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RESEARCH ARTICLE

Exploration of peptide motifs for potent non-viral gene delivery highly selective for dividing cells

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Abstract

Background The immunogenicity of viral DNA vectors is an important problem for gene therapy. The use of peptide motifs for gene delivery would largely overcome this problem, and provide a simple, safe and powerful approach for non-viral gene therapy.

Methods We explored the functional properties of two motifs: the $(Lys)_{16}$ motif (for binding and condensing DNA, and probably also nuclear translocation of plasmids) and the fusogenic peptide motif of influenza virus (for acid-dependent endocytic escape of peptide/DNA particles). The physical properties and gene delivery efficiencies of $(Lys)_{16}$-containing peptides in combination with free fusogenic peptide were evaluated, and compared with a single composite peptide incorporating both moieties. Post-mitotic corneal endothelial cells and growth-arrested HeLa were included, so as not to neglect the question of nuclear translocation of plasmids.

Results The fusogenic moiety in the composite peptide was able to adopt an alpha-helical configuration unhindered by the $(Lys)_{16}$ moiety, and retained acid-dependent fusogenic properties. The composite peptide gave remarkably high levels of gene delivery to dividing cell lines. However, in marked contrast to $(Lys)_{16}$/DNA complexes plus free fusogenic peptide, the composite peptide was *completely* ineffective for gene delivery to post-mitotic and growth-arrested cells.

Conclusions Attachment of the fusogenic peptide to $(Lys)_{16}$ appears to block $(Lys)_{16}$-mediated nuclear translocation of plasmid, but not fusogenic peptide mediated endocytic escape. This strengthens the experimental basis for $(Lys)_{16}$-mediated nuclear translocation of plasmids, and provides a single peptide with potent gene delivery properties, restricted to dividing cells. This property is potentially useful in experimental biology and clinical medicine.
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Keywords gene therapy; non-viral gene delivery; fusogenic peptide; nuclear translocation; oligolysine; mitosis

Introduction

Although viral vectors can deliver genes with high efficiency to particular cell types, depending on viral tropism, their immunogenicity is an inescapable problem. It can give rise to severe and sometimes fatal reactions in both experimental animals [1] and man [2]. Studies of viral infectivity and intracellular protein trafficking have defined key peptide motifs for traversing various cellular and intracellular barriers. The use of synthetic

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peptides corresponding to these motifs for delivering therapeutic DNA is an attractive option for gene therapy. Small synthetic peptides are essentially non-immunogenic, are stable and readily standardised, and are a class of compound already in clinical therapeutics.

Most viruses gain cellular entry via one of the endocytic pathways [3,4]. Viruses entering via clathrin-coated pit endocytosis have evolved acid-dependent mechanisms for escape from endocytic vesicles. For influenza viruses, this is mediated by the fusogenic properties of the amino terminus of the HA2 moiety of the haemagglutinin proteins [5]. This consists of 20 amino acids, all of which are hydrophobic except for five critically spaced glutamates. As pH falls, the gamma carboxyls of the glutamates (which have a pK_a of ~4.0) become protonated, and the amino terminus of haemagglutinin becomes progressively more hydrophobic. At pH 5–6, a conformational change occurs in the haemagglutinin, with the hydrophobic, membrane-active amino terminus protruding from the molecule as an amphipathic alpha helix. Several groups have used influenza haemagglutinin peptides to promote gene delivery by high molecular weight polylysine and cationic lipid based systems (e.g. [6–13]).

Our group has been developing small, $(Lys)_{16}$-containing synthetic peptides as non-viral gene delivery systems [12–23]. An early study, using chloroquine for endocytic escape, demonstrated excellent gene delivery to post-mitotic corneal endothelial cells [14]. More recently, we reported that a simple system, consisting only of a $(Lys)_{16}$ peptide and a 20 amino-acid fusogenic peptide, is able to deliver genes with >95% efficiency to post-mitotic corneal endothelial cells [13]. The fact that these $(Lys)_{16}$-based systems can overcome all cellular barriers for gene delivery, including translocation into the nucleus, strongly suggests that $(Lys)_{16}$ has nuclear translocating ability for plasmid DNA. This is supported by a study evaluating nuclear translocation in digitonin-permeabilised cells [24]. These data are consistent with the fact that nuclear localising signals for proteins are short cationic sequences, such as the well-known PKKKRKV sequence of the SV40 large T antigen [25]. Thus the $(Lys)_{16}$ motif in our system serves two functions – firstly, to bind and condense DNA via electrostatic interactions to form vector/DNA particles, and secondly, once in the cytosol, to translocate the DNA plasmid into the nucleus.

