Membrane penetrating peptides greatly enhance baculovirus transduction efficiency into mammalian cells

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ABSTRACT

The baculovirus group of insect viruses is widely used for foreign gene introduction into mammalian cells for gene expression and protein production; however, the efficiency of baculovirus entry into mammalian cells is in general still low. In this study, two recombinant baculoviruses were engineered and their ability to improve viral entry was examined: (1) cytoplasmic transduction peptide (CTP) was fused with baculovirus envelope protein, GP64, to produce a cytoplasmic membrane penetrating baculovirus (vE-CTP); and (2) the protein transduction domain (PTD) of HIV TAT protein was fused with the baculovirus capsid protein VP39 to form a nuclear membrane penetrating baculovirus (vE-PTD). Transduction experiments showed that both viruses had better transduction efficiency than vE, a control virus that only expresses EGFP in mammalian cells. Interestingly, vE-CTP and vE-PTD were also able to improve the transduction efficiency of a co-transduced baculovirus, resulting in higher levels of gene expression. Our results have described new routes to further enhance the development of baculovirus as a tool for gene delivery into mammalian cells.

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1. Introduction

Baculoviruses are a group of enveloped insect viruses which do not have the potential to replicate in mammalian cells [1]. *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV), the best known baculovirus, is capable of entering a wide spectrum of cells, including cells of non-insect origin [2]. No cytotoxic effects of baculovirus transduction in mammalian cells have been reported [3].

An essential requirement for baculovirus-mediated mammalian protein expression is the ability of the viruses to transport their genomes efficiently into the nuclei of the target cells. Baculovirus entry into mammalian cells is a multi-step process and is thought to be similar to that found in insect cells [4]. The first step in viral entry is the attachment of virions onto the host cell surface. The receptor on mammalian cells for *Ac*MNPV attachment and entry is still unknown but since baculovirus can enter a variety of mammalian cells, Duisit et al. [5] and Tani et al. [6] suggested that it attaches to a common cell-surface component such as an integrin, phospholipid or heparan sulfate proteoglycan [5,6]. The attached virus then enters the cell by endocytosis. Both clathrin-mediated and -independent endocytosis have been suggested as the route by which baculoviruses enter different types of mammalian cells [7,8]. In the next step, a pH-dependent process mediated by baculovirus surface glycopro-

tein GP64 causes fusion between the viral envelop and the endosomal membrane, and releases the viral nucleocapsids into the cytoplasm. Finally, the released nucleocapsids are transported to the nuclear pore complex [9], enter the nucleus and are disassembled. The viral genome with the foreign transgene expression cassette is then released and transcribed [9,10].

Cytomegalovirus immediate early promoter (CMV) is probably the most frequently used promoter for baculovirus-mediated gene transfer into mammalian cells [11]. In addition, several factors have been shown to stimulate CMV promoter activity, such as both the homologous region sequences (*hr*) [12] and the immediate early gene 2 (ie2) of *Ac*MNPV [13]. An alternative method of baculovirus-mediated gene transfer into mammalian cells is to modify the virus particle, specifically the envelope protein, GP64, or the major nucleocapsid protein, VP39, with the aim of improving the binding and entering of baculovirus to mammalian cells, thus delivering more transgene copies into the cell nucleus where they can be transcribed and expressed. Previously, pseudotype baculoviruses have been made by displaying vesicular stomatitis virus G protein (VSVG), the RGD motif, tumor-homing peptides, or avidin on the viral envelope, fused to the surface GP64 glycoprotein [14].

Many synthetic or naturally occurring peptides (often the protein transduction domain [PTD] of a protein) have been proved to have cell-penetrating abilities [15]. Proteins fused with these cell-penetrating peptides can translocate into cells with great efficiency [15]; some examples include antennapedia homeodomain, penetratin, synthetic oligo-Arg peptide sequences and a 14 amino

acid arginine-rich sequence of HIV TAT protein called TAT-PTD. These cell-penetrating peptides can deliver not only proteins but also other macromolecules such as nucleic acids, 200 nm liposomes and some anticancer agents [16,17]. Here, we investigated the potential of two such peptides in enhancing baculovirus entry into mammalian cells.

