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Genetically modified adenoviral vector with the protein transduction domain of Tat improves gene transfer to CAR-deficient cells

Shihai LIU*, Qinwen MAO†, Weifeng ZHANG*, Xiaojing ZHENG*, Ye BIAN*, Dongyang WANG*, Huijin LI*, Lihong CHAI*, Junli ZHAO* and Haibin XIA*[1]

*Laboratory of Gene Therapy, Department of Biochemistry, College of Life Sciences, Shaanxi Normal University, 199 South Chang'an Road, Xi'an 710062, People's Republic of China, and †Department of Pathology, University of Texas Southwest Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390, U.S.A.

Synopsis

The transduction efficiency of Ad (adenovirus) depends, to some extent, on the expression level of CAR (coxsackievirus and Ad receptor) of a target cell. The low level of CAR on the cell surface is a potential barrier to efficient gene transfer. To overcome this problem, PTD.AdeGFP (where eGFP is enhanced green fluorescent protein) was constructed by modifying the HI loop of Ad5 (Ad type 5) fibre with the Tat (trans-activating) PTD (protein transduction domain) derived from HIV. The present study showed that PTD.AdeGFP significantly improved gene transfer to multiple cell types deficient in expression of CAR. The improvement in gene transfer was not the result of charge-directed binding between the virus and the cell surface. Although PTD.AdeGFP formed aggregates, it infected target cells in a manner different from AdeGFP aggregates precipitated by calcium phosphate. In addition, PTD.AdeGFP was able to transduce target cells in a dynamin-independent pathway. The results provide some new clues as to how PTD.AdeGFP infects target cells. This new vector would be valuable in gene-function analysis and for gene therapy in cancer.

Key words: adenovirus (Ad), cancer, fibre protein, gene delivery, protein transduction domain (PTD), Tat

INTRODUCTION

Ad5 [Ad (adenovirus) type 5] has been shown to be able to transduce multiple cell types, including lung epithelial cells [1,2], muscle cells [3,4], endothelial cells [5,6], fibroblasts [7,8] and neuronal cells [9,10], with different transduction efficiencies as a result of variable CAR (coxsackievirus and Ad receptor) expression levels on target cells. A low level of CAR on the cell surface is a potential barrier to efficient gene transfer [11–15]. Development of more efficient vectors to improve gene transfer to CAR-deficient cells, for example, tumorigenic cells and neuronal cells, will be critical for gene therapy of related diseases.

Ad5 infects target cells by a two-step binding process. The first step is the attachment of the virus to a fibre receptor (i.e. CAR) present on the cell surface, which determines the tropism of Ad5. The second step is the interaction between the Ad penton base RGD motif and the integrin $\alpha v\beta 5$ on the target cell surface, leading to endocytic internalization of the Ad [16]. A few laboratories have attempted to find approaches which improve transduction of CAR-deficient cells by Ad. They reported that Ad modified by bi-functional antibodies could improve gene transfer to target cells *in vitro* [17–19]. Experiments also showed that Ad capsids genetically modified to contain a small foreign peptide at the C-terminal or in the HI loop of the Ad5 fibre allowed for efficient transduction of multiple cell types [20–24]. So far, as a result of the structure of the Ad5 fibre knob being solved, modification of the Ad5 fibre is a common method used to alter the tropism of Ad5 [25,26]. The tropism of genetically modified Ad, to some extent, depends on the characteristics of the peptide inserted into the HI loop of the fibre.