We were interested to see if incorporating both the fusogenic and the $(Lys)_{16}$ moieties into a single multifunctional synthetic peptide might influence the physical and gene delivery properties of the peptide/DNA nanoparticles.

Materials and methods

Peptides (Table 1)

Synthesis, cyclisation via cysteine residues (where indicated) and purification by high-performance liquid chromatography (HPLC) techniques were performed by Cambridge Research Biochemicals (Northwich, Cheshire, UK). The peptides were supplied as the trifluoroacetate (TFA) salt, in the form of a dry powder, and stored dessicated at −35 °C. Quantities of 1–2 mg were dissolved in phosphate-buffered saline (PBS) with Ca^{2+} and Mg^{2+} (Invitrogen), 5% dextrose buffered to pH 7.4 in 10 mM Tris (dextrose/Tris), or pure water, and stored in small aliquots at −35 °C. The fusogenic peptide must be dissolved in buffered solutions, in order to maintain the pH at ~7, in which case solubility is not a problem.

Determination of secondary structure by circular dichroism

A volume of 250 µl of peptide at either 0.25 or 1 mg/ml in PBS was scanned through wavelengths 200–250 nm using a Jobin Yvon CD6 instrument and a pathlength of 0.1 cm. The results were recorded as molar ellipticity (with PBS background-subtracted) against wavelength [26,27]. Secondary structure was inferred from comparisons with model structures.

Imaging of individual peptide/DNA nanoparticles in solution

Automated image analysis was performed using a fluid particle image analyser (FPIA 2100, Sysmex Corporation, Kobe, Japan) as previously described [23]. Particles in solution pass through a sheath flow cell that ensures that the largest area of the particle is orientated towards the camera and that all particles are in focus. A CCD microscope illuminated by a stroboscope captures particle images at 30 Hz. A pulsed xenon light source that produces white light was used, giving a lower size detection limit of 0.7 µm. Numerical evaluation of particle shape is derived from measurement of the projected area and perimeter of the particle. Circularity (defined as the ratio of the perimeter of a circle of equivalent projected

Table 1. Synthetic peptides used in this study

Peptide	Amino acid sequence (single letter code)	Molecular weight (Daltons) ♦	Net charge (pH 7)
$(Lys)_{16}$	$(K)_{16}$	2069	+16
$(Lys)_{16}$-fuso*	GLFEALLELLESLWELLLEADP$(K)_{16}$	4565	+10
$(Lys)_{16}$-molossin$^{\phi}$	$(K)_{16}$ICRRARGDNPDDRCT	3797	+17
Fuso*	GLFEALLELLESLWELLLEA	2301	−5

*'Fuso' refers to a modified form of the amino terminal 20 amino acids of the influenza virus haemagglutinin protein [8]. The $(Lys)_{16}$ moiety is at the carboxy terminus in order to maintain the fuso peptide at the amino terminus, to mimic its orientation as in the haemagglutinin protein.

ϕ Molossin refers to the 15 amino acid integrin-binding loop of the snake venom, molossin [37]. This peptide is cyclised via the disulphide bond between the cysteine residues to ensure correct presentation of the integrin-binding RGD domain.

♦ Figures do not include the trifluoroacetate counterion (114 Da), one per positively charged amino group.

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area to the particle, to the perimeter of the particle itself) and diameter (defined as the diameter of a circle of equivalent projected area) can be determined.

Analysis of particle size by dynamic light scattering (DLS)

DLS measurements were performed on a Zetasizer 3000 HS (Malvern Instruments Ltd., Malvern, UK). Data analysis used the non-negatively constrained least-squares (NNLS) method, via software provided by Malvern Instruments. The hydrodynamic radius (Z_{av}) and polydispersity index values of the peptide/DNA nanoparticles were measured at least three times and the average of these measurements is reported.

