* (Dong *et al*. 2010) was cut and inserted downstream of P_{Op166}-*gp64*-SV40 poly(A) cassette, generating pFastBacHTb-BP*gp64*-BP*egfp*. Then the sequence of *gp64* gene was cut off and the GP_{HA} genes were inserted to pFastBacHTb-BP*gp64*-BP*egfp*, generating pFastBacHTb-BPGP_{HA}-BP*egfp*. The donor vector pFastBacHTb-BPGP_{HA}-BP*egfp* was transposed into the AcBacmid or AcBacΔ*gp64* (Wang *et al*. 2008) at the attTn7 integration site and the recombinant bacmids were named as Ac-$ThGP_{HA}$, Ac-$DhGP_{HA}$, AcΔ*gp64*-$ThGP_{HA}$ and AcΔ*gp64*-$DhGP_{HA}$. The donor vector pFB-P_{CMV}-P_{op166}-*egfp* was transposed into the AcBac and the recombinant bacmid was named as Ac-WT. The construction of bacmid Ac-VSVG was based on the same protocol for constructing Ac-$ThGP_{HA}$ and Ac-$DhGP_{HA}$ by inserting the ORF of VSV G into AcBacmid. The recombinant bacmids were used to transfect Sf9 cells and produced the recombinant viruses named correspondingly. The transfection and infection assay was performed as described previously (Li *et al*. 2018).

Immunofluorescence Microscopy

Sf9 cells were seeded into 35 mm glass bottom dish and infected by the recombinant viruses at an multiplicity of infection (MOI) of 5 ($TCID_{50}$ units/cell) for 48 h. Cells were fixed by 4% paraformaldehyde, permeabilized by 0.1% Triton-X 100, washed and blocked by 5% bovine serum albumin (BSA). Then cells were incubated with the anti-HA antibody (diluted 1:1000, Sigma) in 1% BSA at room temperature for 1 h, washed and incubated with Alexa Fluor 647 goat anti-mouse antibody (Abcam) at room temperature for 1 h. Cells were washed and the cell nuclei were stained by Hoechst33258 (Beyotime). Finally, the cells were imaged by a fluorescence microscope (Deltavision softWoRx Imaging Workstation, Applied Precision) at channels Cy5 (red), FITC (green), DAPI (blue) and bright field.

Western Blot Analysis of Thogotovirus GP Incorporation in the Virions

The recombinant viruses were produced by infecting Sf9 cells for 48 h, collected before cells were lysed and purified as previously reported (Wang *et al*. 2010). The purified virus was suspended in PBS and prepared for the following Western blot analysis as previously reported (Zou *et al*. 2016). The anti-HA antibody (diluted 1:2000; Sigma) or the anti-VP39 polyclonal rabbit antibody (diluted 1:3000) (Wang *et al*. 2010) was used as the primary antibody and the horseradish peroxidase (HRP)-conjugated goat anti-rabbit/mouse antibody (Sigma) was used as the secondary antibody. To indicate the protein molecular weight, a PageRuler prestained protein ladder (Thermo Scientific) was used.

One-Step Growth Curve Assay

Sf9 cells were infected with recombinant virus Ac-WT, Ac-$ThGP_{HA}$ and Ac-$DhGP_{HA}$ at an MOI of 5 at 27 °C for 1 h. Then the viruses were removed and the cells were washed three times before addition of 2 mL of Grace's medium supplemented with 10% FBS. Thirty μL of the culture were collected at 0, 24, 48, 72 and 96 h post infection (p.i.) and clarified by centrifugation at 2000 ×*g* for 5 min. The virus titer was determined by endpoint dilution assays.

Transducing Mammalian Cells

Based on the cell size and confluence, cells were seeded into 24 well culture plates at density of 2×10^5 cells/well (A549, HeLa, HMC3, HEK 293, RD, Hep2, HepG2 and SH-SY5Y cells) or 1×10^5 cells/well (HUVEC, SW13, U87MG and Vero cells) and cultured overnight. Cells were incubated with the virus Ac-WT, Ac-$ThGP_{HA}$, Ac-$DhGP_{HA}$ and Ac-VSVG at an MOI of 5 or 20 at 37 °C for 1 h. Then the virus supernatant was removed and the cells were washed three times before addition of fresh culture medium. The cells were imaged by fluorescence microscopy and collected for flow cytometry (FCM) to analyze the EGFP expressing cells after culturing for 24 h.

Quantitative PCR (qPCR) Analysis of Virus Binding and Internalization

To analyze virus binding, HeLa cells seeded in 24 well culture plates (2×10^5 cells/well) were pre-chilled on ice for 30 min and then incubated with the recombinant viruses at an MOI of 5 or 20 on ice for 1 h to allow virus synchronously to bind to cells. The cells were washed with

cold cell culture medium for three times before being collected for total DNA extraction. Using a pair of primers against viral gene VP80, the genomic copies of cell-bound virions were quantified by qPCR (Li *et al*. 2018).

For analysis of virus internalization, the viruses first bound to HeLa cells as described above. Then virus-bound cells were cultured in fresh culture medium at 37 °C for 30 min or 60 min before cells were digested by 0.5% trypsin for 10 min to remove the remaining cell-surface virions. Trypsin was neutralized by culture medium supplemented with 10% FBS and the cells were washed by cold PBS for three times. The total DNA of HeLa cells were extracted and the viral genomic copies were quantified by qPCR as described above. The quantity of internalized virions was normalized to those of cell-bound virions for each recombinant virus.

DiD Labeling of Recombinant Viruses

Sf9 cells cultured in the 75 cm^2 cell culture flasks ($\sim 3 \times 10^7$ cells/flask) were infected by the recombinant viruses at an MOI of 2 for 24 h. Then 1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindodicarbocyanine (DiD) was added into the culture with the final concentration as 100 μmol/L. The cells were cultured for another 36 h keeping in the dark. The culture supernatants were collected, clarified by centrifugation at 2000 $\times g$ for 5 min and filtered by 0.45 μm membrane (Millipore) to remove cell debris. Then the DiD labeled virions were pelleted from the medium by ultracentrifugation through a 20% (W/V) sucrose cushion at 18,000 rpm for 1 h at 4 °C in an SW28 rotor (Beckman Couler). The virus pellet was resuspended in Grace's insect medium and aliquoted before store at − 80 °C.