The Tat (trans-activating) protein from HIV is composed of 86 amino acids encoded by two exons, and plays a critical role in helping the replication of HIV inside infected cells. Interestingly, a Tat protein-derived peptide sequence, also called a PTD (protein transduction domain), has been used to help internalize

Abbreviations used: Ad, adenovirus; Ad5, Ad type 5; CAR, coxsackievirus and Ad receptor; CHO, Chinese-hamster ovary; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; eGFP, enhanced GFP; HEK-293, human embryonic kidney; HUVEC, human umbilical-vein endothelial cell; PTD, protein transduction domain; RSV, Rous sarcoma virus; Tat, trans-activating

[1]To whom correspondence should be addressed (email xiahaibin@snnu.edu.cn).

a number of marker proteins into cells [27–30] by a transporter- or receptor-independent mechanism. In the present study, by genetically modifying the HI loop of Ad with Tat PTD, we proved that Tat PTD could improve gene transfer to CAR-deficient cells by Ad. The present study also provided clues to the possible mechanisms involved.

MATERIALS AND METHODS

Cells and viruses

All cells, except for the HUVECs (human umbilical-vein endothelial cells) and C39 cells (a human fibroblast cell line) were from the A.T.C.C. HUVECs and the C39 cell line were supplied by Dr Beverly L. Davidson (Department of Internal Medicine, University of Iowa, IA, U.S.A.). A CHO (Chinese-hamster ovary) cell line which stably expressed CAR (CHO–CAR) was generated in our laboratory. The AdeGFP virus contained an eGFP [enhanced GFP (green fluorescent protein)] gene in the E1 region under the control of an RSV (Rous sarcoma virus) promoter. E1-deleted Ad5 or AdeGFP was amplified in HEK-293 (human embryonic kidney) packaging cells and purified as described previously by Xia et al. [21].

Virus construction

Tat PTD peptide (YGRKKRRQRRR) was inserted into the HI loop of the Ad5 fibre knob by overlapping PCR as described previously by Xia et al. [21]. PCR was performed using two pairs of primers: F1 (5′-AGAAATGGAGATCTTACTGAAGG-C-3′) and R1 (5′-GCCTCTTCGTCGCGTCTCCGCTTCTTCC-TGCCATAGCCAGTTGTGTCTCCTGTTTCCTGTGTACC-3′) and F2 (5′-GGCTATGGCAGGAAGAAGCGGAGACAGCGA-CGAAGAGGCCCAAGTGCATACTCTATGTCATTTTCA-3′) and R2 (5′-AACACTAGTCTATTCTTGGGCAATGTATGA-AAAAGTGTA-3′), which were used to amplify a 210 bp and a 100 bp fragment of the Ad5 fibre using purified virus genomic DNA as a template. The reaction products were gel purified and mixed, and contiguous sequences were generated by overlapping PCR using primers F1 and R2. The PCR amplification product contained a unique BglII site at the 5′end and a SpeI site at the 3′ end. The digested fragment was cloned into BglII- and SpeI-digested pTM1Ad5 fibre. The resultant plasmid, named pTM1/Ad5 Tat PTD-HI, was digested by SphI and SpeI and the small fragment was purified and cloned into a pBS shuttle vector [21]. The resultant plasmid was named pBS/Ad5 Tat PTD-HI. The large fragments from pBS/Tat PTD-HI digested with EcoR1 and BamHI were co-transformed into *Escherichia coli* BJ5183 cells along with the SwaI-linearized pTG3602 RSVeGFP/SwaI vector, and the positive clones were screened by enzyme digestion and DNA sequencing. The resulting plasmid was designated PTD.AdeGFP. The PTD.AdeGFP virus was produced by transfecting PacI-digested PTD.AdeGFP vector into HEK-293 cells. The virus was propagated in HEK-293 cells and purified by caesium chloride gradient methods. The titres were detected by spectrophotometry at an absorbance (A) of 260 nm.