Erythrocyte lysis assays

Membrane-lytic activity was evaluated by monitoring the release of haemoglobin from freshly isolated rat erythrocytes (RBC). RBC (75 µl) at 7×10^7/ml in PBS of pH 5.0 or 7.4 was added to wells of a 96-well plate. An equal volume of peptide in the same buffer was added and the plate was incubated for 1 h at 37 °C with gentle shaking. The 100% lysis control was obtained by adding 1 µl of 10% Triton X-100. The plate was centrifuged at 1000 *g* for 5 min, and the optical density at 450 nm of the supernatant was measured.

Formation of peptide/DNA nanoparticles

$(Lys)_{16}$-molossin/DNA nanoparticles were prepared at a weight/weight (w/w) ratio of 3 : 1 $(Lys)_{16}$-molossin/DNA, which has previously been shown to be optimal [17]. This corresponds to a + to − charge ratio of 2.8 : 1. $(Lys)_{16}$/DNA and $(Lys)_{16}$-fuso/DNA particles were formed at the same charge ratio.

The pGL3 (Promega, Madison, WI, USA) or pCMVβ (Clontech, Palo Alto, CA, USA) plasmids were used to form the complexes. The plasmids were prepared from bacterial cultures by alkaline lysis followed by column purification under endotoxin-free conditions (Quiagen Ltd., Dorking, UK). Purity was assessed by the ratio of the optical density at 260 nm against that at 280 nm, which was always >1.8. The concentration of DNA in the complexes was 5 µg/ml, unless otherwise stated.

The complexes were incubated for 15 min before adding fusogenic peptide and incubated for a further 15 min before beginning measurements, or adding to target cells for gene delivery.

Formation of poly(ethylenimine) (PEI)/DNA complexes

Branched PEI 25 000 (Sigma Aldrich, Dorset, UK) was used to make PEI/DNA complexes with 5 µg/ml of DNA at a molar amine-to-phosphate (N/P) ratio of 10, as recommended by the manufacturer. The samples were allowed to stand for 30 min before adding to target cells for gene delivery.

Formation of Lipofectamine 2000™/DNA complexes

Lipofectamine 2000™/DNA complexes (Invitrogen, Paisley, UK) were made with 5 µg/ml of DNA at a w/w ratio of 1, as recommended by the manufacturer.

Adenoviral gene delivery to corneal endothelial cells

The adenovirus vector (a kind gift from Dr. S. Salehi) was an E1-deleted adenovirus serotype 5 (AdEasy, Q Biogene, Cambridge, UK) carrying the *E. coli* β-galactosidase gene driven by the cytomegalovirus (CMV) promoter.

Gel electrophoresis to show complex dissociation

$(Lys)_{16}$-molossin/DNA complexes were electrophoresed on a 1% agarose gel (60 min at 100 V) containing ethidium bromide (0.1 µg/ml). DNA migration was assessed using a UV light box.

Cell lines

The HepG2 and T-24 cell lines (European Collection of Cell Cultures, Salisbury, Wiltshire, UK) are adherent lines originally derived respectively from a human hepatocyte carcinoma and a bladder carcinoma. They were maintained under mycoplasma-free conditions in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum (FCS), 2 mM glutamine and 1× non-essential amino acids (Invitrogen).

Transfection of cell lines

Cells were transfected in 24-well plates as previously described [12] and assayed for reporter gene expression after 48 h.

Luciferase reporter gene

Cell lysates were analysed using a luciferase activity kit (Promega, Madison, WI, USA). Total light emission was measured for 10 s in a luminometer (Anthos Lucy 1, Labtech International Ltd., East Sussex, UK). Protein concentrations were determined with a protein assay reagent (Biorad, Hercules, CA, USA). Luciferase enzyme activity was expressed as relative light units (RLU) per mg of protein ± standard error (SE) of the mean.

