THE JOURNAL OF GENE MEDICINE
J Gene Med 2008; **10**: 1210–1222.
Published online 27 August 2008 in Wiley InterScience (www.interscience.wiley.com) **DOI:** 10.1002/jgm.1247

RESEARCH ARTICLE

$S4_{13}$-PV cell penetrating peptide and cationic liposomes act synergistically to mediate intracellular delivery of plasmid DNA

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Received: 18 April 2008
Revised: 16 July 2008
Accepted: 17 July 2008

Abstract

Background Cell penetrating peptides have been successfully used to mediate the intracellular delivery of a wide variety of molecules of pharmacological interest. The main aim of the present work was to evaluate the potential of the $S4_{13}$-PV cell penetrating peptide to mediate the intracellular delivery of plasmid DNA, aiming at its use in gene therapy applications. The $S4_{13}$-PV cell penetrating peptide is a chimeric peptide that results from the combination of a cell penetrating sequence derived from the Dermaseptin S4 peptide with the nuclear localization signal present in the Simian Virus 40 (SV40) large T antigen.

Methods $S4_{13}$-PV cell penetrating peptide and cationic liposomes composed of 1,2-dioleoyl-3-trimethylammonium-propane:1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine were complexed with pDNA at different charge ratios. Complexation of pDNA was assessed by gel electrophoresis. Luciferase assay, fluorescence microscopy and fluorescence-activated cell sorting analysis were used to evaluate reporter gene delivery to TSA and HeLa cells. Cytotoxicity of the pDNA complexes was assessed by Alamar blue assay.

Results Complexes obtained through electrostatic association of the $S4_{13}$-PV cell penetrating peptide with plasmid DNA are able to very efficiently mediate transfection, particularly at high peptide/DNA charge ratios. Additionally, our results clearly demonstrate that, both in HeLa and TSA cells, ternary complexes, resulting from association of cationic liposomes to peptide/DNA complexes, are significantly more efficient in mediating transfection than the corresponding peptide/DNA or cationic liposome/DNA complexes.

Conclusions Overall, our data highlight the potential of cell penetrating peptides for the development of improved nonviral gene delivery systems. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords cationic liposomes; cell penetrating peptide; gene technology; nonviral vectors; nuclear localization signal; plasmid DNA

Introduction

Therapeutic benefit from gene therapy applications relies on the development of delivery systems able to mediate high and sustained levels of gene expression, at the same time as avoiding the safety concerns usually associated with the use of viral vectors. Despite extensive research on the rational

improvement of nonviral gene delivery vectors, such as cationic liposomes or polymers [1], these systems are still far from being viable alternatives to the use of viral vectors in gene therapy [2,3].

Cell penetrating peptides, a group of peptides able to efficiently accumulate inside cells, have been successfully used to mediate the intracellular delivery of a wide variety of molecules of pharmacological interest, particularly proteins, both *in vitro* and *in vivo* [4–14]. Although the mechanisms underlying the cellular uptake of these peptides and of their conjugates remain highly debated, the efficient uptake of these peptides and, most importantly, the ability shared by a considerable number of cell penetrating peptides to accumulate inside the nucleus of cells, render them particularly suited to act as gene delivery vectors *per se*, or in association with other existing nonviral systems. Even though the delivery of peptides and proteins to the cytoplasm has been the major focus of research on cell penetrating peptides, a number of studies have focused on the use of cell penetrating peptides to mediate the intracellular delivery of plasmid DNA [15–26].

Cell penetrating peptides are usually short sequences rich in basic amino acids, in some cases exhibiting the ability to be arranged in amphipathic structures. The peptides derived from the HIV-1 Tat protein and from the homeodomain of the Antennapedia protein of Drosophila (Tat and Penetratin peptides, respectively) [27,28], as well as the synthetic Pep-1 peptide [29], are among the best characterized cell penetrating peptides. The $S4_{13}$-PV cell penetrating peptide is a chimeric peptide that results from the combination of a cell penetrating sequence derived from the Dermaseptin S4 peptide with the well-characterized nuclear localization signal present in the Simian Virus 40 (SV40) large T antigen [30]. Previously, we have demonstrated that the $S4_{13}$-PV peptide is able to very efficiently accumulate inside live cells, and particularly inside the nucleus, through a rapid, dose-dependent and nontoxic process [31]. Moreover, we provided evidence that, rather than endocytosis, the cellular uptake of the $S4_{13}$-PV peptide is a consequence of its direct translocation through cell membranes, following conformational changes induced by peptide–membrane interactions [32,33].

Nevertheless, it should be highlighted that the internalization mechanism of cell penetrating peptides is strongly influenced by the presence of a cargo, which changes the size and charge of the resulting complex. Endocytosis has been implicated as the mechanism responsible for the internalization of different cell penetrating peptides when associated with different types of cargoes, regardless of the uptake mechanism of the cell penetrating peptides alone [13].

The main aim of the present work was to evaluate the potential of the $S4_{13}$-PV peptide to mediate the intracellular delivery of plasmid DNA. The transfection efficiency of $S4_{13}$-PV peptide/DNA complexes, as well as that of ternary systems formed between the peptide/DNA complexes and cationic liposomes, was evaluated, aiming to explore a possible synergistic enhancement of gene delivery mediated by the cationic liposomes. The results obtained demonstrate that highly efficient transfection can be achieved using complexes resulting from electrostatic association of the $S4_{13}$-PV cell penetrating peptide with plasmid DNA. Experiments using peptides derived from the $S4_{13}$-PV peptide demonstrated that the sequence derived from the Dermaseptin S4 peptide, rather than the nuclear localization signal (NLS), is crucial for efficient transfection. Ternary complexes obtained by association of cationic liposomes to peptide/DNA complexes, prepared with the different peptides, mediated transfection more efficiently than the corresponding peptide/DNA or cationic liposome/DNA complexes. Notably, the transfection efficiency of these ternary complexes was not inhibited by the presence of serum, suggesting that these systems may be suitable for *in vivo* administration.

Overall, our results strongly indicate that cell penetrating peptides, including the $S4_{13}$-PV peptide, can be of great interest for the development of improved nonviral gene delivery systems.

Materials and methods

Cells

HeLa cells (human epithelial cervical carcinoma) and TSA cells (mouse mammary adenocarcinoma) were maintained at 37 °C, under 5% CO_2, in Dulbecco's Modified Eagle's medium-high glucose (DMEM; Sigma, St Louis, MO, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Biochrom KG, Berlin, Germany.), and with 100 units of penicillin and 100 μg of streptomycin (Sigma) per ml.

For flow cytometry experiments, 0.8×10^5 HeLa cells/well or 1.1×10^5 TSA cells/well were seeded onto 12-well plates; for fluorescence microscopy studies, 0.8×10^5 HeLa cells/well or 0.9×10^5 TSA cells/well were seeded onto 12-well plates containing 16 mm glass coverslips; and for the luminescence experiments (luciferase activity) 4.5×10^4 cells/well (HeLa or TSA) were seeded onto 48-well plates. Cells were plated 24 h prior to incubation with complexes.