Ad transduction

In experiments to detect gene delivery to CAR-positive and CAR-deficient cells [31–33] by PTD.AdeGFP, CAR-positive A549 cells and CAR-deficient cells indicated in the Figure legends [10^5 cells in 1 ml of DMEM (Dulbecco's modified Eagle's medium) containing 2% (v/v) FBS (fetal bovine serum) in a 60-mm-diameter dish] were incubated with PTD.AdeGFP or unmodified AdeGFP (5000 particles/cell) at 37°C for 1 h. The cells were washed and incubated at 37°C for 48 h. GFP expression was evaluated by FACS analysis. In experiments to test the effect of the soluble Ad5 fibre knob on PTD.AdeGFP infection, 10^5 CAR-positive A549 cells or CAR-deficient cells were pre-incubated with 5 μg/ml soluble Ad5 fibre knob for 1 h at 4°C. The cells were washed and then incubated with PTD.AdeGFP (5000 particles/cell) at 37°C for 1 h. The cells were washed and incubated at 37°C for 48 h. A549 cells were infected by unmodified AdeGFP (5000 particles/cell) as a positive control in the Ad5 fibre-blocking assay experiments. Representative areas were then photographed by microscopy at ×20 magnification, except for C39 cells and HUVECs (×10 magnification). To compare gene transduction of calcium phosphate precipitated AdeGFP and PTD.AdeGFP, calcium phosphate precipitated AdeGFP and PTD.AdeGFP (8000 particles/cell) were incubated with NIH 3T3 cells at 4°C for 1 h, then the cells were washed in 2% (v/v) DMEM medium or treated by trypsin for 3–5 min at room temperature (25°C). The trypsin-treated cells were re-plated in 60-mm-diameter plates and incubated at 37°C for 48 h. Representative areas were photographed using a microscope at ×20 magnification. The cells were then detached by trypsinization for FACS analysis. In experiments to determine the effect of heparinase 1 (Sigma) on PTD.AdeGFP infection, enzyme digestion was performed in 150 mM Tris/HCl (pH 7.5) at 25°C for 1 h in the presence of different concentrations of heparinase 1. The indicated cells were then washed and incubated with PTD.AdeGFP (5000 particles/cell) at 37°C for 48 h. The GFP expression was assayed by FACS analysis. In experiments to test the effect of mutant dynamin on PTD.AdeGFP infection, 5×10^4 A549 cells were infected with mutant dynamin Ad or E1-deleted Ad5 (5000 particles/cell) and, after 48 h, the cells were washed and incubated with PTD.AdeGFP or AdeGFP (5000 particles/cell) at 37°C for 1 h. The cells were washed and incubated at 37°C for a further 48 h. GFP expression was analysed by FACS. In experiments to determine the effect of the Tat PTD peptide on PTD.AdeGFP infection, 5×10^4 CHO cells were pre-incubated at 4°C for 1 h in the presence or absence of the Tat PTD peptide (Genosys Biotechnologies) (2 μg/ml). The cells were washed and incubated with PTD.AdeGFP (5000 particles/cell) at 37°C for 1 h. The cells were washed again and incubated at 37°C for a further 48 h. GFP expression was analysed by FACS. In experiments to determine gene transduction of CHO–CAR cells by PTD.AdeGFP, 10^5 CHO–CAR or CHO cells were incubated with PTD.AdeGFP (5000 particles/cell) at 37°C for 1 h, then the