Baculoviruses have to overcome at least two physical barriers during viral transduction into target cells: the cell membrane and nuclear membrane. To enhance baculovirus entry into cells, we fused a cell membrane translocation peptide, cytoplasmic transduction peptide (CTP) with GP64. CTP is a short peptide derived from the PTD of HIV TAT protein which can deliver macromolecules into the cytosol [18]. To enhance baculovirus nuclear entry, we fused the TAT-PTD peptide with VP39, the major capsid protein of baculovirus [19]. Our results showed that both CTP and PTD peptides can enhance the transduction efficiency of the recombinant baculoviruses. Interestingly, we also found that the recombinant baculoviruses with fused peptides can enhance the entry of co-transduced baculoviruses into mammalian cells.

2. Materials and methods

2.1. Vector constructions

Viruses vAtLUC (vLUC) and vAtE (vE), and plasmids pAtEp64S488 and pAtE, were constructed as reported previously [13]. The hsp70 promoter of pABpaR2hX was constructed from pKih35hN [12,20].

For the ligation of CTP to GP64, the CTP fragment was first generated by PCR-mediated synthesis using CTP forward primer and CTP reverse primer (Table 1). The CTPHA fragment was cloned into pAtEp64S488 through the two SfiI sites to replace the SARS CoV spike N-terminal ectodomain S488 [21]. The CTPHA-GP64 (including the GP64 signal peptide) fragment was then sub-cloned into pABpaR2hX between StuI and PacI restriction sites thus obtaining pABpaR2-hCTPHA-GP64. The hCTPHA-GP64 fragment was cut out and placed within pAtE to obtain pAtE-hCTPHA-GP64 (pE-CTP). PTD-VP39 forward primer and a VP39HA reverse primer (Table 1) were used to ligate the PTD-VP39HA DNA fragment from the wild-type AcMNPV genome. The resulting PTD-VP39HA fragment was cloned into pABpaR2hX within the XmaI and NotI sites to get pABpaR2-hPTD-VP39HA. Finally the hPTD-VP39HA DNA fragment was placed into pAtE between the XhoI and NotI sites to get pAtE-hPTD-VP39HA (pE-PTD).

2.2. Baculovirus production in insect cells

Recombinant baculoviruses were generated and propagated in Sf21 cells according to the standard protocols of O'Reilly et al. [22]. Baculovirus transfer plasmids pE-CTP and pE-PTD were used to generate recombinant baculoviruses vE-CTP and vE-PTD using the BaculoGold system (Invitrogen). Cells were cultured at 26 °C in TC100 insect medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). Titers of virus were determined by quantitative PCR [23] or end point dilution [22].

2.3. Western blot

Recombinant baculovirus stocks vE-CTP and vE-PTD (50 μl) were precipitated with acetone and resuspended in 8 M urea. These samples were then subjected to Western blotting with an anti-HA tag antibody to detect the peptide-fusion proteins (1:5000; Sigma).

2.4. Transmission electron microscope

For immunogold labeling, baculoviruses were loaded onto a collodion-coated electron microscopy (EM) grid for 1 min. After the removal of excess sample solution by gently blotting with filter paper at the edge of the grid, the grid was immersed in blocking buffer (3% normal goat serum) for 1 h at room temperature. A mouse monoclonal anti-HA antibody (Sigma) was then added onto the grid and incubated for 1 h at room temperature. Grids were then washed six times for 10 s each in Sorensen's phosphate buffer at room temperature and incubated with 12 nm gold conjugated anti-mouse IgG for 1 h. After six 10 s washes in Sorensen's phosphate buffer, the samples were stained with 2% PTA for 1 min, then drained and examined under the EM (Tecnai G2 Spirit TWIN, FEI Company) [24].

2.5. Transduction of mammalian cells

Vero E6 cells were grown in minimal essential medium (MEM) containing 10% FBS at 37 °C with 5% CO_2. U-2OS cells were grown in McCoy's medium containing 10% FBS at 37 °C with 5% CO_2. For transduction, 2.5×10^4 cells in 500 μl medium were added directly into one well of a 24-well plate. As for 96-well plate, 5×10^3 cells with 50 μl medium were seeded per well. After cells attached to the bottom, the culture medium was removed, and fresh medium with virus was added. After gentle mixing, the transduced cells were incubated at 37 °C with 5% CO_2 for 48 h.

2.6. Flow cytometry

Transduced cells were washed with Dulbecco's PBS (DPBS, Invitrogen) twice, harvested with trypsin and resuspended in 200 μl of DPBS. Cells were collected and analyzed using FACScan flow cytometer (Becton–Dickinson, San Jose, CA). The EGFP protein within the cells was excited at 488 nm and detected at 525 nm. About 10^4 cells were collected per specimen with three repeats.