Peptides

Peptide $S4_{13}$-PV (Table 1) was prepared as a C-terminal amide by solid-phase methodologies based on classical Fmoc (1-(9H-fluoren-9-yl)-methoxycarbonyl)/tert-Butyl chemistry [34]. Briefly, the peptide sequence was assembled on a Fmoc-Rink-MBHA (*p*-{(R,S)-α-[1-(9H-fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl}-phenoxyacetic acid-4-methylbenzhydrylamine) resin (0.30 mmol/g), using DIC (diisopropylcarbodiimide)/HOBt (1-hydroxybenzotriazole) in dichloromethane

DOI: 10.1002/jgm

to promote coupling of the Fmoc-AA-OH (N^{α}-Fmoc-protected amino acid), and 20 vol-% piperidine in *N*,*N*-dimethylformamide for Fmoc-removal cycles. After acetylation of its *N*-terminal amino acid, the peptide was fully deprotected and cleaved from resin by acidolytic treatment with a TFA/TIS/H_2O 95 : 2.5 : 2.5 (v/v/v) cocktail for 3 h, after which the crude product was precipitated with methyl*tert*butylether. The target peptide was isolated in high purity (>95%) from the crude mixture by preparative reverse phase-high performance liquid chromatography and successfully identified by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry) (not shown). NLS and scrambled peptides (Figure 1A) of identical purity levels were obtained from Thermo Electron GmbH, (Ulm, Germany), also as C-terminal amides. In the reverse NLS peptide, the sequence corresponding to the nuclear localization signal of the SV40 large T antigen (amino acids 14–20) is inverted; the scrambled peptide was generated on the basis of $S4_{13}$-PV peptide sequence, so that the resulting peptide had the same amino acid composition and overall charge, but a distinct primary sequence. Both peptides were also acetylated at their N-terminus. Freeze-dried peptides were reconstituted in high purity water.

Concentration of $S4_{13}$-PV and related peptides was determined by amino acid analysis and light absorption at 280 nm. Amino acid analysis was performed in a Beckman 6300 automatic analyser, (CA, USA), following acid hydrolysis of the peptide.

Tat peptide 48–60 (CGRKKRRQRRRPP) was kindly provided by Professor Bernard Lebleu (University of Montpellier II, France).

Preparation of cationic liposomes

Small unilamellar vesicles (SUVs) were prepared by extrusion of multilamellar vesicles composed of the cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP; Avanti Polar Lipids, AL, USA) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE; Avanti Polar Lipids), at a 1 : 1 molar ratio.

Lipid solutions in chloroform were mixed at the desired molar ratio, and dried under vacuum, at room temperature, using a rotary evaporator. The dried lipid films were then hydrated with 1.0 ml high purity water, and the multilamellar vesicles obtained were briefly sonicated and extruded 21 times through two stacked polycarbonate filters (pore diameter 50 nm) using a Liposofast device (Avestin, Ontario, Canada.). The lipid concentration of the resulting SUVs was determined by the Bartlett method [35].

Preparation of complexes

Peptide/DNA complexes were prepared by mixing 1.0 μg of pEGFP-C1 plasmid DNA (Clontech, CA, USA.) (fluorescence microscopy and flow cytometry) or 0.25 μg of pCMVluc encoding luciferase (a gift of Dr P. Felgner, Vical, San Diego, CA, USA) (luciferase assay) in HEPES-buffered saline solution (HBS; 140 mM NaCl, 10 mM HEPES, pH 7.4) with different amounts of peptide (depending on the desired charge ratio). The mixtures were incubated for 15 min at room temperature to allow formation of the complexes.

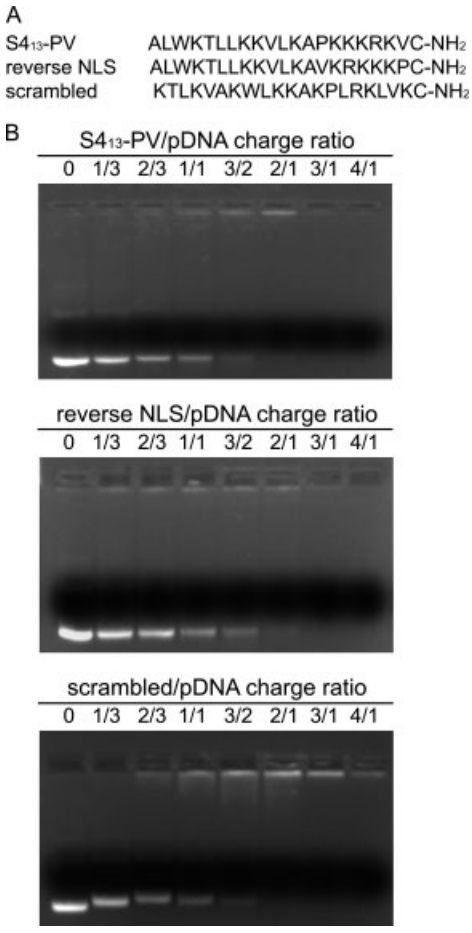

Figure 1. Complexation of plasmid DNA by $S4_{13}$-PV, reverse NLS and scrambled cell penetrating peptides. (A) Comparison of the sequences of the $S4_{13}$-PV, reverse NLS and scrambled peptides used in the present study. (B) Peptide/DNA complexes were prepared by mixing plasmid DNA (pEGFP-C1) with increasing amounts of $S4_{13}$-PV, reverse NLS or scrambled peptides, at room temperature for 15 min. Formation of peptide/plasmid DNA complexes was monitored by evaluating the electrophoretic mobility of the different mixtures in a 1% agarose gel (0.5 μg of plasmid DNA per lane). Free plasmid DNA is shown for comparison

Complexes of peptide, DNA and cationic liposomes (ternary complexes) were obtained by mixing peptide/DNA complexes, prepared at different charge ratios, with the cationic liposomes. Mixtures were then incubated for 15 min, at room temperature.

Cationic liposome/DNA complexes were prepared by mixing 1.0 μg of pEGFP-C1 plasmid DNA or 0.25 μg of pCMVluc plasmid DNA with the cationic liposomes, in order to achieve the desired charge ratio. The mixtures were incubated for 15 min at room temperature to allow formation of the complexes.

DOI: 10.1002/jgm

Peptide/DNA, peptide/DNA/cationic liposome and cationic liposome/DNA complexes were used immediately after their preparation.

Complexes of plasmid DNA with Lipofectamine 2000 (Invitrogen, CA, USA.) were prepared accordingly to manufacturer's instructions, using 1.0 μg of pEGFP-C1 plasmid DNA.

Physicochemical characterization of complexes

Complexes were characterized with respect to their size using a Coulter N4 Plus (Coulter Corporation, Miami, FL, USA). The Coulter N4 Plus uses autocorrelation spectroscopy of scattered laser light to determine the rate of diffusion or Brownian motion of particles in suspension and hence their size. The analysis was performed at 20 °C in HBS and the detection angle was fixed at 90°. Complexes were prepared immediately before analysis.

Complexation of plasmid DNA by the $S4_{13}$-PV cell penetrating peptide and derived peptides

Complexation of plasmid DNA by the $S4_{13}$-PV, reverse NLS and scrambled peptides was evaluated by monitoring the electrophoretic mobility of complexes of the peptides and pEGFP-C1 plasmid, prepared at increasing peptide/DNA charge ratios, as described above. The different complexes were separated on a 1% agarose gel in Tris-acetate buffer (0.5 μg of plasmid DNA per lane). The agarose gel was stained with ethidium bromide.

Transfection studies

HeLa and TSA cells were incubated with either free plasmid DNA or the different complexes (1.0 μg of pEGFP-C1 or 0.25 μg of pLuc per well) at 37 °C for 4 h, in the presence or absence of 10% (v/v) FBS. After this period, the medium was replaced with fresh medium containing 10% (v/v) FBS, and the cells were further incubated for 48 h to allow gene expression. The transfection efficiency mediated by the different complexes was evaluated by analysing GFP expression by fluorescence microscopy and/or flow cytometry, and by assessing luciferase expression, by luminescence measurements.

For analysis of GFP expression by fluorescence microscopy, 48 h post-transfection the cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min at room temperature, and rinsed with PBS. The coverslips were then inverted and mounted on glass slides with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA). The cells were observed using a fluorescence microscope, at ×200 magnification.