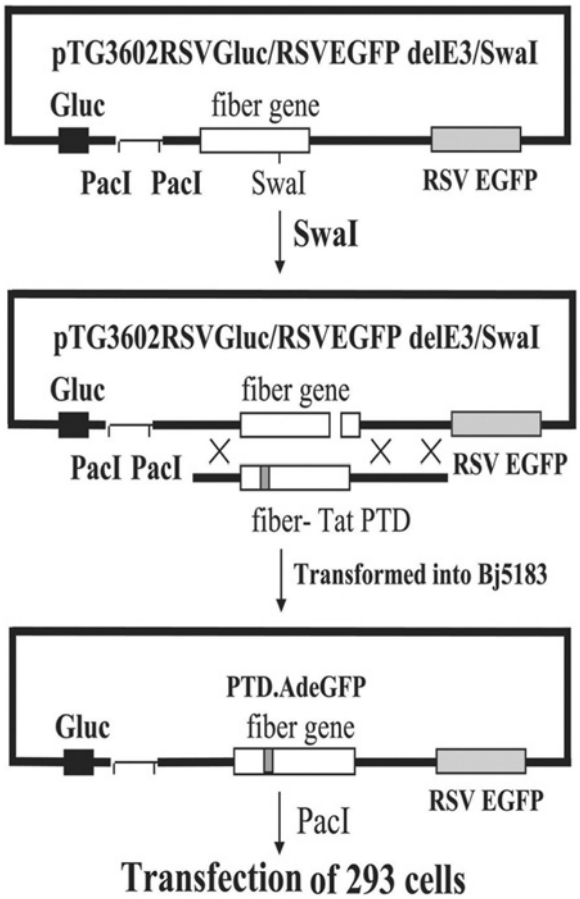

Figure 1 Schematic diagram of PTD.AdeGFP production
PTD.AdeGFP contains the eGFP gene in the E1 region under the control of the RSV promoter. The pBS shuttle vector containing the Tat PTD motif in the HI loop of Ad5 fibre linearized by BamHI and EcoRI was co-transformed into *E. coli.* BJ5183 cells along with SwaI linearized pTG360RSVeGFP/*SwaI*. The positive clones were screened by enzyme digestion and DNA sequencing. Appropriate recombinants were digested by PacI and transfected into HEK-293 cells. Viruses were harvested, amplified and purified using standard methods. Gluc, glucuronidase.

cells were washed and incubated at 37°C for 48 h. GFP expression levels were evaluated by FACS.

RESULTS

Efficient gene transfer into CAR-deficient cells is mediated by PTD.AdeGFP

Using a homologous strategy in *E. coli* BJ5185 cells, Ad5 was modified with the Tat PTD in the HI loop of the fibre to obtain a new construct, PTD.AdeGFP. The PTD.AdeGFP virus was then produced by transfecting PacI-linearized PTD.AdeGFP plasmids into HEK-293 cells (Figure 1). PTD.AdeGFP was first tested in A549 (a human lung epithelial cancer cell line), a CAR-positive cell line. The results indicated that PTD.AdeGFP infected A549 cells less efficiently when compared with the unmodified control virus AdeGFP (Figure 2A). Flow cytometry showed that 53% of A549 cells were infected by PTD.AdeGFP (revealed by GFP expression) and 91% of the cells were infected by AdeGFP. However, in contrast with the control virus AdeGFP, PTD.AdeGFP infected A549 cells in a fashion that was not blocked by the presence of the soluble fibre knob (Figure 2A). We next tested if PTD.AdeGFP could transduce CAR-deficient cells by using CHO cells, and the results revealed that 31% of the cells were infected, which was not reversed by pre-treatment with the soluble Ad5 fibre knob (results not shown). In addition, there were no significant differences in transduction efficiency between CHO and CHO–CAR cells infected by PTD.AdeGFP ($P > 0.05$) (Figure 2B). These results suggested that PTD.AdeGFP tranduces cells in a CAR-independent manner. Other CAR-deficient cells, for example, NIH 3T3 (mouse fibroblast cell line), C39 (human fibroblast cell line), T24 (human prostate cancer cell line) and HUVECs all showed efficient transduction (30%–50% GFP expression in cells) by PTD.AdeGFP (Figures 2C and 2D). To test whether the enhanced gene transfer mediated by PTD.AdeGFP in CAR-deficient cells is the result of the insertion of the Tat PTD into the HI loop of the adenoviral vector, PTD.AdeGFP was incubated with CHO cells in the presence or absence of the Tat PTD peptide. The results indicated that the Tat PTD peptide could block the infection of CHO cells by PTD.AdeGFP by 50% (Figure 2E), suggesting that the entry of PTD.AdeGFP into target cells could be the result of an interaction between the Tat PTD and molecules present on the surface of target cells.