Table 1
Primers for amplification and real-time PCR quantitation.

DNA fragment	Primer	Sequence
CTPHA	CTP forward	5′-ATTCGGCCTTTGCGGCC**TACGGCCGCCGCGCCCGCCGCCGCCGCCGCCGC**-3′ (SfiI site underlined and CTP in bold)
	CTP reverse	5′-TGAAGGCCACCGTGGCCGGCAGAGTCTGGGACGTCATATGGATAG**CGGCGGCGGCGGCGGC**-3′ (SfiI site underlined, HA taq italicized and CTP in bold)
PTD-VP39HA	PTD-VP39 forward	5′-ATCCCCCGGGATG**TACGGCCGCAAAAAACGCCGCCAACGCCGCCGC**ATGGCGCTAATGCCCGTGGGTATGG-3′ (XmaI sites underlined, and PTD in bold)
	VP39HA reverse	5′-TTAATTAAGCGGCCGCTTA*GGCAGAGTCTGGGACGTCATATGGATA*GGCGGCTACACCTCCGCCTGCTTCG-3′ (NotI and PacI sites underlined and HA tag italicized)
ie1 (Q-PCR)	ie1 forward	5′-CCCGTAACGGACCTCGTACTT-3′
	ie1 reverse	5′-TTATCGAGATTTATTTGCATACAACAAG-3′
Luciferase (Q-PCR)	luc forward	5′-ATTCTATCCGCTGGAAGAT-3′
	luc reverse	5′-TTTCACTGCATACGACGA-3′

The cell sample without virus transduction served as a negative control. The transduction efficiency was presented as a percentage calculated according to the following formula: number of EGFP positive cells/total number of cells collected.

2.7. Viral DNA extraction and copy number determination by Q-PCR

Viruses were transduced into mammalian cells. At 48 h post-transduction (hpt), mammalian cells were washed with DPBS three times and harvested by trypsin treatment. Viral DNA extraction was performed with a High Pure Viral Nucleic Acid Kit (Roche) according to the manufacturer's protocol. Quantitative real-time PCR (Q-PCR) was performed using FastStart DNA Master^{plus} SYBR Green I (Roche) to determine copies of *ie1* or *luc* (luciferase) DNA compared to viral titers. Primers used for Q-PCR are listed in Table 1, and real-time PCR was performed by LightCycler 1.5 (Roche).

2.8. Luciferase activity

Transduced cells were washed with DPBS, lysed with culture cell lysis reagent and centrifuged to remove cell debris. Finally, samples were mixed with luciferase activity reagent and luciferin (Promega) and luciferase activity was measured with a luminometer (Victor 3; Perkin-Elmer). Detailed procedures have been described previously [25]. The protein concentration of each well was measured using a Coomassie protein assay kit (Pierce). Luciferase activity data (average ± SD) were collected from triplicate assays of three independent transductions.

3. Results

3.1. Modification and integration of CTP and PTD into baculoviral particles

Previously, Kost and colleagues reported that certain cell lines, such as Chinese hamster ovary (CHO) cells, were inefficiently transduced by baculovirus, [26]. In an attempt to address this problem, we engineered two recombinant baculoviruses: the first expressing an extra copy of modified GP64 protein, CTP-GP64, to enhance virus penetration into mammalian cells; and the second expressing an extra copy of modified VP39 protein, PTD-VP39, to enhance baculovirus nucleocapsid transport into cell nuclei (Fig. 1A). In order to determine whether these modified proteins could be expressed and presented on baculovirus particles, the recombinant viruses were purified and analyzed by Western blot. CTP-GP64 and PTD-VP39 fusion proteins of predicted sizes were identified in the purified vE-CTP and vE-PTD viral particles, respectively (Fig. 1B). Therefore not only were the proteins successfully modified; they were also able to integrate into the baculovirus envelope or capsid. To further characterize the location of these engineered proteins, immunogold labeling was performed (Fig. 1C). As expected, CTP-GP64 was distributed on one tip of the baculovirus; and PTD-VP39 was distributed on the circumference of the viral particle.