For flow cytometry analysis of GFP expression, 48 h post-transfection, the cells were washed once with PBS and detached with trypsin (10 min at 37 °C). The cells were then further washed, resuspended in PBS, and immediately analysed. Flow cytometry analysis was performed, in live cells, using a Becton Dickinson, (NJ, USA), FACSCalibur flow cytometer. Data were obtained and analysed using CellQuest software. Live cells were gated by forward/side scattering from a total of 10 000 events.

The quantification of luciferase expression in cell lysates was evaluated by measuring light production by luciferase in a luminometer (Lmax II^{384}; Molecular Devices, CA, USA.). Forty-eight hours post-transfection, the cells were washed twice with PBS and lysis buffer [1 mM dithiothreitol; 1 mM ethylenediaminetetraacetic acid; 25 mM Tris-phosphate (pH 7.8); 8 mM $MgCl_2$; 15% glycerol; 1% (v/v) Triton X-100] was added to each well. The protein content of the lysates was measured by the DC Protein Assay reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. The data were expressed as RLU of luciferase per mg of total cell protein.

Cell viability studies

Cell viability under the different experimental conditions was assessed, in parallel experiments, by a modified Alamar Blue assay [36]. Forty-seven hours post-transfection, the cells were incubated with DMEM containing 10% (v/v) Alamar Blue dye. After a 1-h incubation period at 37 °C, the absorbance of the medium was measured at 570 nm and 600 nm. Cell viability was calculated, as a percentage of the nontransfected control cells, according:

$$\text{Cell viability(\% of control)} = [(A_{570} - A_{600})/(A'_{570} - A'_{600})] \times 100$$

where A_{570} and A_{600} are the absorbances of the samples, and A'_{570} and A'_{600} those of control cells, at the indicated wavelengths.

Statistical analysis

All data are presented as mean ± SD. Data were analysed using GraphPad Software, Inc., CA, USA. Statistical significances of differences between data were evaluated by *t*-test.

Results

Complexation of plasmid DNA by the $S4_{13}$-PV cell penetrating peptide and derived peptides

Given the polycationic nature of the $S4_{13}$-PV cell penetrating peptide, its ability to bind and form complexes with plasmid DNA was evaluated. Parallel experiments

DOI: 10.1002/jgm

were performed with the reverse NLS and scrambled peptides (Figure 1A), two peptides derived from the $S4_{13}$-PV peptide, with similar physico-chemical properties (peptide length, mass and charge).

Comparative analysis of the electrophoretic mobility of complexes of plasmid DNA with the different peptides, prepared at increasing peptide/DNA charge ratios, showed that complexes prepared at charge ratios higher than 3 : 2 do not migrate in an agarose gel (Figure 1B), indicating a total neutralization of the DNA negative charges by the peptides. At peptide/DNA charge ratios below 3 : 2, a decrease in the intensity of the bands corresponding to plasmid DNA was observed for increasing amounts of peptide (Figure 1B), most likely reflecting a partial complexation of the plasmid DNA molecules by the different peptides.

Overall, these results demonstrate that the $S4_{13}$-PV, reverse NLS or scrambled peptides are able to form complexes through electrostatic interactions with plasmid DNA, although an excess of peptide positive charge is required for complete complexation of DNA.

Efficiency of $S4_{13}$-PV cell penetrating peptide to mediate gene delivery

To evaluate the potential of the $S4_{13}$-PV cell penetrating peptide to mediate the intracellular delivery of DNA, the transfection efficiency of complexes of this peptide with plasmid DNA encoding GFP, prepared at different charge ratios, was determined in HeLa and TSA cells. Because total complexation of plasmid DNA was achieved only at peptide/DNA charge ratios higher than 3 : 2 (Figure 1B), complexes prepared at 2 : 1 or higher charge ratios were selected for these studies.

Fluorescence microscopy and flow cytometry analysis of GFP expression revealed that the efficiency of the different complexes to transfect HeLa cells is highly dependent on their charge ratio. Complexes prepared at a peptide/DNA charge ratio of 2 : 1 mediated low efficiencies of transfection (approximately 10% of cells transfected), whereas higher transfection efficiencies (approximately 50% of cells transfected) were obtained for those prepared at higher charge ratios (10 : 1) (Figure 2A). Although lower levels of transfection were observed in TSA cells for the same charge ratios (less than 20% of cells transfected at a peptide/DNA charge ratio of 10 : 1), a similar dependence on the charge ratio was observed (Figure 2B).

These results demonstrate that complexes formed upon electrostatic interaction between the $S4_{13}$-PV peptide and plasmid DNA, particularly at high peptide/DNA charge ratios, are able to efficiently transfect both HeLa and TSA cells.

Effect of peptide sequence on the transfection efficiency mediated by peptide/DNA complexes

To investigate the relevance of the Dermaseptin-derived sequence and of the nuclear localization signal to the efficiency of the overall process of gene delivery, a comparative analysis of the transfection efficiency mediated by the $S4_{13}$-PV peptide and by the reverse NLS and scrambled peptides (Figure 1A) was performed.

The results obtained from these experiments demonstrated that, in both cell lines and at the different peptide/DNA charge ratios tested, the reverse NLS peptide mediated transfection at efficiencies comparable to those observed with the $S4_{13}$-PV peptide. On the other hand, transfection mediated by the scrambled peptide was consistently less efficient than that obtained for the $S4_{13}$-PV and reverse NLS peptides (Figure 3).

Interestingly, a general trend was observed for the three peptides used in the experiments performed with HeLa cells, showing increasing transfection efficiencies as a result of the increase in the peptide/DNA charge ratio at which the different complexes were prepared (Figures 3A to 3C). In TSA cells, the same trend was observed for the $S4_{13}$-PV and reverse NLS peptides, but not for the scrambled peptide, for which very low efficiencies of transfection were observed at any of the peptide/DNA charge ratios tested (Figures 3B and 3C).

Overall, these results demonstrate that the Dermaseptin-derived sequence present in the $S4_{13}$-PV and reverse NLS peptides (amino acids 1–13) plays an important role in the ability of the peptide/DNA complexes to mediate transfection.

Transfection efficiency of peptide/DNA/cationic liposome complexes

Cationic liposomes have been extensively used to promote the intracellular delivery of plasmid DNA, although the efficiency of this process is rather low. To explore whether association of cationic liposomes to peptide/plasmid DNA complexes would result in an enhancement of gene delivery, the transfection efficiency mediated by complexes resulting from the interaction of these three components (ternary complexes) was evaluated.