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Culturing and transfection of rabbit corneas

Eyes were obtained from male New Zealand White rabbits of ~4.5 kg weight (Harlan Sera-Lab Ltd., Loughborough, Leicestershire, UK), placed in sterile, ice-cold saline, and used within 4 h of death. The cornea was cut into six pieces, and transfections were carried out in 24-well plates as previously described [13]. Reporter gene analysis was performed 48 h later.

β-Galactosidase reporter gene

Corneal lysates were assayed using the Galactostar kit (Applied Biosystems, Boston, MA, USA) [13].

Cell cycle arrest of HeLa cells

Double thymidine block was used to arrest cells at the G1/S border [28]. Briefly, cells were plated at a density of 5×10^4 cells/ml in DMEM in 24-well plates, and allowed to adhere for 3–4 h. Cells were then washed with PBS, and cultured in DMEM containing 2 mM thymidine for 19 h at 37 °C. Cells were then washed with PBS, and cultured in DMEM without thymidine for 9 h. The cells were then washed with PBS, and cultured in DMEM containing 2 mM thymidine for a further 16 h. Subsequent transfection of the arrested cells (including all wash steps using PBS) was performed in the presence of 2 mM thymidine.

Assay for cell viability

Gene delivery was performed under standard conditions to HeLa cells using $(Lys)_{16}$-fuso/DNA particles, $(Lys)_{16}$/DNA particles ± fuso peptide and $(Lys)_{16}$-molossin/DNA particles ± fuso peptide. At 24 h, MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2*H*-tetrazolium bromide, Sigma Aldrich) was added to a final concentration of 0.5 mg/ml, and the reaction product measured as recommended by the manufacturer.

Results

Interaction of free fusogenic peptide with $(Lys)_{16}$/DNA particles

The results given in Figure 1a demonstrate that as little as 2.5 μg/ml of fusogenic peptide, corresponding to a w/w ratio of $(Lys)_{16}$-molossin/DNA/fusogenic peptide of 3 : 1 : 0.5, caused measurable dissociation of the $(Lys)_{16}$-molossin/DNA complexes. At 5 μg/ml of fusogenic peptide, much of the DNA has been dissociated. However, optimal gene transfection of cell lines [12] and cornea [13] occurs at w/w ratios of 3 : 1 : 1 or 3 : 1 : 2 of $(Lys)_{16}$-molossin/DNA/fusogenic peptide. Thus, although the fusogenic peptide appears to displace much of the DNA from the complexes, sufficient must remain to mediate high levels of gene expression.

Given the surprisingly high level of DNA dissociation, the effect of the fusogenic peptide on the size and number of $(Lys)_{16}$-molossin/DNA particles was of interest. Figure 1b demonstrates that the size of the particles *increases* with increasing concentrations of the fusogenic peptide, while fluid particle image analysis (FPIA) (Figure 1c, upper 2 rows) shows that particle number was not significantly affected. Taken together with our previous report that free fusogenic peptide neutralises the net positive charge of $(Lys)_{16}$-molossin/DNA nanoparticles [13], these data suggest that the fusogenic peptide both displaces and replaces DNA in the particle. In fact, the fusogenic peptide and $(Lys)_{16}$ peptides do interact with one another (unpublished data).

Direct visualisation of $(Lys)_{16}$-molossin/DNA particles (Figure 1c, upper 2 rows) shows that the addition of fusogenic peptide causes a remarkable and highly reproducible change in particle shape, from spherical to more 'doughnut' shaped. Of particular interest is the effect of the fusogenic peptide on $(Lys)_{16}$/DNA particles. We have previously reported the very loose structure of $(Lys)_{16}$/DNA particles [23] (Figure 1c, lower 2 rows). The fusogenic peptide caused a dramatic change to smaller, regular spheres, and an ~8-fold increase in particle number. The data in Figure 1a show virtually complete displacement of DNA from $(Lys)_{16}$-molossin/DNA particles by the fusogenic peptide. This suggests that the remarkable changes in particle morphology caused by the addition of the fusogenic peptide might be a consequence of the replacement of DNA by the fusogenic peptide in $(Lys)_{16}$/DNA particles.