3.2. CTP and PTD improve baculoviral anchoring or entry into mammalian cells

Transduction enhancement tests in Vero E6, U-2OS and CHO-RD cells were then performed by flow cytometry (FACS), as both viruses also contained an EGFP reporter gene (Fig. 2). An EGFP-expressing virus, vE, made from the same vector backbone as the vE-CTP and vE-PTD viruses, was used as a control. Transduction efficiency was determined by using the recombinant baculoviruses at different multiplicities of infection (MOIs), and calculating the
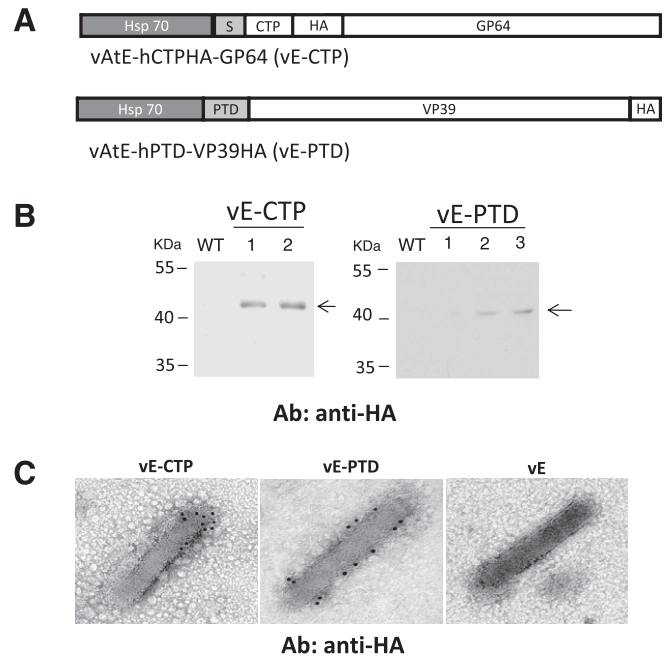

Fig. 1. Fusion of membrane-penetrating peptides CTP and PTD onto the surface of baculoviruses. (A) Schematic representation of recombinant baculoviruses vE-CTP (CTPHA-GP64) and vE-PTD (PTD-VP39HA). Hsp70, heat shock 70 promoter; S, GP64 protein signal peptide; HA, HA tag; GP64, AcMNPV major envelope protein GP64. VP39, AcMNPV major capsid protein VP39. (B) Western blot analysis of CTPHA-GP64 and PTD-VP39HA proteins isolated from the membrane of purified baculoviruses. The 42.3 kDa CTPHA-GP64 (left panel) and 41.8 kDa PTD-VP39HA (right panel) proteins were detected by anti-HA monoclonal antibody. (C) Ultra-thin structural characterization of recombinant virus. Immunogold labeling of HA tagged recombinant viruses. vE, a virus without surface modification, served as a negative control.

percentage cell transduction rate (Fig. 2). The results showed that vE-PTD transduced Vero E6 and CHO-RD cells more efficiently; and vE-CTP transduced U-2OS cells more efficiently. These results suggest that both modifications can efficiently improve baculovirus transduction into some mammalian cells.

We then determined the efficiency of viral entry into various cells. One way to measure the efficiency of viral entry is to determine the amount of viral DNA in cells after transduction. Either vE-CTP or vE-PTD was transduced into three types of mammalian cells, Vero E6, U-2OS and CHO-RD at MOI = 10, and washed three times. The eGFP-expressing recombinant baculovirus vE was used as a control. Viral DNAs from the virus transduced mammalian cells were collected at 48 hpt. These DNA samples were then subjected to Q-PCR quantification to obtain the number of virus particles within transduced cells (2.5×10^4 cells per sample). vE-CTP and vE-PTD showed an increased amount of viral DNA within all three types of cells in comparison with cells transduced with vE (Fig. 3A). Vero E6 and CHO-RD cells in particular were very susceptible to vE-CTP and vE-PTD anchoring and/or penetration. This result indicates that VP39 fused with PTD, or GP64 fused with CTP, can indeed help baculovirus translocation into cells. We thus went onto investigate whether a greater number of viral particles entering cells actually led higher levels of transgene expression.