Comparative analysis of the transfection efficiency mediated by ternary complexes and the corresponding peptide/DNA complexes, performed by fluorescence microscopy and flow cytometry, revealed that, in general, addition of cationic liposomes to pre-formed peptide/DNA complexes potentiates transfection. In HeLa cells the enhancing effect of the addition of cationic liposomes was relevant for peptide/DNA complexes prepared at the lower charge ratios tested (2 : 1 and 5 : 1) (compare Figures 3A and 4A). The transfection efficiency obtained

DOI: 10.1002/jgm

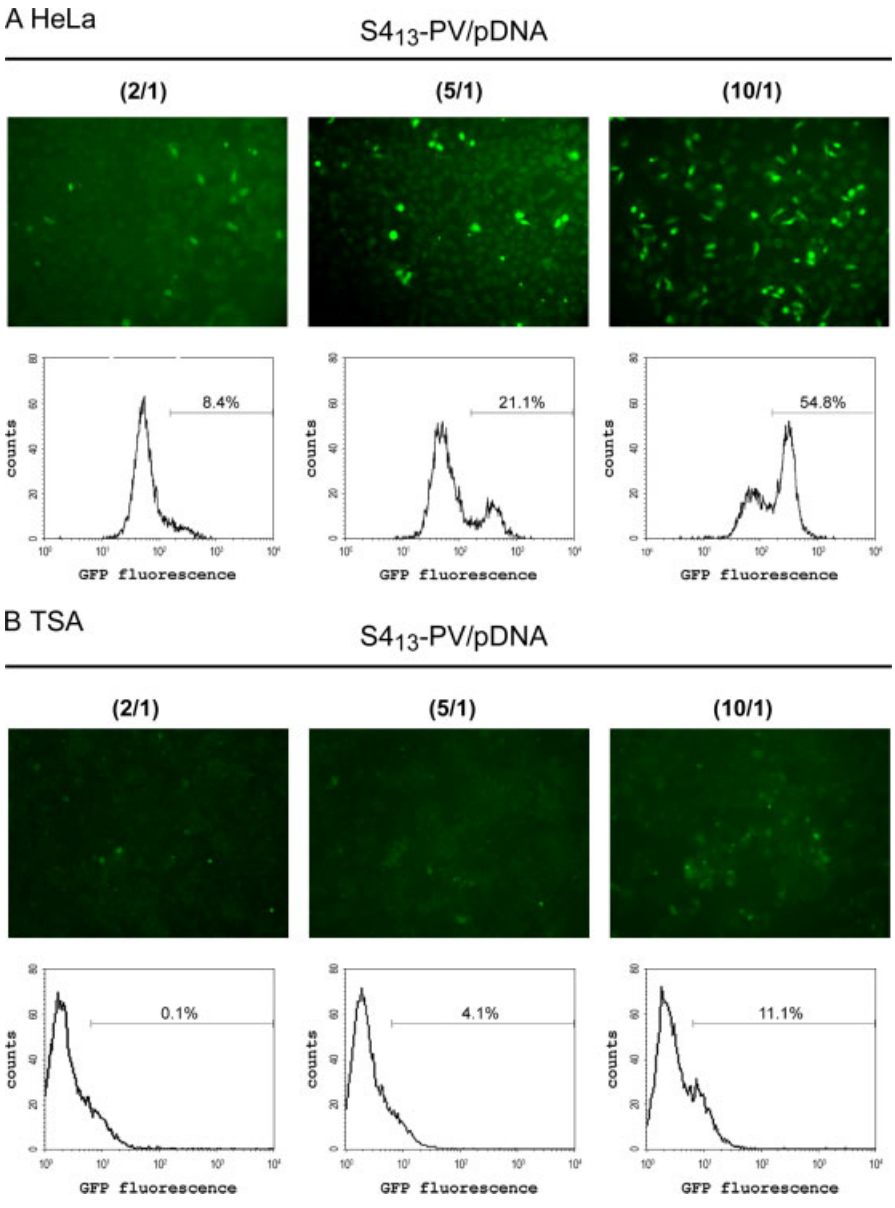

Figure 2. Effect of charge ratio on the transfection efficiency mediated by $S4_{13}$-PV peptide/plasmid DNA complexes in HeLa and TSA cells. (A) HeLa and (B) TSA cells were incubated with $S4_{13}$-PV peptide/plasmid DNA complexes prepared at different charge ratios, for 4 h at 37 °C, as described in the Materials and Methods. Transfection efficiency mediated by the different complexes was evaluated, 48 h post-transfection, by fluorescence microscopy and flow cytometry analysis of GFP expression

for the ternary complexes was independent of the peptide used in their preparation and of peptide/DNA charge ratio (Figure 4A). In TSA cells, the transfection efficiency of the ternary complexes was slightly dependent on the peptide/DNA charge ratio for the complexes prepared with the $S4_{13}$-PV and the reverse NLS peptides (Figure 4B). On the other hand, complexes prepared with the scrambled peptide showed lower transfection efficiency, which was independent of the peptide/DNA charge ratio (Figure 4B).

It is important to emphasize that, under the same experimental conditions and in both cell lines, the efficiency of transfection mediated by the different ternary complexes used in the present study was higher than that achieved by standard cationic liposome/DNA complexes prepared at an equivalent charge ratio, and comparable to that obtained by the commercially available transfection reagent Lipofectamine 2000 (Figures 4A and 4B). These results clearly demonstrate that association of the $S4_{13}$-PV cell penetrating peptide to plasmid DNA may constitute a valuable approach to improve the efficiency of gene delivery mediated by cationic liposomes.

Effect of peptide/DNA and ternary complexes on cell viability

The toxicity of the peptide-based vectors used in the present study was monitored by a modified Alamar Blue assay, 48 h after transfection. As shown in Figure 5, no significant toxicity was observed upon incubation of HeLa or TSA cells with complexes prepared at the 2 : 1 and