Comparison of gene transduction by PTD.AdeGFP and calcium phosphate precipitated AdeGFP

Interestingly, PTD.AdeGFP was found to form aggregates when observed by electron microscopy (results not shown). Normally Ad particles will not aggregate unless they are exposed to some specific physical condition, for example, low pH or low ionic strength [34]. Fasbender et al. [35] found that adenoviral aggregates precipitated by calcium phosphate could enhance gene transfer into airway epithelia deficient in CAR expression. To test whether high-efficiency gene transfer into CAR-deficient cells by PTD.AdeGFP is the result of aggregate formation, we incubated NIH 3T3 cells at 4°C for 1 h with PTD.AdeGFP and calcium phosphate precipitated AdeGFP. The results showed that calcium phosphate precipitated AdeGFP markedly enhanced gene transfer (Figure 3A) and this effect was markedly blocked by treatment with trypsin ($P < 0.01$) (Figure 3). Notably, trypsin had no significant effect on gene delivery mediated by PTD.AdeGFP ($P > 0.05$) (Figure 3). Precipitation of PTD.AdeGFP by calcium phosphate was also performed and showed no effects on gene transfer to NIH 3T3 cells compared with untreated PTD.AdeGFP (results not shown). These results suggested that PTD.AdeGFP infects target cells in a different manner compared with calcium phosphate precipitated AdeGFP.

The effect of heparinase 1 and mutant dynamin on gene delivery by PTD.AdeGFP

Most mammalian cell types express heparin-containing cellular receptors that could bind to charged amino acids [36,37]. Previous

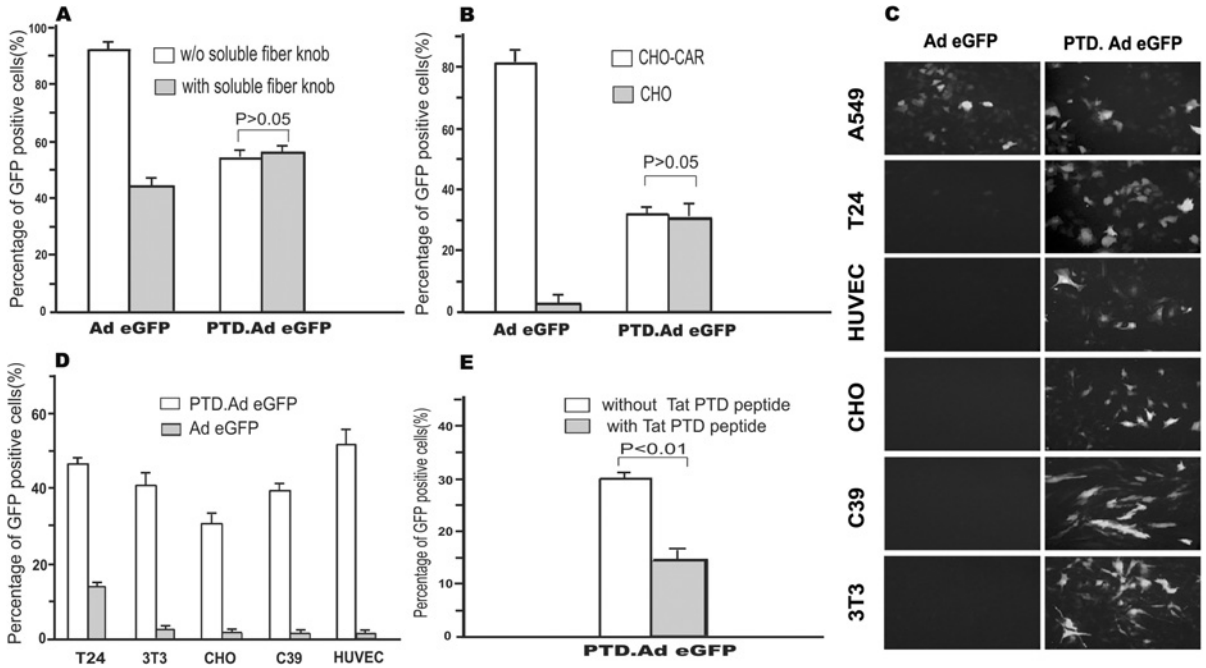

Figure 2 **Comparison of gene transduction by PTD.AdeGFP and control virus AdeGFP**