3.3. Improved viral entry leads to higher levels of transgene expression in baculovirus susceptible cells

We next tested the levels of transgene expression of vE-CTP, vE-PTD and vE in Vero E6, U-2OS and CHO-RD cells by flow cytometry. The level of transgene expression in vE-transduced cells serves as a control. The total fluorescence intensities of each transduced cell sample are shown in Fig. 3B.
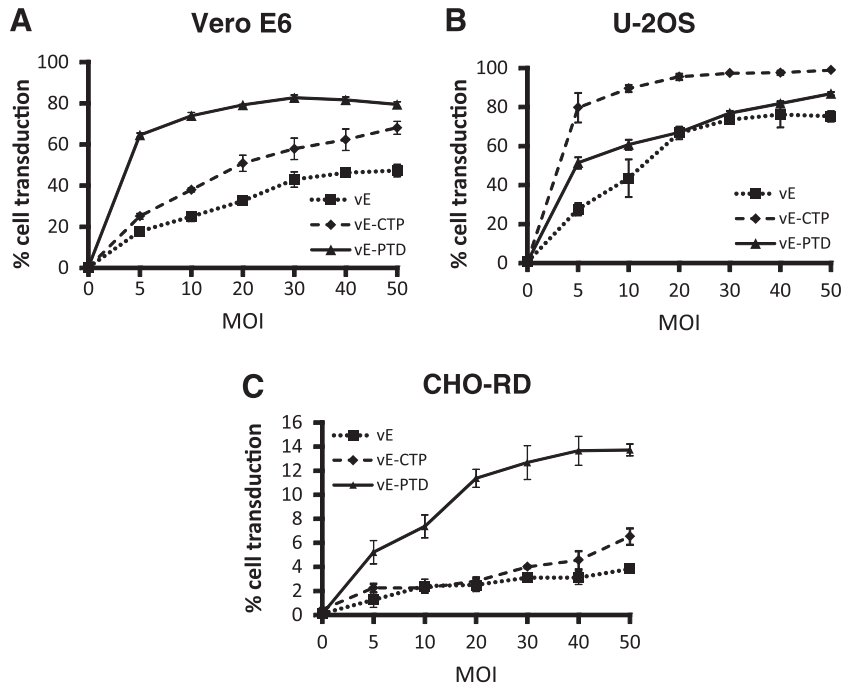
Fig. 2. Determination of transduction efficiency of vE, vE-CTP and vE-PTD in Vero E6, U-2OS and CHO-RD cells. Vero E6 (A), U-2OS (B) and CHO-RD (C), cells were transduced at various MOIs with indicated virus, and the transduction ratios were determined by the emission of green fluorescence of the cells by flow cytometry at 2 days post infection.
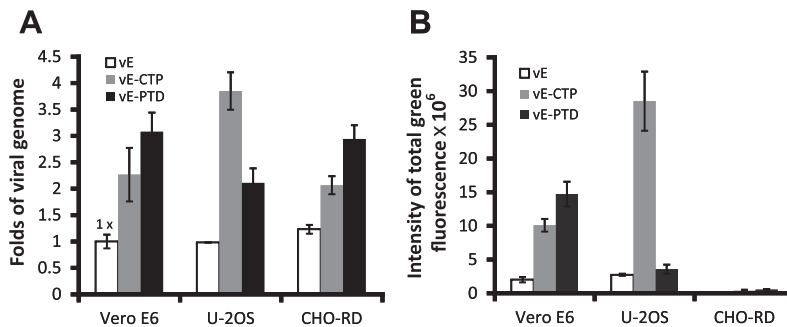

Fig. 3. Determination of viral genomic copy number and transduction efficiency in transduced cells. (A) Determination of viral genome copy number in transduced cells. Viruses were transduced into each cell type at MOI = 10. At 2 days post-transduction (dpt), transduced cells were collected for viral DNA extraction. Viral DNAs were subjected to Q-PCR to estimate the baculovirus *ie1* gene copy numbers as described previously. The DNA amount of *ie1* gene, determined from the Vero E6 cells transduced with vE, was set as one. Total EGFP fluorescence intensity (B) of each transduced cell type was determined by flow cytometry at 2 dpt. vE, vE-CTP and vE-PTD were transduced into each cell type at MOI = 10.