DOI: 10.1002/jgm

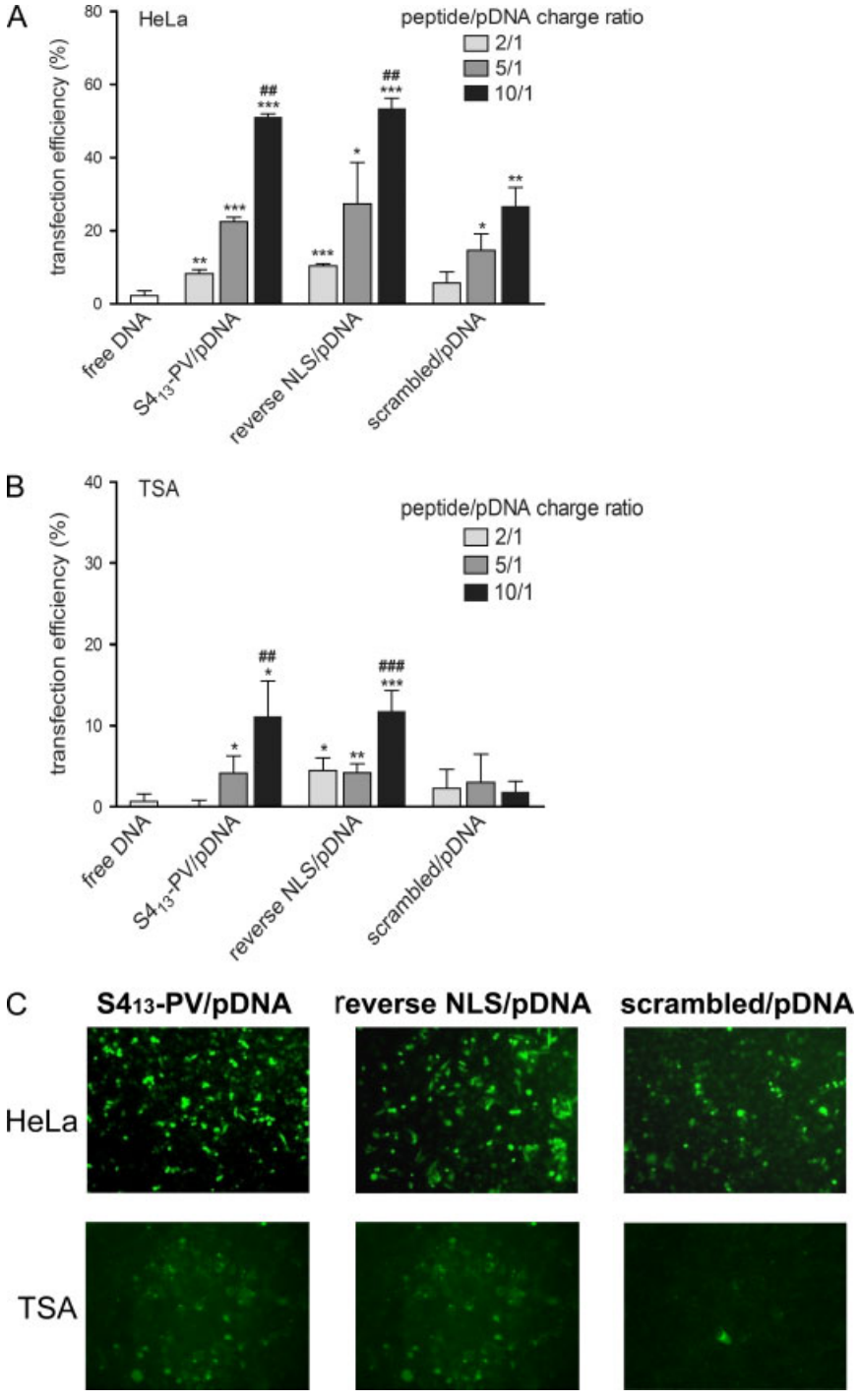

Figure 3. Effect of peptide sequence on the efficiency of transfection mediated by peptide/plasmid DNA complexes in HeLa and TSA cells. (A) HeLa and (B) TSA cells were incubated with free plasmid DNA and complexes of $S4_{13}$-PV, reverse NLS or scrambled peptides with plasmid DNA, prepared at different charge ratios, for 4 h at 37 °C, as described in the Materials and Methods. Forty-eight hours post-transfection, the efficiency of transfection was evaluated by flow cytometry. (C) Representative images from fluorescence microscopy analysis of cells transfected with complexes of $S4_{13}$-PV, reverse NLS or scrambled peptides with plasmid DNA, prepared at the 10 : 1 peptide/DNA charge ratio. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to free DNA, and $^{\#\#}p < 0.01$ compared to 10 : 1 scrambled/DNA

5 : 1 peptide/DNA charge ratios, independently of the peptide used. Complexes resulting from the interaction of the $S4_{13}$-PV or reverse NLS peptides with DNA prepared at the highest charge ratio (10 : 1), which mediated the highest efficiencies of transfection, induced a significant decrease in cell viability (Figures 5A and 5B), presumably caused by interaction of the large excess of free peptide with cell membranes.

Incubation of HeLa cells with ternary complexes induced levels of toxicity slightly higher than those observed for the corresponding peptide/DNA complexes (Figure 5C). Of note, a reduction in cell viability

DOI: 10.1002/jgm

induced by the ternary complexes prepared at the 10 : 1:1 charge ratio was similar to that obtained by the Lipofectamine 2000 complexes (Figure 5C). Parallel experiments performed with TSA cells showed that the levels of toxicity induced by ternary complexes were similar to those observed for the corresponding peptide/DNA complexes (Figure 5D). Decrease in cell viability was only significant for complexes prepared with the reverse NLS peptide at the highest charge ratio (Figure 5D).

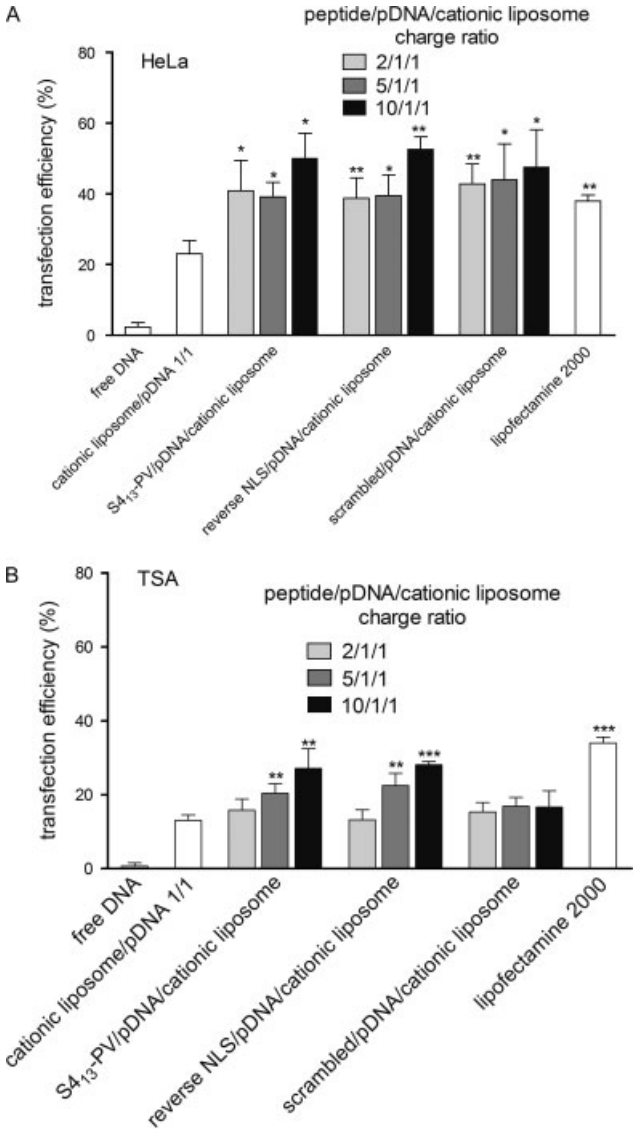

Figure 4. Efficiency of transfection mediated by peptide/plasmid DNA/cationic liposome complexes (ternary complexes) in HeLa and TSA cells. (A) HeLa and (B) TSA cells were incubated with free plasmid DNA, cationic liposome/DNA complexes, Lipofectamine 2000-based complexes and the different ternary complexes for 4 h at 37 °C, as described in the Materials and Methods. Transfection efficiency was evaluated, 48 h later, by flow cytometry analysis of GFP expression. Ternary complexes were obtained by the addition of cationic liposomes composed of DOTAP : DOPE (1 : 1 molar ratio) to complexes of $S4_{13}$-PV, reverse NLS or scrambled peptides with plasmid DNA, prepared at different peptide/DNA charge ratios. *$p < 0.05$, **$p < 0.01$ compared to cationic liposome/DNA 1 : 1 complexes

Effect of serum on the transfection activity of $S4_{13}$-PV peptide/DNA/cationic liposome complexes

The inhibition of gene delivery by serum is known as one of the major limitations associated with the *in vivo* application of nonviral vectors. To evaluate the effect of serum on the stability of the systems described in the present study, transfection experiments were performed in both HeLa and TSA cells, in the presence or absence of 10% serum, and the transfection activity of the different complexes was assessed by measuring luciferase activity (Figures 6A and 6B).

The results obtained from these experiments demonstrated that the transfection efficiency of the different delivery systems is not inhibited by the presence of serum (Figures 6A and 6B). In some cases, the transfection efficiency of the complexes in the presence of serum was even potentiated (Figures 6A and 6B). Importantly, in the presence of serum the toxicity induced by ternary complexes was maintained or even reduced, depending on the cell line and charge ratio tested (Figures 6C and 6D).

Overall, these results demonstrate the suitability of the carrier systems developed in the present study for the delivery of therapeutically relevant plasmid DNA *in vivo*.

Comparative analysis of the transfection activity mediated by ternary complexes prepared with the $S4_{13}$-PV and Tat peptides

To assess the potential of the $S4_{13}$-PV peptide with respect to other more extensively studied cell penetrating peptides, the biological activity of the most effective $S4_{13}$-PV-based formulations was compared with that of equivalent formulations prepared with the Tat peptide. These studies were performed in TSA cells due to the lower toxicity observed in this cell line (Figure 5).