Detection of Viral Fusion by DiD Dequenching Assay

To stain the cell cytoplasm, HeLa cells were incubated with 2 μmol/L Calcein blue, AM (Invitrogen) at 37 °C for 1 h. Then the cells were prechilled on ice for 30 min and washed two times. DiD labeled viruses were added to the cells and incubated for 1 h and unbound virions were washed away by cold cell culture medium. The cells were imaged by fluorescence microscopy immediately and finished in 5 min at channels DAPI (Calcein bule, AM), Cy5 (DiD) and bright field. The time to start imaging was set as 0 min. After imaging at 0 min, the cells were culture at 37 °C for another 30 or 60 min and imaged.

Imaging Analysis

The fluorescence intensity of DiD of each virus particle was analyzed by ImageJ software (NIH). For the fluorescence intensity of DiD at 0 min, five randomly selected fields of each recombinant virus were analyzed and the mean intensity was calculated. Two fold of the mean intensity, which beyond the max intensity at 0 min, was set as the threshold (I_{th}) to define dequenched DiD. To calculate the percentage of dequenched DiD at 30 and 60 min, cells was outlined based on the cytoplasm staining by Calcein blue, AM and only the fluorescent DiD dots in the cytoplasm were analyzed. The percentage of dequenched DiD was determined by dividing the number of DiD dots with intensity higher than I_{th} by the total number of DiD dots. For each recombinant virus at each time points, more than ten randomly selected cells were analyzed.

Neutralization Assay

The anti-GP64 mouse monoclonal antibody AcV1 is a neutralizing antibody of AcMNPV (Zhou and Blissard 2006). Fifty μL of recombinant viruses Ac-WT, Ac-$ThGP_{HA}$ and Ac-$DhGP_{HA}$ were mixed with AcV1 and diluted to a total volume of 100 μL with Grace's insect medium. The final concentration of AcV1 was 31.3, 62.5 and 125 μg/mL. The viruses were incubated with AcV1 at 27 °C for 1 h and then added to HeLa cells at an MOI of 40 (Ac-WT) or 20 (Ac-$ThGP_{HA}$ and Ac-$DhGP_{HA}$). After virus attachment at 37 °C for 1 h, the virus-antibody mixture was removed and cells were washed once before addition of fresh medium. Cells were cultured at 37 °C for 24 h and imaged by fluorescence microscopy.

Low pH Triggered Infection Through Direct Fusion Pathway

Low-pH triggered virus infection through direct fusion pathway was performed according to a previous report (Dong *et al*. 2010). HeLa cells were incubated with 40 nM bafilomycin A1 (BFA1) at 37 °C for 30 min and then were chilled on ice for 30 min. Recombinant viruses Ac-WT, Ac-$ThGP_{HA}$ and Ac-$DhGP_{HA}$ were added to the cells at an MOI of 10. After the cells were incubated with the viruses on ice for 2 h, the virus containing supernatant was removed and the cells were incubated with Grace's medium with pH 5.0, 5.4, 5.8 or 6.2 for 5 min to trigger virus fusion at plasma membrane. The Grace's medium was replaced with EMEM supplemented with 10% FBS and 40 nM BFA1 and the cells were cultured at 37 °C for another 4 h. Then the BFA1 containing medium was removed and the cells were washed twice before addition of fresh EMEM supplemented with 10% FBS. The cells were collected for FCM at 24 h post transdction.

Results

Construction of Recombinant Baculoviruses Expressing ThGP and DhGP

The coding sequences of ThGP and DhGP were inserted into the AcMNPV bacmid under the control of *polyhedrin* promoter (P_{Polh}) and Orgyia pseudotsugata multicapsid polyhedrosis virus (OpMNPV) *gp64* promoter (P_{Op166}) (Blissard and Rohrmann 1991), which would ensure high expression of the GPs in insect cells (Fig. 1A). To facilitate the detection of protein expression, a HA tag was fused with the C-terminal of GPs (GP_{HA}). An *egfp* reporter gene was inserted to the virus genome under the control of promoters P_{CMV} and P_{Op166}, which allows the EGFP expression in both mammalian- and insect cells. Although it was reported that the incorporation of ThGP could not rescue the infectivity of AcMNPV when the *gp64* gene was knocked out (Lung *et al*. 2002), there is no study on the incorporation of DhGP in baculoviruses. In this work, both *ThGP* and *DhGP* and the reporter *egfp* genes were inserted to a previously constructed *gp64*-null bacmid AcΔ*gp64* (Wang *et al*. 2008).

Insect Sf9 cells were transfected by the recombinant bacmids. For Ac-WT, Ac-$ThGP_{HA}$ and Ac-$DhGP_{HA}$, infectious virions were produced and resulted in significant secondary infection at 96 h post transfection (Fig. 1B). The supernatants were collected and further infection assay indicated that Ac-$ThGP_{HA}$ and Ac-$DhGP_{HA}$ showed infectivity similar to Ac-WT. For AcΔ*gp64*-$ThGP_{HA}$ and AcΔ*gp64*-$DhGP_{HA}$, there were no infectious progeny virions produced as the amount of fluorescent cells did not increase even at 96 h post transfection and there was no fluorescent cells in the infection assay, suggesting that neither $ThGP_{HA}$ nor $DhGP_{HA}$ alone could rescue the infectivity of the *gp64*-null AcMNPV.

Thogotovirus GPs Were Incorporated into the Baculovirus Particles

The expression and localization of $ThGP_{HA}$ and $DhGP_{HA}$ in infected Sf9 cells was detected by immunofluorescence microscopy. As shown in Fig. 2A, both $ThGP_{HA}$ and