Physical properties of the $(Lys)_{16}$-fusogenic peptide

Given the dissociation of much of the DNA from $(Lys)_{16}$-molossin/DNA complexes by free fusogenic peptide, we were interested to study a single synthetic peptide which combined the $(Lys)_{16}$ moiety for binding and condensing DNA with the fusogenic moiety (Table 1).

We included an acid-labile bond (Asp–Pro) between the fusogenic and $(Lys)_{16}$ moieties in case dissociation of the fusogenic peptide in the acidic endocytic compartments was important for its membrane-disrupting properties. However, incubation of the peptide at pH 5 for 4 h, followed by analyses using matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-TOFMS; Dr. M. Ward, Institute of Psychiatry, London, UK), showed <5% cleavage, so it is unlikely that this is important (data not shown).

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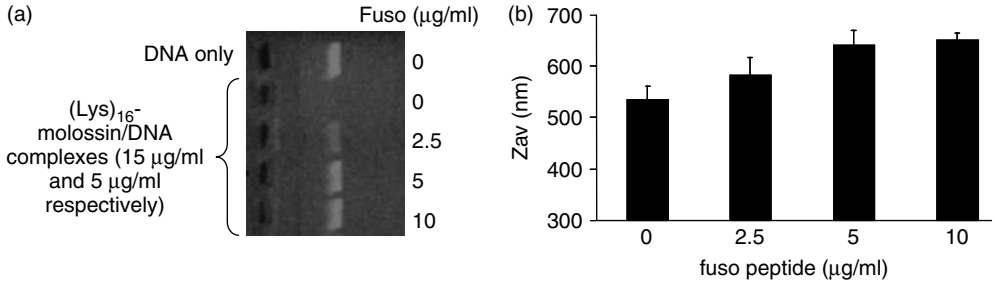

(c)

Peptide in particle	Photograph of particles	Mean diameter (μm)	Mean circularity	Number of particles per ml
$(Lys)_{16}$-molossin		0.8	0.766	2.1×10^7
$(Lys)_{16}$-molossin +fuso		0.84	0.782	2.8×10^7
$(Lys)_{16}$		1.9	0.629	1.3×10^6
$(Lys)_{16}$+ fuso		1.57	0.684	1×10^7

Figure 1. The influence of free fusogenic peptide on $(Lys)_{16}$ peptide/DNA complexes. (a) DNA alone or $(Lys)_{16}$-molossin/DNA complexes were electrophoresed in 1% agarose gels. The complexes were incubated in different concentrations of fusogenic peptide for 15 min prior to electrophoresis, as indicated. The pGL3 plasmid was at 5 μg/ml in PBS in all lanes. (b) $(Lys)_{16}$-molossin/DNA complexes as above were incubated for 30 min with varying concentrations of free fusogenic peptide, and DLS was performed to measure particle formation. The hydrodynamic radius, or Z_{av}, is given. (c) Fluid particle image analysis was performed on $(Lys)_{16}$-molossin/DNA (row 1) or $(Lys)_{16}$/DNA complexes (row 3) at 3 : 1 and 2.1 : 1 w/w ratios, respectively (which corresponds to a + to − charge ratio of 2.8 : 1) 30 min after complex formation. When the fusogenic peptide was used, it was added at 20 μg/ml 30 min after complex formation and the analysis performed 15 min later. DNA concentration was 10 μg/ml, and analyses were in PBS. The photographs of a random selection of 12 particles are shown. Physical parameters were means of 500 particles. Particle diameter is defined as the diameter of a circle of the equivalent projected area. Mean circularity is defined as the ratio of the perimeter of a circle of equivalent projected area to the perimeter on the particle

(a) Circular dichroism

Figure 2a gives values for molar ellipticity of model peptides in the left-hand panel, and then the values for the four peptides used in this study. The $(Lys)_{16}$-molossin peptide and the $(Lys)_{16}$ peptide (middle panel) assume a random configuration, as expected for small peptides in aqueous solutions. However, both the fusogenic peptide and the $(Lys)_{16}$-fusogenic peptide adopt an alpha-helical secondary structure in solution in PBS, at pH of 6.9 and 7.2, respectively. From the molar ellipticity at 220 nm [26,27], it can be calculated that ~60% of the free fusogenic peptide and ~30% of the $(Lys)_{16}$-fusogenic peptide adopt an alpha-helical structure. As the fusogenic moiety comprises only around half of the $(Lys)_{16}$-fusogenic peptide, the ability of the fusogenic moiety to assume an alpha helix appears unimpeded in the composite peptide.