The results obtained from these experiments showed that ternary complexes prepared with the $S4_{13}$-PV or Tat peptides mediate transfection at comparable levels, although at the lowest charge ratio the former displayed a slightly better performance (Figure 7A). Interestingly, the transfection activity of ternary complexes containing the Tat peptide increased with increasing charge ratios, similarly to what was observed for $S4_{13}$-PV peptide/DNA/cationic liposome complexes (Figure 7A).

As shown in Figure 7B, no significant toxicity was observed upon incubation of TSA cells with the ternary complexes, independently of the peptide used.

Physicochemical characterization of complexes

Because the efficacy to deliver genetic material inside target cells is strongly dependent on the physicochemical

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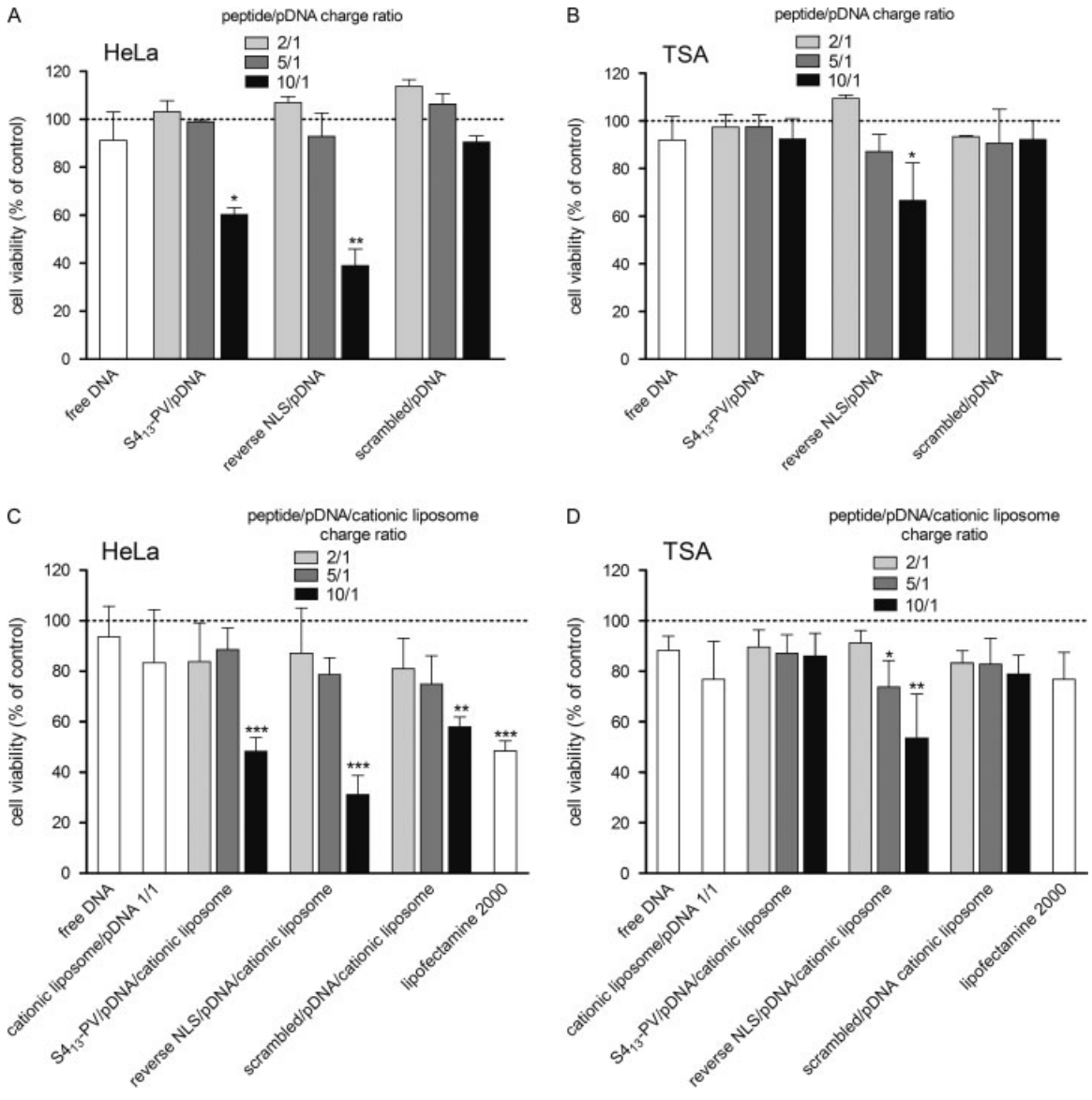

Figure 5. Effect of peptide/DNA and peptide/plasmid DNA/cationic liposome complexes on cell viability. (A, C) HeLa and (B, D) TSA cells were incubated with free plasmid DNA, cationic liposome/DNA complexes, Lipofectamine 2000-based complexes and the different (A, B) peptide/plasmid DNA complexes, or (C, D) peptide/plasmid DNA/cationic liposome complexes, for 4 h at 37 °C, as described in the Materials and Methods. Forty-eight hours post-transfection, cell viability was evaluated by the Alamar Blue assay. Cell viability is expressed as the percentage of control (nontransfected cells). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to free DNA

properties of the carrier systems, the different complexes used in the present study were characterized with respect to their size.

As shown in Table 1, lipoplexes prepared from DOTAP : DOPE (1 : 1) cationic liposomes at the 1/1 lipid/DNA (+/−) charge ratio exhibited the smallest mean diameter (approximately 640 nm). On the other hand, complexes prepared with the S4$_{13}$-PV, reverse NLS and scrambled peptides at different charge ratios (2 : 1, 5 : 1 and 10 : 1) exhibited the largest mean diameters (1900–2900 nm).

Addition of DOTAP : DOPE (1 : 1) cationic liposomes to any of the peptide/DNA complexes resulted in a significant decrease of the size of the resulting complexes (approximately 600–1200 nm).

Discussion

Accumulating evidences have demonstrated the capacity of cell penetrating peptides to mediate intracellular delivery of a wide variety of exogenous molecules, especially peptides, proteins and small nucleic acid molecules (oligonucleotides, siRNA). A number of studies have also addressed the potential of cell penetrating peptides for the delivery of plasmid DNA, either alone or in association with existing nonviral gene delivery vectors, such as cationic liposomes and cationic polymers [15–26].

In the present study, we evaluated the potential use of the S4$_{13}$-PV cell penetrating peptide for the intracellular delivery of plasmid DNA.