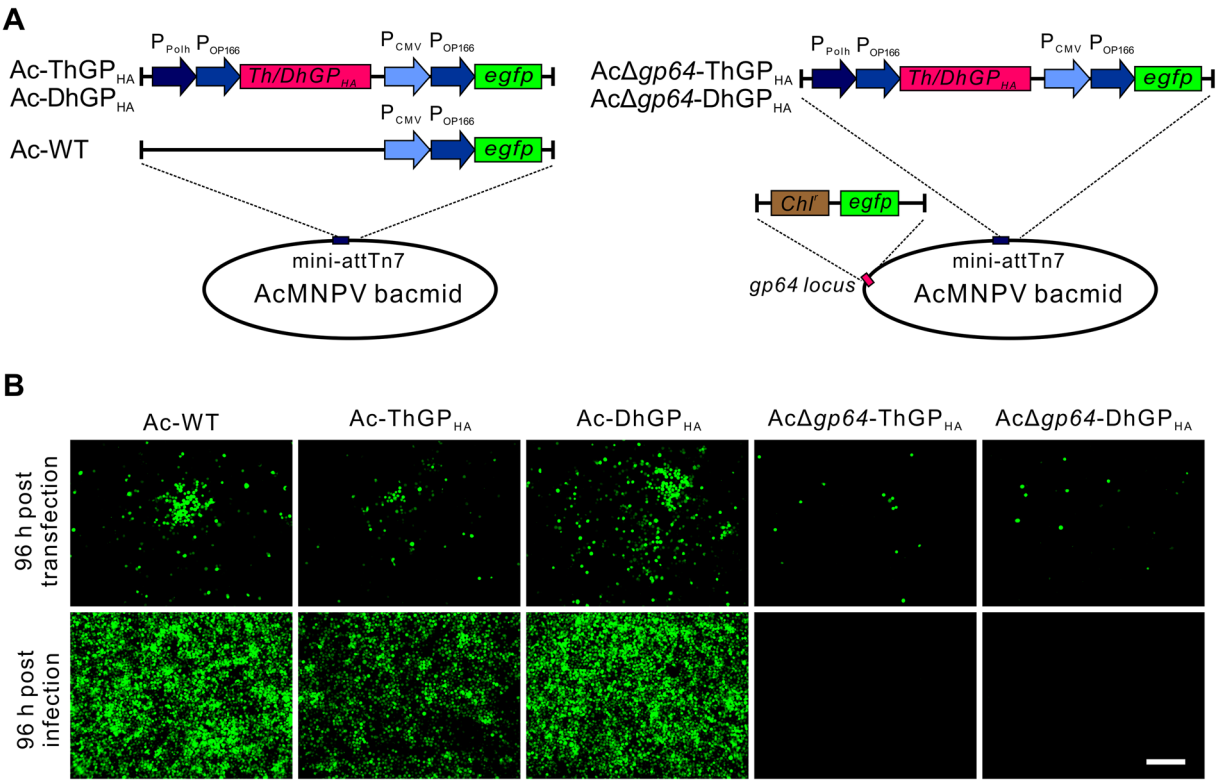


Fig. 1 Construction of recombinant baculoviruses expressing thogotovirus GPs. **A** The schematics of recombinant viruses. The left panel shows the schematic representation of recombinant AcMNPV bacmids carrying the reporter gene *egfp* and the thogotovirus *GPs* fused with HA tag at C-terminal. The right panel shows the baculoviruses presenting thogotovirus *GPs* in a *gp64*-null bacmid, where the *gp64* gene was substituted by chloramphenicol resistance gene (*Chl*^{r}) and *egfp*. **B** Transfection and infection assay of recombinant viruses. Sf9 cells were transfected by the recombinant bacmid DNA of Ac-WT, Ac-$ThGP_{HA}$, Ac-$DhGP_{HA}$, AcΔ*gp64*-$ThGP_{HA}$ and AcΔ*gp64*-$DhGP_{HA}$. The cells were imaged at 96 h post transfection and the supernatants were collected to infect Sf9 cells and imaged at 96 h p.i. Bar, 200 μm.

$DhGP_{HA}$ detected by anti-HA tag antibody was significantly localized in the perinuclear region and on plasma membrane, where the virions are enveloped and budded from. The incorporation of $ThGP_{HA}$ and $DhGP_{HA}$ in budded viruses was further detected by Western blot assay (Fig. 2B). Both $ThGP_{HA}$ and $DhGP_{HA}$ were detected in the virions of Ac-$ThGP_{HA}$ and Ac-$DhGP_{HA}$, but not Ac-WT, with the molecular weight of ~ 68 kDa and ~ 63 kDa, respectively, which are in line with the previous reports that the thogotovirus GPs were expressed in insect cells (Jones *et al*. 1995; Peng *et al*. 2017).

To investigate the effect of the incorporation of $ThGP_{HA}$ and $DhGP_{HA}$ on virus infection in insect cells, one-step growth curve analysis was performed. The result showed that there was no significant difference in the infectious budded virus productions between Ac-WT and Ac-$DhGP_{HA}$ ($P > 0.05$), while the production of Ac-$ThGP_{HA}$ increased to ~ 1.9 fold of Ac-WT ($P < 0.05$) (Fig. 2C).

The GPs of Thogotoviruses Enhanced AcMNPV Transduction into Mammalian Cells

The transduction efficiency of Ac-WT, Ac-$DhGP_{HA}$ and Ac-$ThGP_{HA}$ was first measured in HeLa cells. Furthermore, the transduction efficiency of recombinant virus Ac-VSVG, which incorporates the GP of VSV and significantly improves the baculovirus transduction in mammalian cells was compared in parallel experiments (Barsoum *et al*. 1997; Tani *et al*. 2001). HeLa cells transduced by Ac-$DhGP_{HA}$ and Ac-$ThGP_{HA}$ at an MOI of 5 or 20 showed more EGFP positive cells and more EGFP expression than cells transduced by Ac-WT and Ac-VSVG (Fig. 3A). To further compare the transduction efficiency of Ac-WT, Ac-$DhGP_{HA}$, Ac-$ThGP_{HA}$ and Ac-VSVG, eleven human cell lines (including HeLa) and a non-human primate cell (Vero) were transduced by the recombinant viruses and the transduction efficiency was analyzed by