(A) PTD.AdeGFP-infected A549 cells compared with unmodified control virus AdeGFP in the presence of soluble fibre knob (with soluble fiber knob) (5 μg/ml) or absence of soluble fibre knob (w/o soluble fiber knob). (B) Comparison of gene transduction by PTD.AdeGFP and AdeGFP in CHO–CAR or CHO cells. (C) Comparison of gene transduction by PTD.AdeGFP and AdeGFP in different cell lines. Representative areas were imaged at ×20 magnification, except for C39 and HUVEC cells (×10 magnification) using an Olympus IX70 microscope. (D) Comparison of gene transduction by PTD.AdeGFP and AdeGFP in CAR-deficient cells derived from different tissues. (E) Effect of the presence or absence of the Tat PTD peptide on PTD.AdeGFP transduction in CHO cells. In (A, B, D and E), results are means ± S.D. ($n = 3$). Statistical significance was determined by the Student's *t* test. 3T3, NIH 3T3.

studies indicated that adenoviral capsids modified to contain a C-terminal poly-lysine tract allowed for an efficient transduction of multiple cell types via facilitated binding to cell surface heparan sulfate proteoglycans [20]. The Tat PTD is enriched in positively charged amino acids (YGKKKRRQRRR), and, in order to rule out the possibility that the improvement of gene transfer is a result of increased charge-directed binding between viruses and cell surface, the cells were treated with heparinase 1 for 1 h at 25°C prior to incubation with PTD.AdeGFP. The results indicated that heparinase 1 had no effect on the infection of HUVECs or T24 cells by PTD.AdeGFP (Figure 4A). After attaching to the fibre receptor on the cell surface, Ad5 is internalized through the clathrin-coated-pit pathway, and dynamin is required for the pathway [16]. To explore whether PTD.AdeGFP entered into the cells via the clathrin-coated-pit pathway, A549 cells were treated by Ad expressing mutant dynamin or E1-deleted Ad prior to incubation with AdeGFP (control virus) or PTD.AdeGFP. The results demonstrated that mutant dynamin inhibited transduction by control viruses significantly ($P < 0.01$), but had no effects on transduction by PTD.AdeGFP (Figure 4B), indicating that PTD.AdeGFP infected the cells by a dynamin-independent pathway.

DISCUSSION

The present study demonstrates that the Tat PTD-modified Ad can effectively transduce CAR-deficient cells. The infection of target cells by PTD.AdeGFP is not via a pathway that Ad5 usually utilizes, i.e. via clathrin-mediated endocytosis. The increased gene delivery to CAR-deficient cells by PTD.AdeGFP is not the result of the positive charges that the Tat PTD contains. Although PTD.AdeGFP forms aggregates, it infects target cells in a different manner compared with AdeGFP aggregates precipitated by calcium phosphate.

A truly targeted Ad is one which is devoid of the native-binding ability of CAR [38] that is difficult to construct and to propagate in regular HEK-293 cells. As a result of the insight into the structure of the Ad5 fibre knob [25,26], an alternative approach used is to incorporate a small foreign peptide into the HI loop of the wild-type Ad5 fibre to test its new tropism. Recently, Kurachi et al. [39] generated Ad5 vectors containing a slightly different Tat peptide (GRKKRRQRRRPQ) compared with the one used in the present study, and the peptide was inserted into the HI loop or the C-terminus of the fibre knob. The modified virus