Interestingly, the similar levels of viral genomic copies within the CHO-RD cells did not translate into comparable levels of transgene expression. In these cells, the total fluorescence intensity was extremely weak compared to that in U-2OS cells (Fig. 3). This indicates that the baculovirus may encounter problems after entering CHO-RD cells and ultimately fail to express the transgene. In the cell lines which were already relatively susceptible to baculovirus transduction, Vero E6 and U-2OS, the improvements in transduction efficiencies were very significant. Recombinant vE-PTD virus (MOI = 10) raised the transduction rate from 24.9% to 73.9% in Vero E6 cells, while vE-CTP virus (MOI = 10) raised the transduction rate from 43.5% to 89.5% in U-2OS cells (Fig. 2). The preference of a virus in different cell lines points out where is the major bottle neck for baculovirus transduction into these cells. In this case, both vE-CTP and vE-PTD worked fairly well in Vero E6, while only vE-CTP showed a significant improvement in U-2OS. This could mean that baculovirus, once penetrated U-2OS cellular membrane, would have no difficulty entering the nuclei. In CHO-RD cells, however,

baculovirus may either encounter difficulty further entering the nucleus or may have difficulty expressing the target gene.

3.4. vE-CTP and vE-PTD improve transduction and expression of a co-transduced baculovirus

Our data above shows that CTP or PTD peptides fused to the surface of baculovirus can improve viral transduction efficiency. To test whether vE-CTP and vE-PTD could also improve transgene expression of a co-transduced baculoviruses, a reporter virus vLUC (containing a *luciferase* gene driven by CMVie promoter) was used. The vLUC was co-transduced with vE-CTP or vE-PTD, and luciferase levels in each transduced cell sample were measured at 48 hpt. Comparing with vE, both vE-CTP and vE-PTD were able to enhance the transduction efficiency of the co-transduced baculovirus, vLUC, into target cells (Fig. 4A). The recombinant baculovirus vLUC generated 1.7- and 2.4-fold more luciferase activities in Vero E6 cells when it was co-transduced with vE-CTP and vE-PTD, respectively,
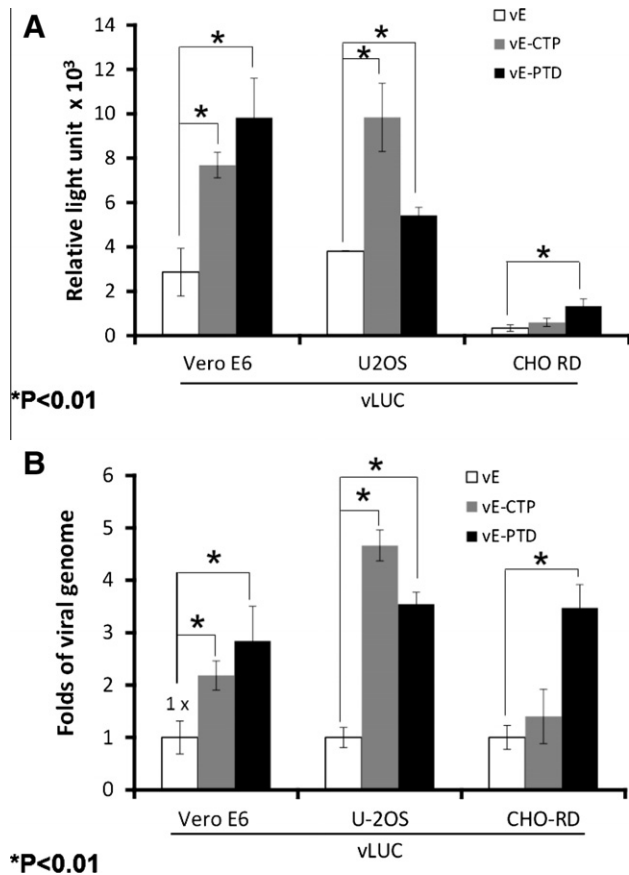
Fig. 4. Enhancement of gene expression and entry of the co-transduced baculovirus. (A) Determination of luciferase activities in the transduced cells. Reporter virus (vLUC, MOI = 10) was co-transduced with viruses vE, vE-CTP or vE-PTD (MOI = 10), respectively, in the indicated cells. Luciferase activity was determined at 2 dpt. (B) Amounts of viral genomic DNA determined by Q-PCR. Viral genomes in the transduced cells were quantified based on the sequence of *luc* gene. The DNA amount of *luc* gene, determined from the Vero E6 cells co-transduced with vLUC and vE, was set as one. The mean values and standard deviation (SD) of three independent experiments are shown. All data were analyzed by *t*-test using independent samples. A *P*-value of less than 0.01 was considered significant. *$P \leqslant 0.01$.

as compared to the control virus vE. In U-2OS cells, vE-CTP and vE-PTD enhanced the luciferase activity by 1.6- and 0.4-fold, respectively, comparing to the virus vE. In CHO-RD cells, enhancement of luciferase activity by vE-CTP and vE-PTD were 0.7- and 2.9-fold, respectively, to the virus vE. To confirm that this phenomenon was caused by an increased entry of vLUC virus, we performed Q-PCR using luciferase gene-specific primers (Table 1) to quantitate the copy of luciferase gene in the transduced cells. The result revealed that the copy number of vLUC DNA in the cells increased significantly when this virus is co-transduced with vE-CTP or vE-PTD (Fig. 4B).