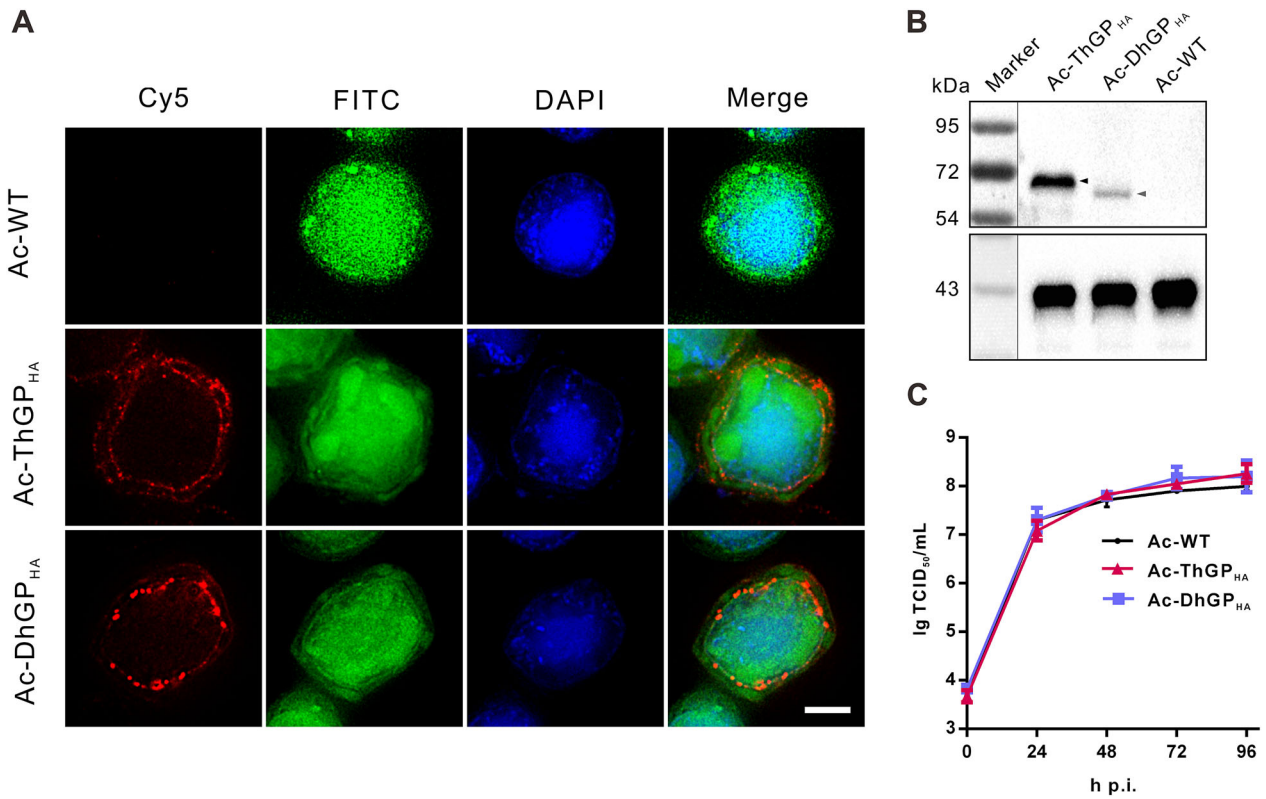
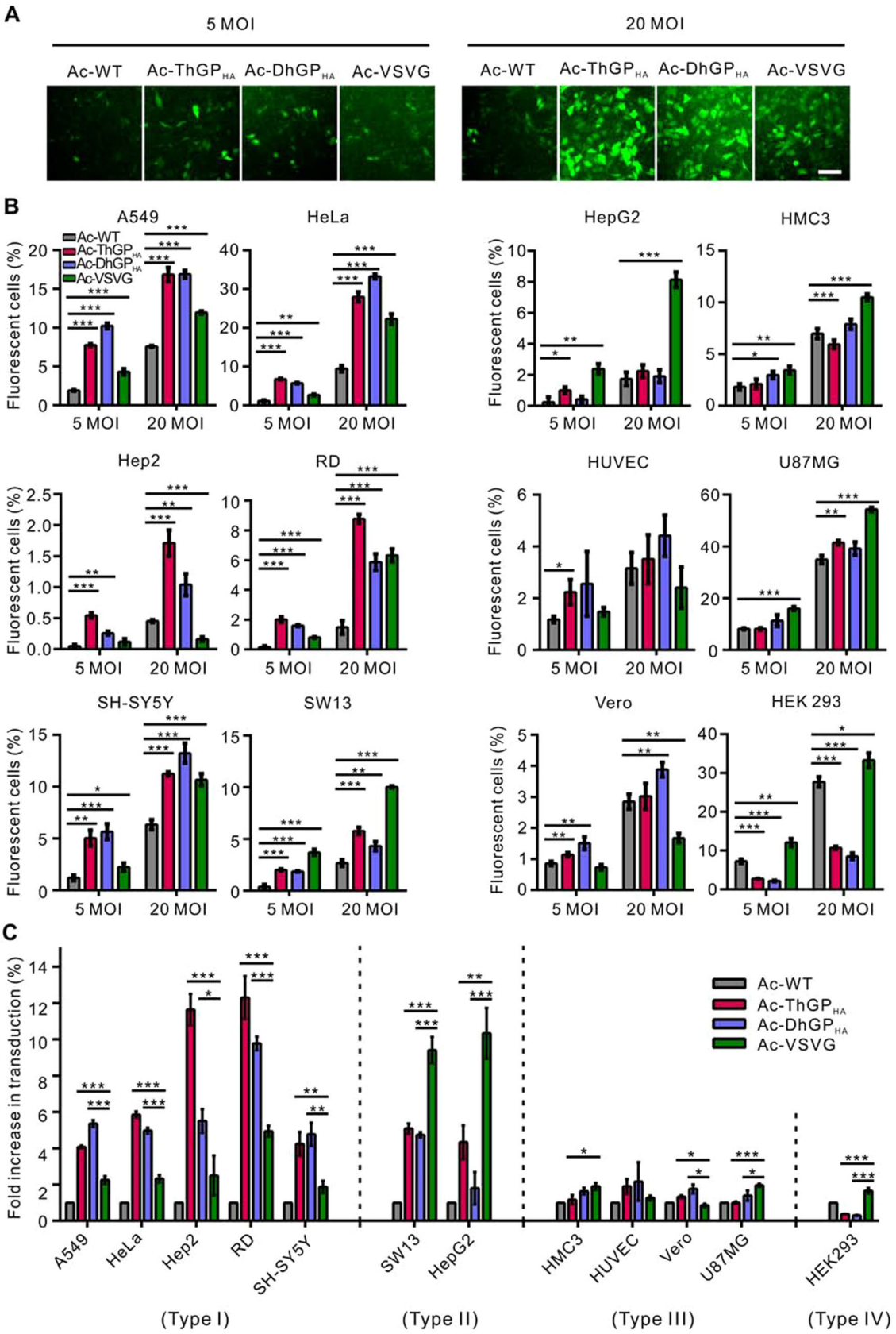


Fig. 2 The incorporation of thogotovirus GPs in the AcMNPV virions and its impact on virus infection. **A** The subcellular localization of thogotovirus GPs in infected cells. Sf9 cells were infected by Ac-WT, Ac-$ThGP_{HA}$ or Ac-$DhGP_{HA}$ and fixed at 48 h p.i. The thogotovirus GPs were detected by anti-HA antibody and Alexa flour 647 goat anti-mouse antibody (Cy5). The cell nuclei were stained by Hoechst33258 (blue). FITC channel indicates the EGFP expression in the virus infected cells. Bar, 5 μm. **B** Detection of the incorporation of thogotovirus GPs in the virions. The virions of recombinant viruses were purified and analyzed by Western blot assay using anti-HA antibody. The major viral capsid protein VP39 was detected by anti-VP39 antibody as control. **C** One-step growth curve analysis of recombinant viruses. Sf9 cells were infected by Ac-WT, Ac-$ThGP_{HA}$ and Ac-$DhGP_{HA}$ at an MOI of 5 $TCID_{50}$ units/cell. The supernatants were collected at indicated time points and the virus titer was determined by endpoint dilution methods. The results are mean ± SD (n = 3 experimental replicates).