(b) Membrane-active properties

Neither the fusogenic nor the $(Lys)_{16}$-fusogenic peptide was able to lyse erythrocytes at pH 7.4 (Figure 2b). This is important from the point of view of the safety of these peptides, as will be discussed later. At pH 5, the fusogenic peptide was ~10-fold more effective (w/w), which represents a ~5-fold superiority on a molar basis.

(c) Formation of peptide/DNA particles

Using DNA electrophoresis as in Figure 1a, $(Lys)_{16}$-fuso/DNA at w/w ratios of 2 : 1 and 3 : 1 showed partial and complete DNA retardation, respectively (data not shown). This suggested effective peptide/DNA interactions. Using dynamic light scattering (DLS), particle formation was readily observed in PBS and dextrose/Tris. After incubation for 30 min at room temperature, $(Lys)_{16}$/DNA, $(Lys)_{16}$-fuso/DNA and $(Lys)_{16}$-molossin/DNA particles formed at a +/− charge ratio of 2.8 and a DNA concentration of 5 μg/ml had Z_{av} diameters (mean ± SD) of 1134 ± 90, 1037 ± 56 and 745 ± 32 nm, respectively, in PBS, and 107 ± 1, 69 ± 1 and 145 ± 1 nm when formed in dextrose/Tris. It is interesting that $(Lys)_{16}$-fuso/DNA particles were larger than $(Lys)_{16}$-molossin/DNA particles in PBS, but significantly smaller when formed in dextrose/Tris.

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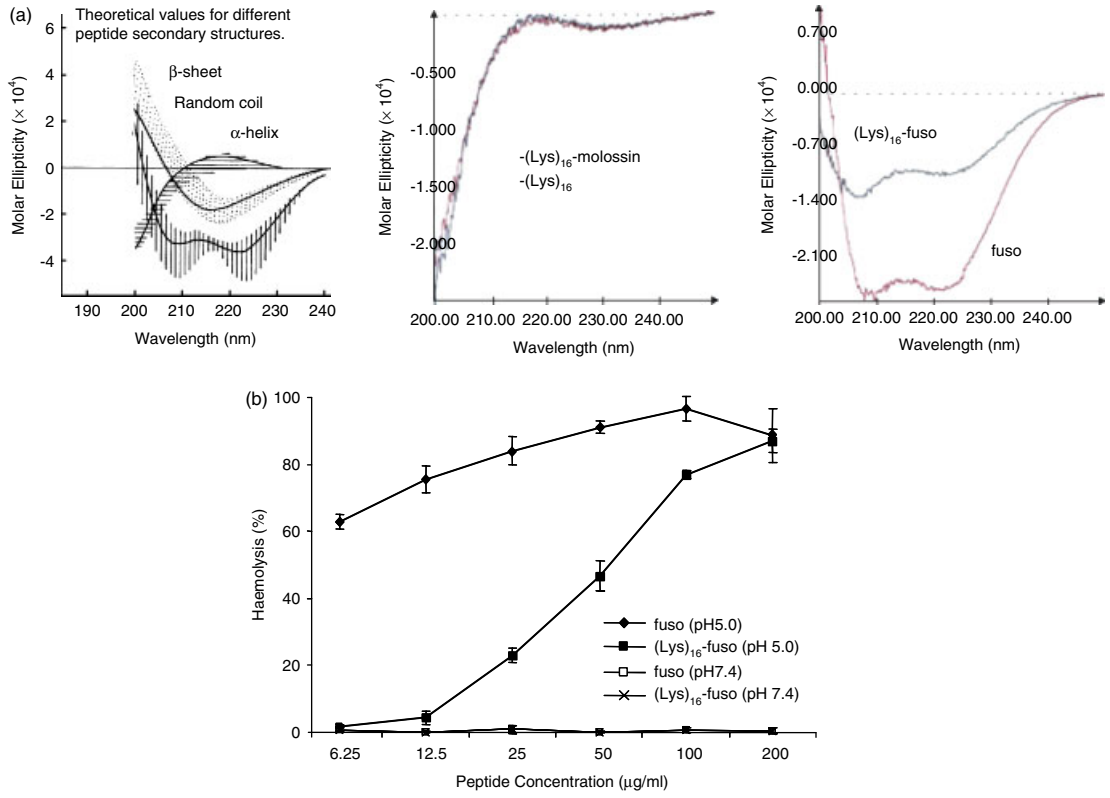