DOI: 10.1002/jgm

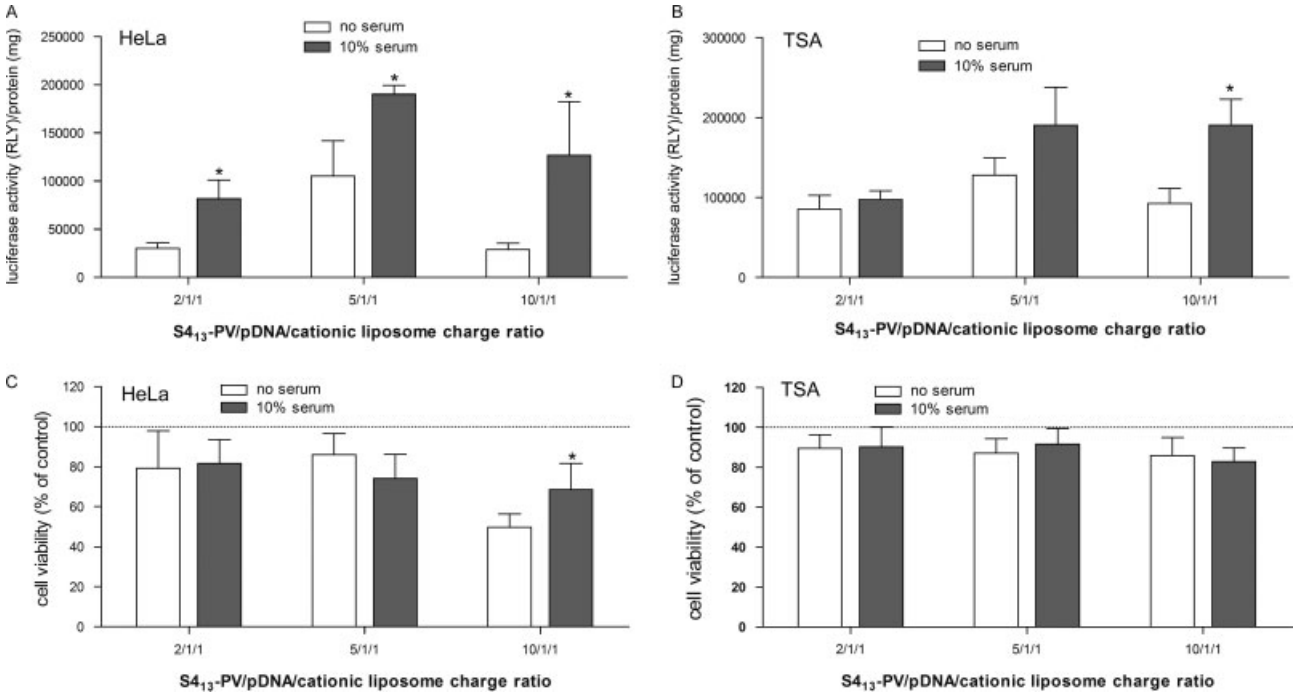

Figure 6. Effect of serum on the efficiency of transfection of ternary complexes based on the $S4_{13}$-PV peptide in HeLa and TSA cells. (A, C) HeLa and (B, D) TSA cells were incubated with ternary complexes for 4 h at 37 °C, in the presence or absence of 10% serum, as described in the Materials and Methods. Ternary complexes were obtained by the addition of cationic liposomes composed of DOTAP : DOPE (1 : 1 molar ratio) to complexes of $S4_{13}$-PV peptide with plasmid DNA, prepared at different peptide/DNA charge ratios. (A, B) Transfection efficiency was evaluated, 48 h later, by measuring luciferase activity. (C, D) In parallel experiments, cell viability was evaluated by the Alamar Blue assay. Cell viability is expressed as the percentage of control (nontransfected cells). *$p < 0.05$, compared to cells treated with the same formulation but in the absence of serum

Assessment of the biological activity of complexes resulting from the electrostatic interaction between the $S4_{13}$-PV cell penetrating peptide and plasmid DNA clearly demonstrated that these complexes are able, *per se*, to efficiently mediate transfection in two cell lines (HeLa and TSA cells). The additional observation that transfection efficiency was significantly improved with an increase of the charge ratio at which peptide/DNA complexes were prepared suggests that an excess of positively charged peptide promotes transfection, most likely due to transient membrane destabilization induced by the peptide. Interestingly, Deshayes *et al*. have demonstrated that the intracellular delivery of DNA oligonucleotides by the MPG peptide, a chimeric peptide that results from the combination of a hydrophobic domain and the SV40 large T antigen nuclear localization signal, involves the formation of pore-like structures consisting of peptide molecules inserted into the lipid bilayer [37]. Although we have previously demonstrated that the cellular uptake of the $S4_{13}$-PV cell penetrating peptide occurs mainly through a non-endocytic mechanism involving direct translocation of the peptide across membranes [31–33], it is likely that alternative mechanisms, the most prominent being endocytosis, are involved in the cellular uptake of peptide/DNA complexes.

In both HeLa and TSA cell lines, complexes prepared with the $S4_{13}$-PV or reverse NLS peptides mediated transfection at significantly higher efficiencies than those containing the scrambled peptide, demonstrating the importance of the cell penetrating sequence derived from the Dermaseptin S4 peptide (amino acids 1–13) to the transfection process. In this context, it should be noted that the $S4_{13}$-PV and reverse NLS peptides, which contain the Dermaseptin-derived sequence, undergo significant conformational changes in the presence of target membranes, which are intricately related to the ability of these peptides to translocate across membranes [32,33].

Moreover, these results strongly suggest that the NLS present in the C-terminus of the $S4_{13}$-PV peptide (amino acids 14–20) is not critical to the process of intracellular gene delivery. However, because these experiments were performed in dividing cells, the active nuclear import of the plasmid DNA mediated by the NLS may have been overridden by the nuclear membrane disassembly events that occur during mitosis, which facilitate the nuclear transport of the delivered molecules [38–40]. Independently of its role in promoting the nuclear import of plasmid molecules, the NLS confers a high positive charge density to the $S4_{13}$-PV peptide and its derivatives, a feature that is, most likely, crucial to the efficient complexation of DNA molecules by these peptides.

Different efficiencies of transfection were observed in the two cell lines. Approximately 50% of HeLa cells were transfected, while no more than 15% of transfection was observed in TSA cells. Differences in the composition of the extracellular matrix, which is known to

DOI: 10.1002/jgm

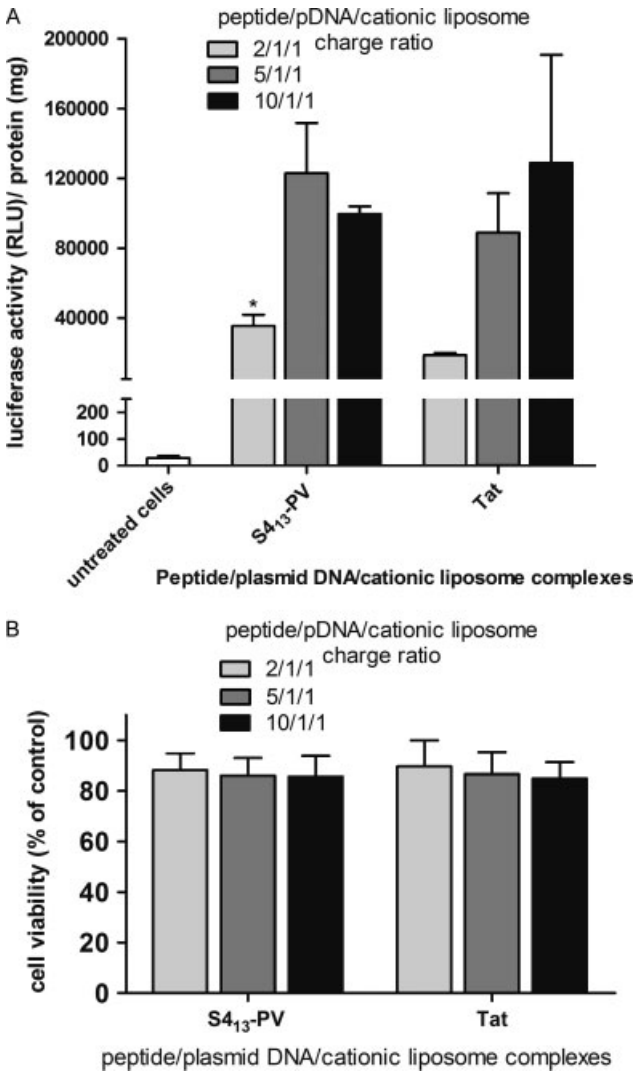

Figure 7. Transfection activity of ternary complexes based on S4$_{13}$-PV and Tat peptides. TSA cells were incubated with the different ternary complexes for 4 h at 37 °C, as described in the Materials and Methods. Ternary complexes were obtained by the addition of cationic liposomes composed of DOTAP : DOPE (1 : 1 molar ratio) to complexes of S4$_{13}$-PV, or Tat peptides with plasmid DNA, prepared at different peptide/DNA charge ratios. (A) Transfection activity was evaluated, 48 h later, by measuring luciferase activity. (B) In parallel experiments, cell viability was evaluated by the Alamar Blue assay. Cell viability is expressed as the percentage of control (nontransfected cells). *$p < 0.05$, compared to cells treated with Tat/DNA/cationic liposome complexes of the same charge ratio

contain molecules that interact with the delivery systems facilitating their uptake, in the efficiency of intracellular trafficking or in the mitotic rate of these cells may explain the observed differences in transfection efficiencies.