A
5 MOI
20 MOI
Ac-WT
Ac-ThGP$_{HA}$
Ac-DhGP$_{HA}$
Ac-VSVG
B
A549
HeLa
HepG2
HMC3
Hep2
RD
HUVEC
U87MG
SH-SY5Y
SW13
Vero
HEK 293
Fluorescent cells (%)
5 MOI
20 MOI
C
Fold increase in transduction (%)
A549
HeLa
Hep2
RD
SH-SY5Y
SW13
HepG2
HMC3
HUVEC
Vero
U87MG
HEK293
(Type I)
(Type II)
(Type III)
(Type IV)

◀**Fig. 3** Incorporation of thogotovirus GPs improved the transduction of AcMNPV in mammalian cells. **A** HeLa cells were transduced by Ac-WT, Ac-ThGP$_{HA}$, Ac-DhGP$_{HA}$ and Ac-VSVG at an MOI of 5 or 20 $TCID_{50}$ units/cell and imaged by fluorescence microscopy at 24 h p.t. Bar, 100 μm. **B** Twelve mammalian cell lines were transduced by the recombinant viruses at an MOI of 5 or 20 $TCID_{50}$ units/cell and analyzed by flow cytometry (FCM) at 24 h p.t. The transduction efficiency of Ac-ThGP$_{HA}$ and Ac-DhGP$_{HA}$ was significant higher than Ac-WT in six cell lines (the left panel) and was equal to, slightly higher, or even lower than Ac-WT in other six kinds of cells (the right panel). The results are mean ± SD (n = 3 experimental replicates). Significant difference relative to Ac-WT was performed by student's *t* test: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. **C** The comparison of Ac-ThGP$_{HA}$, Ac-DhGP$_{HA}$ and Ac-VSVG transduction into different cell lines. Data of **B** at an MOI of 5 $TCID_{50}$ units/cell was analyzed and the fold increase of Ac-ThGP$_{HA}$ and Ac-DhGP$_{HA}$ comparing to Ac-WT was calculated. The cells were divided into four group in which the transduction efficiency of Ac-ThGP$_{HA}$ and Ac-DhGP$_{HA}$ > Ac-VSVG (type I), Ac-ThGP$_{HA}$ and Ac-DhGP$_{HA}$ < Ac-VSVG (type II), Ac-ThGP$_{HA}$, Ac-DhGP$_{HA}$ and Ac-VSVG is equal or slightly higher than Ac-WT (type III) and Ac-ThGP$_{HA}$ and Ac-DhGP$_{HA}$ < Ac-WT (type IV). Significant difference relative to Ac-VSVG was performed by student's *t* test: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

FCM (Fig. 3B). For Ac-DhGP$_{HA}$ and Ac-ThGP$_{HA}$, the transduction efficiency of A549, HeLa, Hep2, RD, SH-SY5Y, and SW13 was significantly higher than those of Ac-WT at both MOI of 5 and 20, with the highest increase (∼ 12 and ∼ six-fold compared to Ac-WT at an MOI of 5 and 20, respectively) was observed in RD cells transduced by Ac-ThGP$_{HA}$. For the other five cell lines (HepG2, HMC3, HUVEC, U87MG, and Vero cells), the transduction efficiency of Ac-DhGP$_{HA}$ was equal to or slightly higher (less than two-fold) than Ac-WT. The transduction of Ac-ThGP$_{HA}$ was similar to Ac-DhGP$_{HA}$ in these five cell lines, with an exception for HepG2 cells, in which the transduction efficiency was ∼ four-fold of Ac-WT at an MOI of 5. Unexpectedly, the transduction efficiency of Ac-DhGP$_{HA}$ and Ac-ThGP$_{HA}$ in HEK 293 cells was notably lower than that of Ac-WT.

The transduction efficiency of Ac-VSVG was significantly higher than Ac-WT in nine cell lines, with 1.6–2.5 fold increase in A549, HeLa, SH-SY5Y, HMC3, U87MG and HEK 293 cells and 5–10 fold increase in RD, SW13 and HepG2 cells at an MOI of 5. In HUVEC cells, there was no difference between Ac-VSVG and Ac-WT, while in Hep2 and Vero cells the transduction efficiency of Ac-VSVG is lower than that of Ac-WT.

Ac-DhGP$_{HA}$, Ac-ThGP$_{HA}$ and Ac-VSVG showed significant difference in cell tropism when transducing mammalian cells as shown in Fig. 3C. In A549, HeLa, Hep2,RD and SH-SY5Y cells (type I), the transduction of Ac-DhGP$_{HA}$ and Ac-ThGP$_{HA}$ was 1.8–4.7 fold higher than Ac-VSVG. In contrast, Ac-VSVG showed (1.8–5.8 fold) higher transduction efficiency than Ac-DhGP$_{HA}$ and Ac-ThGP$_{HA}$ in SW13 and HepG2 cells (type II). Ac-DhGP$_{HA}$, Ac-ThGP$_{HA}$ and Ac-VSVG all showed no or only slight improvement of transduction than Ac-WT in HMC3, HUVEC, Vero and U87MG cells (type III), while Ac-DhGP$_{HA}$ and Ac-ThGP$_{HA}$ even showed reduced transduction efficiency in HEK 293 cells (type IV).

The GPs of Thogotoviruses Elevated Virus-Endosome Fusion

To explain why incorporation of GPs of thogotoviruses improved the baculovirus transduction, we first detected the virus binding on HeLa cells. The results showed that the binding of Ac-DhGP$_{HA}$ and Ac-ThGP$_{HA}$ was only half of Ac-WT at both MOI of 5 and 20 (Fig. 4A). Since after virus binding, the virions will be internalized by endocytosis (Kataoka *et al*. 2012; Long *et al*. 2006), we next determined whether the internalization of recombinant viruses was enhanced. The results showed that Ac-DhGP$_{HA}$ entered HeLa cells with similar levels to Ac-WT at 30 and 60 min post transduction (p.t.) (Figure 4B). While for Ac-ThGP$_{HA}$, the virus internalization was ∼ 30% lower than Ac-WT at 30 min p.t. and was equal to Ac-WT at 60 min p.t. These results suggested that the virus binding and internalization were not improved by incorporation of thogtovirus GPs.