The above results show that CTP and PTD peptides can be successfully incorporated onto the baculovirus surface as part of GP64 and VP39, respectively. These modified baculoviruses were able to transduce Vero E6 and U-2OS cells more efficiently than baculoviruses without surface modifications. In CHO-RD cells, although the overall levels of baculovirus gene expression were low, significant improvement in virus entry and gene expression were still evident. Interestingly, these membrane penetrating baculoviruses were able to improve not only their own entry into the susceptible cell lines, but also the transduction rate of a co-transduced baculovirus. This suggests the potential use of vE-CTP and vE-PTD as enhancers of baculovirus transduction in mammalian cells.

4. Discussion

In this study we investigated whether the membrane penetrating peptides, cellular membrane penetrating CTP and nuclear membrane penetrating PTD, were able to aid entry of baculovirus into several types of mammalian cells.

With CTP fused with GP64 (vE-CTP), U-2OS cells showed the most improvement in transduction rate, although Vero E6 and CHO-RD cells also contained high numbers of viral genome copies per cell (Fig. 3A). The increased copy number of viral DNA within U-2OS cells were successfully translated into more reporter gene expression, as shown in Fig. 3B. The vE-CTP virus also significantly improved the transduction of Vero E6 cells; the transduction rate was more than double and the intensity of EGFP expression increased 5-fold in comparison with the control. On the other hand, although CHO-RD cells showed an increased baculovirus vDNA within the cell, this DNA was not available for transcription.

Recombinant baculovirus vE-PTD contained a HIV TAT-derived PTD peptide, which was fused with the major viral capsid protein VP39. Although vE-PTD entered cells more efficiently than unmodified baculovirus vE (Fig. 3A) in all cell lines tested, PTD led to no significant improvement in transgene expression in U-2OS cells (Fig. 3B). A previous report by Li et al. [27] showed that expression levels of genes delivered by the assistance of PTD were cell-type specific. Although we found that PTD improved the transduction efficiency of baculovirus in CHO cells (Fig. 2), the level of gene expression in this cell type is also very low (Fig. 3B). Further studies showed that baculovirus actually enters CHO-RD cells quite well, indicating that baculovirus may encounter other problems perhaps relating to nuclear entry, difficulty in uncoating, poor gene expression, etc., after entering these cells.

In this study, we also found that vE-CTP or vE-PTD can improve the transduction efficiency of an accompanying baculovirus without surface modifications (Fig. 4). Electron microscopic study showed that vE-CTP or vE-PTD enters Vero E6 cells separately, without having an obvious physical association with the co-transduced reporter vLUC virus (data not shown). In other words, baculoviruses appear to enter the cells individually rather than as a group. Recently Laakkonen et al. had reported that baculovirus triggers non-phagocytic human cells uptake of *Escherichia coli* by clathrin-independent entry pathway [8]. We speculated that vE-CTP and vE-PTD, with their better transduction efficiency, may penetrate into mammalian cells quicker than un-modified ones and trigger these cells to take up vLUC more efficiently. Higher viral DNA contents of the reporter virus vLUC in the co-transduced cells also agreed with this speculation (Fig. 4B).

In conclusion, our results showed that the presence of large numbers of virus particles within cells in general correlate with higher levels of baculovirus transgene expression in mammalian cells. Further study will be required to determine the mechanism(s) that block transgene expression mediated by baculovirus transduction in different cells. We also demonstrated that the presence of an engineered membrane-penetrating baculovirus can enhance the transduction efficiency of a co-transduced baculovirus. As the uses of the recombinant baculovirus as gene delivery vectors for mammalian cells become ever more diverse, increased understanding of the interactions between baculoviruses and various mammalian cell types are crucial for foreign gene introduction into these cells.

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