To further evaluate the potential of the S4$_{13}$-PV cell penetrating peptide for gene delivery applications, we assessed the transfection efficiency of complexes containing cationic liposomes, which have been extensively used for nonviral gene delivery [2,41], and the different peptides. Overall, ternary complexes obtained by the addition of cationic liposomes to peptide/DNA complexes

Table 1. Mean diameter of the complexes

Complexes[a]	Charge ratio[b]	Mean diameter (nm)[c]
DOTAP : DOPE/DNA	1 : 1	638 ± 346.3
S4$_{13}$-PV/DNA	2 : 1	2718 ± 131.7
	5 : 1	2237 ± 660.93
	10 : 1	2648 ± 414.7
S4$_{13}$-PV/DNA/DOTAP : DOPE	2 : 1 : 1	784 ± 555.34
	5 : 1 : 1	680 ± 619.61
	10 : 1 : 1	863 ± 378.27
Reverse NLS/DNA	2 : 1	2358 ± 388.82
	5 : 1	2424 ± 407.04
	10 : 1	2881 ± 112.85
Reverse NLS/DNA/DOTAP : DOPE	2 : 1 : 1	599 ± 337.84
	5 : 1 : 1	968 ± 340.32
	10 : 1 : 1	1066 ± 117.16
Scrambled/DNA	2 : 1	1927 ± 617.43
	5 : 1	2599 ± 409.22
	10 : 1	2839 ± 92.82
Scrambled/DNA/DOTAP : DOPE	2 : 1 : 1	976 ± 87.62
	5 : 1 : 1	1180 ± 216.35
	10 : 1 : 1	845 ± 267.9

[a]DOTAP : DOPE (1 : 1) liposomes were prepared as described in the Materials and Methods.
[b]Peptide/DNA (+/−) or peptide/DNA/cationic liposome (+/−/+) charge ratios, where applicable; [c]results represent the mean ± SD from three independent experiments.

were significantly more efficient than the corresponding peptide/DNA complexes, suggesting a synergistic effect between the cationic liposomes and the peptides to enhance gene delivery. In HeLa cells, however, the peptide/DNA complexes prepared at the highest charge ratio exhibited the highest transfection activity, showing no improvement upon the addition of the cationic liposomes. Importantly, in both cell lines, transfection efficiencies obtained with ternary complexes containing the S4$_{13}$-PV peptide were comparable with those obtained with Lipofectamine 2000.

Similar transfection efficiencies were observed in HeLa cells, for the ternary complexes independently of the charge ratio and the peptide used in their preparation. A possible explanation is that the addition of cationic liposomes to peptide/DNA complexes induces changes in the overall structure of the complexes making endocytosis the main route of uptake and masking the cell penetrating properties of the peptides. In TSA cells, the same dependence on the charge ratio was observed for the peptide/DNA and ternary complexes prepared with S4$_{13}$-PV and reverse NLS peptides, the more positively charged complexes being the most efficient to deliver plasmid DNA. It is possible that the main mechanisms responsible for internalization of the complexes in these cells are different from those in HeLa cells. Given the high endocytic capacity of HeLa cells, it is reasonable to assume that in these cells the ternary complexes are mainly internalized by endocytosis, whereas, in TSA cells, in which endocytosis may not have such an important role, the presence of the peptide plays a determinant role in the uptake of the complexes.

Remarkably, the efficiency of transfection achieved by most ternary complexes used in the present study

DOI: 10.1002/jgm

was significantly higher than that obtained by cationic liposome/DNA complexes. These results suggest that condensation of DNA by cell penetrating peptides, in particular the $S4_{13}$-PV and derivative peptides, prior to the addition of cationic liposomes, constitutes a very promising approach to enhance transfection mediated by cationic liposomes. The lower transfection efficiencies observed when ternary complexes were prepared by addition of the different peptides to cationic liposome/DNA complexes reinforce this possibility (M. Mano, A. Henriques, A. Paiva, S. Simões and M.C. Pedroso de Lima, unpublished work). Changes in the physicochemical properties of peptide/DNA complexes upon addition of cationic liposomes, which may confer extra protection and/or facilitate the intracellular traffic of the carried DNA molecules, may also account for the observed differences in transfection efficiency. In this regard, it is important to refer that size determination revealed important differences among the different complexes described in the present study. The smaller size exhibited by the ternary complexes is probably critical for their improved efficacy in comparison to the peptide/DNA systems, of larger sizes. Most likely there is a threshold size above which internalization of complexes by the target cells becomes less efficient.

Complexes containing the $S4_{13}$-PV and reverse NLS peptides prepared at the highest charge ratio tested induced significant levels of cytotoxicity, presumably reflecting membrane destabilization. Nonetheless, the high transfection efficiencies (approximately 50% of cells transfected, for HeLa cells) achieved by ternary complexes with charge ratios at which they have no significant effect on cell viability (2 : 1:1 and 5 : 1:1 charge ratios), are noteworthy, especially when put in perspective with other existing nonviral gene delivery vectors.

The transfection efficiency of the ternary complexes described in the present study was not inhibited (in some cases was even potentiated) by the presence of 10% serum, and their cytotoxicity was, in general, decreased, demonstrating the suitability of these systems for *in vivo* applications. The possibility of an immunological response against these complexes should, however, be taken into account when considering *in vivo* experiments. Although no studies on this issue have been addressed subject for the $S4_{13}$-PV peptide, it has been shown that other cell penetrating peptides containing positively charged amino acids do not induce inflammatory cytokines or anti-peptide antibodies when administered *in vivo* [42].

Although conflicting results have been reported regarding the transfection ability of complexes prepared by electrostatic association of the Tat peptide with plasmid DNA [16–18,43], it is interesting to note that the addition of cationic liposomes or cationic polymers to peptide/DNA complexes was shown to improve transfection [16,18,20,25]. In the present study, no significant transfection was observed for Tat peptide/DNA complexes (data not shown). However, as reported here for the $S4_{13}$-PV-based systems, the addition of cationic liposomes to Tat peptide/DNA complexes enhanced the efficacy of these systems. Comparison of the ternary complexes based on the $S4_{13}$-PV and Tat peptides demonstrated that the complexes based on the $S4_{13}$-PV cell penetrating peptide described in the present study have a potential equivalent or superior to the systems based on the Tat peptide to mediate intracellular gene delivery.

Overall, the results gathered in the present manuscript demonstrate the capacity of the $S4_{13}$-PV cell penetrating peptide, either *per se* or in association with cationic liposomes, to very efficiently mediate the intracellular delivery of a transgene, highlighting the potential use of cell penetrating peptides for the development of improved nonviral gene delivery vectors.

Acknowledgements

We thank Professor F. Regateiro, Head of the Centro de Histocompatibilidade de Coimbra (Portugal), for scientific collaboration. The present study was supported by grants from the Portuguese Foundation for Science and Technology (POCTI/CVT/44854/2002 and PTDC/BIO/65627/2006). S. Trabulo, M. Mano, A.L. Cardoso, S. Duarte, H. Faneca are recipients of a fellowship from the Portuguese Foundation for Science and Technology.

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DOI: 10.1002/jgm