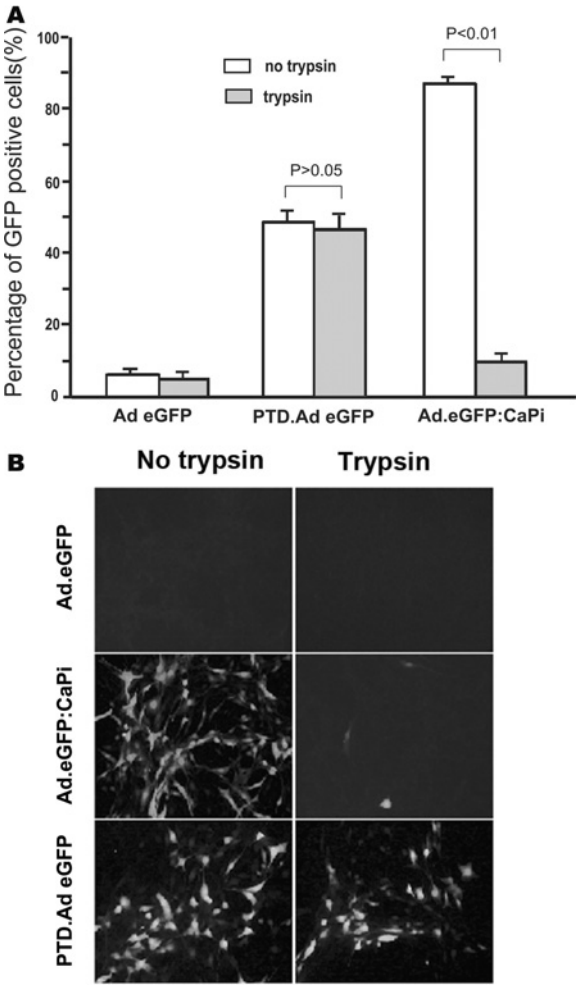

Figure 3 Comparison of gene transduction by PTD.AdeGFP and control virus precipitated by calcium phosphate

(A) NIH 3T3 cells were incubated with PTD.AdeGFP, Ad.eGFP:CaPi (calcium phosphate precipitated AdeGFP) and AdeGFP at 4°C for 1 h. The cells were washed by 2% (v/v) DMEM medium or treated with trypsin. The cells were incubated for a further 48 h at 37°C and the cells were analysed by FACS. Results are means ± S.D. ($n = 4$) and statistical significance was determined by the Student's t test. (B) Cells treated as in (A) were imaged at ×20 magnification using an Olympus IX70 microscope.

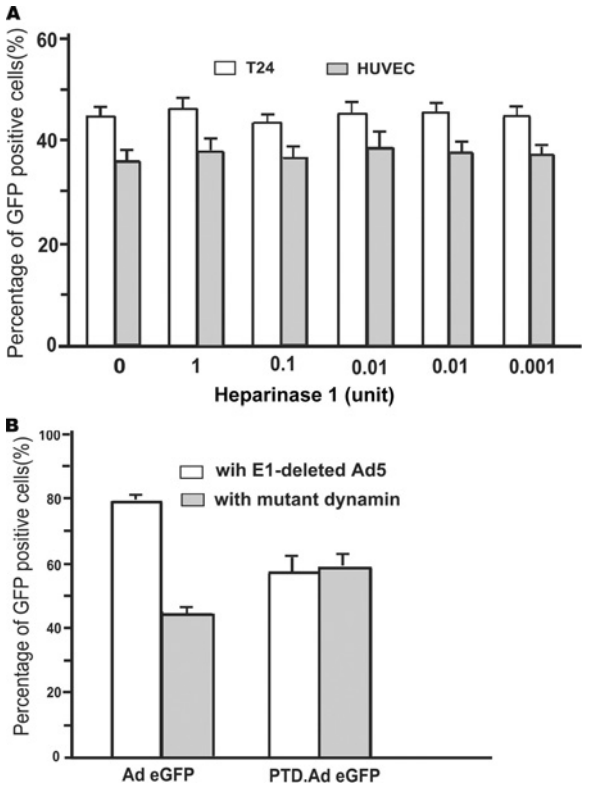

Figure 4 The effect of PTD.AdeGFP infection on cells by heparinase 1 or mutant dynamin