For most of enveloped viruses, a prerequisite for successful infection is escaping from endosomes by fusion of viral envelope and endosomal membrane (Yamauchi and Helenius 2013). Here, we investigated the virus-endosome fusion of Ac-WT, Ac-ThGP$_{HA}$ and Ac-DhGP$_{HA}$ by labeling the viruses with DiD, a lipotropic dye which will dequench and show brighter fluorescence when the dye is diluted as a result of virus-endosome fusion (Spence *et al*. 2016). The DiD labeled viruses showed only weak fluorescence at 0 min p.t. (Figure 4C, the upper panel). While at 60 min p.t., the viruses were endocytosed into cytoplasm and fused with endosomes, thus showing brighter fluorescence, especially the viruses Ac-ThGP$_{HA}$ and Ac-DhGP$_{HA}$ (Fig. 4C, the lower panel). When cells were treated with bafliomycin A1 (BFA1), an inhibitor of endosome acidification, the virus-endosome fusion was blocked, resulting in almost no increase in DiD fluorescence intensity. The fluorescence intensity of each DiD labeled virus was analyzed (Fig. 4D). The DiD fluorescence intensity of both Ac-ThGP$_{HA}$ and Ac-DhGP$_{HA}$ is obviously higher than Ac-WT at 60 min p.t. The percentage of dequenched DiD of Ac-WT is ∼ 16% at 60 min p.t. and BFA1 reduced it to ∼ 4% (Fig. 4E). For both Ac-ThGP$_{HA}$ and Ac-DhGP$_{HA}$, the percentage of dequenched DiD increased to ∼ 40%. The results indicate that the AcMNPV fusion was enhanced by incorporation of the thogotovirus GPs.

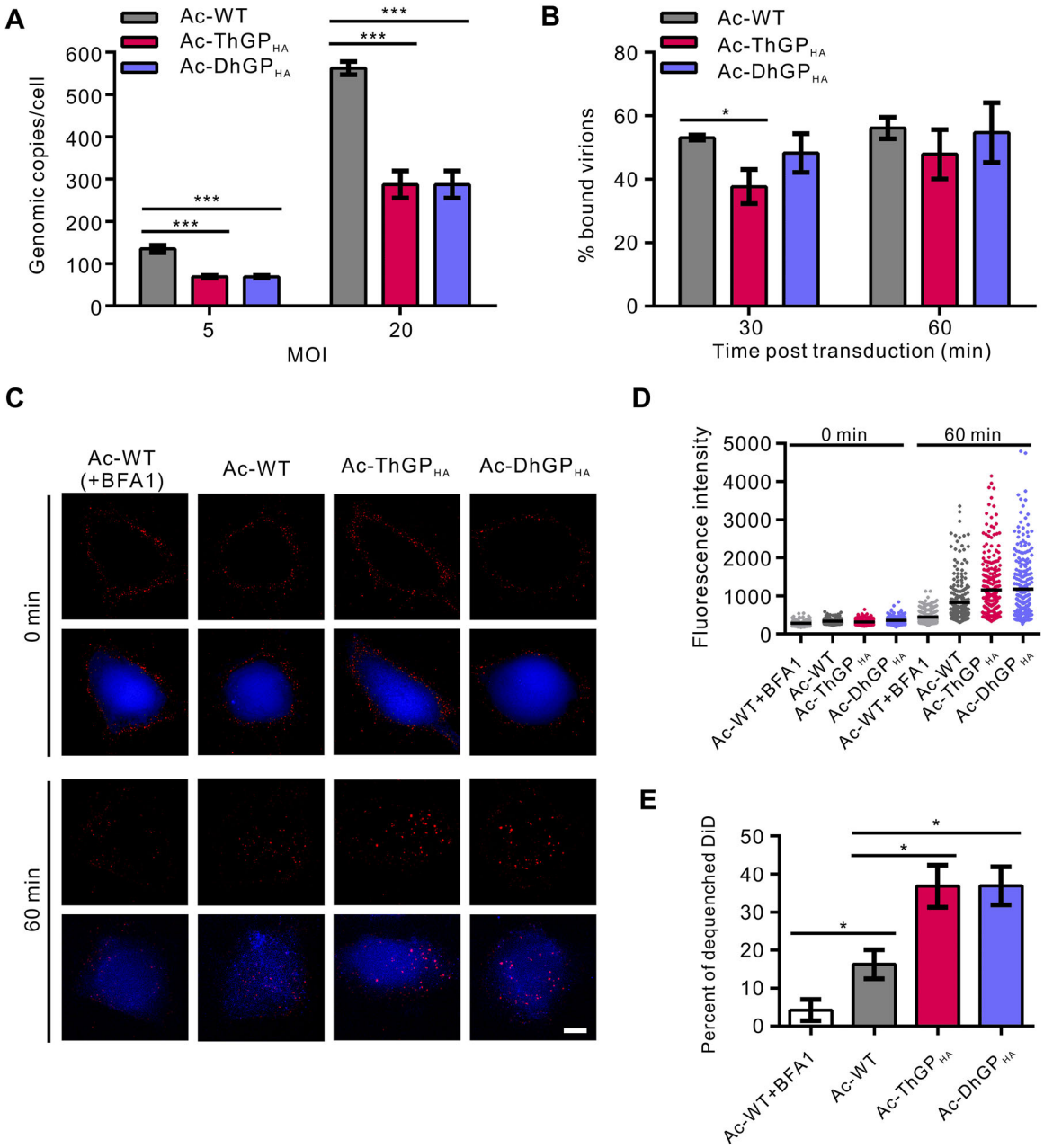


Fig. 4 Thogotovirus GPs enhanced the virus-endosome fusion in mammalian cells. HeLa cells were incubated with Ac-WT, Ac-ThGP$_{HA}$ and Ac-DhGP$_{HA}$ at an MOI of 5 or 20 $TCID_{50}$ units/cell at 4 °C to allow the virions binding to cells. After unbound virions being washed away, the virus-bound cells were collected for DNA extraction and quantitative PCR (qPCR) to quantify the cell-bound virions (**A**), or cultured at 37 °C for 30 or 60 min, then cells were digested by trypsin to remove the virions bound on cell surface and the total DNA was extracted for qPCR to quantify the virions internalized into cells (**B**). The genomic copies were normalized to that of cell bound virions of each recombinant virus determined in **A** separately. The results are mean ± SD (n = 3 experimental replicates). **C** DiD dequeching experiment to investigate virus-endosome fusion. The cytoplasm of HeLa cells was stained by calcein AM and the cells were incubated with DiD labeled viruses at 4 °C to allow synchronous virus binding. Unbound virions were washed away and the cells were imaged immediately by fluorescence microscopy at channels DAPI (calcein AM, blue) and Cy5 (DiD, red). Then cells were cultured at 37 °C for 60 min and imaged again. For the control group (Ac-WT + BFA1), 40 nmol/L BFA1 was added in the whole experiment. Bar, 10 μm. **D** The analysis of fluorescence intensity of DiD. Images of **C** were analyzed to calculate the fluorescence intensity of each DiD labeled virions. The black lines indicate the mean fluorescence intensity. The results are represented of two randomly selected fields. **E** Percentage of dequenched DiDs. Images of **C** were analyzed to calculate the fluorescence intensity of DiD. Three fold of the mean values at 0 min was set as the threshold to define dequenched DiD and the percentages of dequenched DiD of each recombinant viruses were calculated. For each group, more than 10 randomly selected fields were analyzed. The results are mean ± SD (n = 2 independent replicates). Significant difference was analyzed by student's *t* test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