(A) The effect of heparinase 1 on infection of T24 or HUVEC cells by PTD.AdeGFP. GFP-positive cells was detected as described in the Materials and methods section. (B) The effect of mutant dynamin on infection of A549 cells by PTD.AdeGFP or AdeGFP. A549 cells were pre-treated with mutant dynamin virus (5000 particles/cell) or E1-deleted Ad5 (empty vector, 5000 particles/cell). After 48 h, cells were infected with PTD.AdeGFP or AdeGFP and then washed and incubated at 37°C for a further 48 h, after which the GFP-positive cells were detected as described in the Materials and methods section. Results are means ± S.D. ($n = 3$). Statistical significance was determined using the Student's t test.

transduced efficiently into both the CAR-negative or -positive cells [39]. However, the Ad5 virus produced in the present study by inserting the Tat PTD sequence (YGKKKRRQRRR) into the HI loop showed a decrease in the transduction efficiency of CAR-positive cells. The discrepancy in these results might be associated with the differences in the sequences and insertion sites of the Tat peptides between the two methods used. In addition, the results from the present study also imply that conformational changes resulting from foreign peptide insertion into the HI loop of the fibre might impair the CAR-binding ability of PTD.AdeGFP.

Another interesting phenomenon is the formation of viral aggregates of PTD.AdeGFP. The enrichment of positively charged amino acids in the Tat PTD (YGKKKRRQRRR) is probably not the sole cause of aggregate formation. The Ad5 constructed by our laboratory previously did not form aggregates when the HI loop of the Ad5 fibre was inserted with a positively charged peptide [21]. It was reported that adenoviral aggregates precipitated by calcium phosphate enhanced gene transfer to airway epithelia deficient in CAR expression [35]. However, by using trypsin digestion, the present study showed that PTD.AdeGFP infected CAR-deficient cells in a manner which was different from the one used by calcium phosphate precipitated AdeGFP.

Most mammalian cell types express heparin-containing cellular receptors that could bind to charged amino acids [36,37]. Adenoviral capsids modified to contain a C-terminal poly-lysine tract allow for efficient transduction of multiple cell types via facilitated binding to cell-surface heparan sulfate proteoglycans [20]. As mentioned above, the Tat PTD is enriched in positively charged amino acids. But, as indicated by the results of the present

study, heparinase 1 digestion did not reverse the improved gene transfer of target cells by PTD.AdeGFP, which implies that the increased transduction is not caused by charge-directed binding between viruses and the cell surface. This result is consistent with the recent study by Kurachi et al. [39]. Our results also demonstrate that the Tat PTD peptide could block infection of CHO cells by PTD.AdeGFP, suggesting that the interaction of Tat PTD with the molecules present on target cells mediates PTD.AdeGFP entry into cells. After attaching to the fibre receptor on the cell surface, Ad5 is internalized through the clathrin-coated-pit pathway [16]. Dynamin, a 100 kDa GTPase, is involved in this pathway [16]. Overexpressed mutant dynamin did not lower the gene transfer efficiency mediated by PTD.AdeGFP, suggesting that PTD.AdeGFP infects the cells in a dynamin-independent pathway. We propose that the interaction between the Tat PTD and certain molecules on cell membranes may be involved in the mechanism of PTD.AdeGFP entry into the target cells, which needs to be elucidated further.

In conclusion, Tat PTD-modified Ad could effectively transduce CAR-deficient cells. The improvement of gene transfer is not the result of charge-directed binding between the virus and the cell surface. The entry into target cells of PTD.AdeGFP is via a dynamin-independent pathway. Although PTD.AdeGFP forms aggregates, it infects target cells in a different manner from AdeGFP aggregates precipitated by calcium phosphate. The new construct would be very useful in gene function analysis and gene therapy.

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