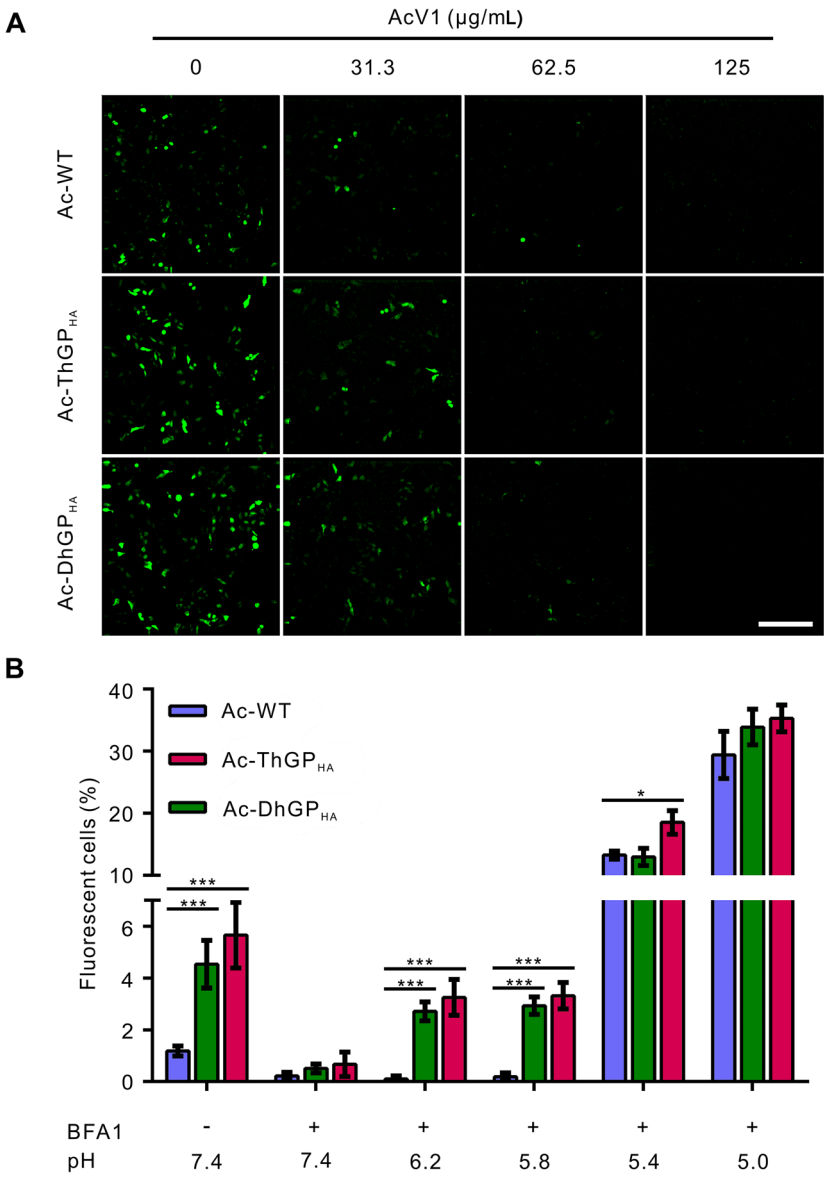


Fig. 5 Thogotovirus GPs enhanced the GP64-mediated membrane fusion at higher pH conditions. **A** The transduction of recombinant viruses were blocked by neutralizing antibody against the fusion activity of GP64. Ac-WT, Ac-ThGP$_{HA}$ and Ac-DhGP$_{HA}$ were incubated with neutralizing antibody AcV1 at indicated concentration and then used to transduce HeLa cells. The cells were imaged for EGFP expressing at 24 h p.t. The results are represent of two independent experiments. Bar, 200 μm. **B** Thogotovirus GPs enhanced the viral fusion at relative high pH condition. HeLa cells were incubated with the recombinant virus at 4 °C to allow virus binding. The virion-bound cells were treated by medium with indicated pH to trigger virus-plasma membrane fusion. Forty nmol/L BFA1 was used to block virus entry through the endocytosis pathway. Cells were analyzed by FCM at 24 h p.t. for EGFP expression. The results are mean ± SD (n = 3 experimental replicates). Significant difference relative to Ac-WT was performed by student's *t* test: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

The GPs of Thogotoviruses Enhanced the GP64-Mediated Membrane Fusion at Mildly Acidic Condition

As ThGP and DhGP are responsible for the viral fusion of THOV and DHOV, respectively, we analyzed the role of ThGP and DhGP in mediating the fusion of AcMNPV. AcV1, a neutralizing antibody of GP64, was used to block the fusion activity of GP64 (Zhou and Blissard 2006). The transduction in HeLa cells of Ac-WT, Ac-ThGP$_{HA}$ and Ac-DhGP$_{HA}$ was significantly reduced by AcV1 at concentration of 31.3 μg/mL and was totally blocked at 125 μg/mL (Fig. 5A). As AcV1 inhibits the fusion activity, but not the cell binding ability of GP64, the results suggested that the GPs of thogotoviruses could not mediate the fusion of AcMNPV independently.

GP64 is a low-pH dependent viral fusion protein, with optimal pH lower than 5.5 (Blissard and Wenz 1992). It is previously reported that a short time treatment with low-pH (pH 4.8) after binding of AcMNPV to mammalian cells can trigger virus fusion at plasma membrane, resulting in high efficient baculovirus transduction of mammalian cells (Dong *et al*. 2010). We tested whether the GP64 mediated fusion was enhanced by the GPs of thogotoviruses by a similar experiment. When the cellular endocytosis pathway was blocked by BFA1, a short time treatment of pH 5.4 and 5.0 increased the transduction rate of Ac-WT from 0.2% (pH 7.4 + BFA1) to 13% and 29%, respectively, and the transduction rate of Ac-ThGP$_{HA}$ and Ac-DhGP$_{HA}$ was equal to or slightly higher than Ac-WT (Fig. 5B). Interestingly, when treated with higher pH condition (pH 6.2 and 5.8), there was no increase in transduction of Ac-WT,

while the transduction of Ac-ThGP$_{HA}$ and Ac-DhGP$_{HA}$ was increased from ~ 0.5% (pH 7.4 + BFA1) to ~ 3%. The results indicates that the GPs of thogotoviruses enhanced the GP64 mediated membrane fusion at mildly acidic condition with pH higher than 5.4.

Discussion

AcMNPV is a promising gene therapy vector as it can transduce a wide variety of human cells with advantages over the traditional viral vectors (Hu 2010). However, the low transduction efficiency of most mammalian cells is one of the major obstacles that limits the clinical application of AcMNPV (Mansouri and Berger 2018). Although a short time low pH treatment can greatly improve the virus transduction, it is not exercisable for *in vivo* application (Dong *et al*. 2010). Incorporation of GPs from other viruses into the virions of AcMNPV seems to be the most commonly used and effective method to enhance baculovirus transduction both *in vitro* and *in vivo* (Ono *et al*. 2018). In this study, the GPs of THOV and DHOV were successfully incorporated into viral envelope of AcMNPV and their transduction efficiency was determined in a wide range of mammalian cell lines with comparison of VSV G incorporated AcMNPV.

ThGP and DhGP improved the baculovirus transduction efficiency from 4 to 12 fold compared to Ac-WT in six of the 12 selected cell lines we tested at an MOI of 5 and showed tropism of wide range of cell types, including the epithelial cells (A549, HeLa and Hep2), muscle cells (RD), bone marrow neuroblast cells (SH-SY5Y) and adrenal adenocarcinoma cells (SW13). VSV G has been widely used to enhance baculovirus transduction efficiency and expand the cell tropism (Barsoum *et al*. 1997; Kolangath *et al*. 2014). Here, we found that the transduction efficiency of Ac-VSVG is higher than Ac-WT in nine cell lines, but increased by more than four-fold only in three of them at an MOI of 5. The transduction of AcMNPV in five cell lines, including A549, HeLa, RD, SW13 and SH-SY5Y, was significantly improved by both thogotovirus GPs and VSV G at an MOI of 5. Among these, the transduction efficiency of Ac-ThGP$_{HA}$ and Ac-DhGP$_{HA}$ is higher than Ac-VSVG in four kinds of cells (A549, HeLa, RD and SH-SY5Y) and lower in one cell line (SW13) (Fig. 3B). Additionally, the baculovirus transduction in Hep2 cells was significantly improved only by thogotovirus GPs, but not by VSV G. The results indicate that thogotovirus GPs and VSV G render baculovirus different cell tropism, and thogotovirus GPs seems to more effective in increasing baculovirus transduction in some cell lines compared to VSV G, such as A549, HeLa, Hep2, RD, and SH-SY5Y cells; Whereas VSV G exihibt better performance than thogotovirus GPs in SW13, HMC3, HepG2, and U87MG cells. Therefore, recombinant virus with different GPs should be considered for improving baculovirus transduction into different human cells.

To elucidate the mechanism that how GPs of thogotoviruses enhance baculovirus transduction in mammalian cells, virus binding was first detected. Unexpectedly, the binding of Ac-ThGP$_{HA}$ and Ac-DhGP$_{HA}$ on HeLa cells was only about 50% to that of Ac-WT. This may be due to the incorporation of ThGP/DhGP impaired the amounts of GP64 assembled on the viral envelope or interfered the receptor binding activity of GP64. Following cell attachment, AcMNPV will be internalized into cells through clathrin-mediated endocytosis pathway (Kataoka *et al*. 2012). The results showed that the internalization of Ac-ThGP$_{HA}$ and Ac-DhGP$_{HA}$ was almost equivalent to Ac-WT at 60 min p.t. As the ThGP/DhGP did not enhance virus binding and internalization, it was speculated that the endosomal escaping was augmented. This idea was confirmed by the DiD dequenching experiments that indicated the virus-endosome fusion. The percentages of dequenched DiD of Ac-ThGP$_{HA}$ and Ac-DhGP$_{HA}$ were 2.3 fold of Ac-WT at 60 min p.t., suggesting more efficient viral fusion and endosome escaping (Fig. 4E).

However, when the fusion activity of GP64 was blocked, Ac-ThGP$_{HA}$ and Ac-DhGP$_{HA}$ failed to transduce mammalian cells, indicating that ThGP and DhGP could not mediate the viral fusion of baculoviruses independently. A possible explanation is that the incomplete glycosylation of the GPs impaired the fusion activity, as the molecular weight of ThGP expressed in insect cells was ~ 68 kDa (Fig. 2B) and is significantly smaller than that expressed in mammalian cells (~ 75 kDa) (Agustin *et al*. 1992). Interestingly, the GP64 mediated fusion was enhanced by ThGP and DhGP at pH 5.8 and 6.2. It was reported that a less than 21-amino-acid ectodomain together with the transmembrane and cytoplasmic tail domain of VSV G could augment the membrane fusion activity of some heterologous viral envelope proteins, including AcMNPV GP64 (Jeetendra *et al*. 2002; Kaikkonen *et al*. 2006). Both THOV and DHOV possess only one envelope GP and the conformational change of the GP was observed upon low pH treatment, with the highest haemolysis activity at pH 6.0–6.2 and cell–cell fusion at pH 6.0 (Agustin *et al*. 1992; Peng *et al*. 2017). Although pH < 5.5 is essential for the fusion activity of GP64 measured by cell–cell fusion, weak fusion activity was observed by virus-liposome fusion at pH 5.5–6.5 (Kamiya *et al*. 2010). It was reported that the baculovirus transduction in HeLa cells dependent on early endosomes but not late endosomes (Liu *et al*., 2014), of which the pH are 6.8–5.9 and 6.0–4.9, respectively (Huotari and Helenius 2011). We speculate that the GP64 mediated virus-endosome fusion in the

mildly acidic early endosomes of mammalian cells is augmented by ThGP and DhGP.

In conclusion, the GPs of two thogotoviruses were expressed and incorporated into the virions of AcMNV. By incorporating these GPs into AcMNPV envelope, the baculovirus transduction in a variety of human cells was significantly improved. The improvement of transduction is probably a result of enhanced GP64 mediated virus-endosome fusion by the incorporation of thogotovirus GPs. These viruses showed higher transduction efficiency than Ac-VSVG in many cells, providing us more choices to improve baculovirus transduction for gene delivery.

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Compliance with Ethical Standards

Conflict of interest The authors declare no competing interests.

Animal and Human Rights Statement This article does not contain any studies with human or animal subjects performed by any of the authors.

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