Figure 2. Physical properties of peptides. (a) Circular dichroism of model secondary structures (left panel) and analysis of peptides in PBS (pH ~7) (middle and right panels) are shown. Left panel is reproduced with permission from Gratzer and Beaven [27]. (b) Erythrocyte lysis assay of fusogenic and $(Lys)_{16}$-fusogenic peptides at pH 5 and 7.4, as indicated

Gene delivery

(a) Cell lines

$(Lys)_{16}$-fuso/DNA complexes give excellent gene delivery to the T-24 cell line, ~10^4 times higher than $(Lys)_{16}$, ~500 times higher than $(Lys)_{16}$-molossin, and equivalent to Lipofectamine 2000 (Figure 3a). On the HepG2 cell line, the $(Lys)_{16}$-fusogenic peptide is also highly effective, and only slightly less effective than Lipofectamine 2000 (Figure 3b). At the end of the 4-h incubation with vector/DNA complexes, and at the time of harvest after 48 h in culture, the cells looked in excellent condition using phase-contrast microscopy, and the protein content per well was the same as in the DNA-only control (data not shown). An MTT assay performed 24 h after exposure of HeLa cells to $(Lys)_{16}$-fuso/DNA particles showed excellent viability (>90% in comparison with cells not exposed to peptide/DNA particles, data not shown). This single synthetic peptide is therefore a highly effective, simple and non-toxic gene delivery agent.

The addition of free fusogenic peptide to promote endocytic escape greatly augments the effectiveness of $(Lys)_{16}$/DNA and $(Lys)_{16}$-molossin/DNA complexes (Figure 4a). However, it does not significantly augment the gene delivery efficiency of $(Lys)_{16}$-fuso/DNA complexes. $(Lys)_{16}$-fuso/DNA complexes alone, in comparison with $(Lys)_{16}$/DNA or $(Lys)_{16}$-molossin/DNA complexes with fusogenic peptide, are ~500-fold and ~30-fold more effective, respectively.

Chloroquine is widely used as an agent to augment gene delivery by promoting escape of DNA complexes from endocytic residues. On entering acidic compartments (such as endocytic vesicles), chloroquine becomes protonated, and no longer membrane-permeable. This has a buffering effect, and also induces osmotic swelling. Chloroquine powerfully inhibits gene delivery by $(Lys)_{16}$-fuso/DNA complexes (Figure 4b), consistent with the dependence of fusogenic activity on acidic pH (Figure 2b).

(b) Post-mitotic corneal endothelial cells

We have recently reported highly efficient (>95%) gene delivery to endothelial cells in rabbit cornea using $(Lys)_{16}$-molossin/DNA or $(Lys)_{16}$/DNA complexes with free fusogenic peptide [13]. $(Lys)_{16}$-fuso/DNA complexes, quite remarkably, are not at all effective, with efficiency similar to the DNA-only control (Figure 5). Neither PEI nor Lipofectamine 2000 was effective on the cornea (Figure 5). Interestingly, the level of gene delivery with the adenovirus vector (used at a multiplicity of infection of >100) was 5–10-fold lower than with the $(Lys)_{16}$/DNA and $(Lys)_{16}$-molossin/DNA complexes with fusogenic peptide.

(c) Cell cycle arrested cell lines

The marked difference in effectiveness of the $(Lys)_{16}$-fuso peptide on cell lines in comparison with post-mitotic corneal endothelial cells might have been a

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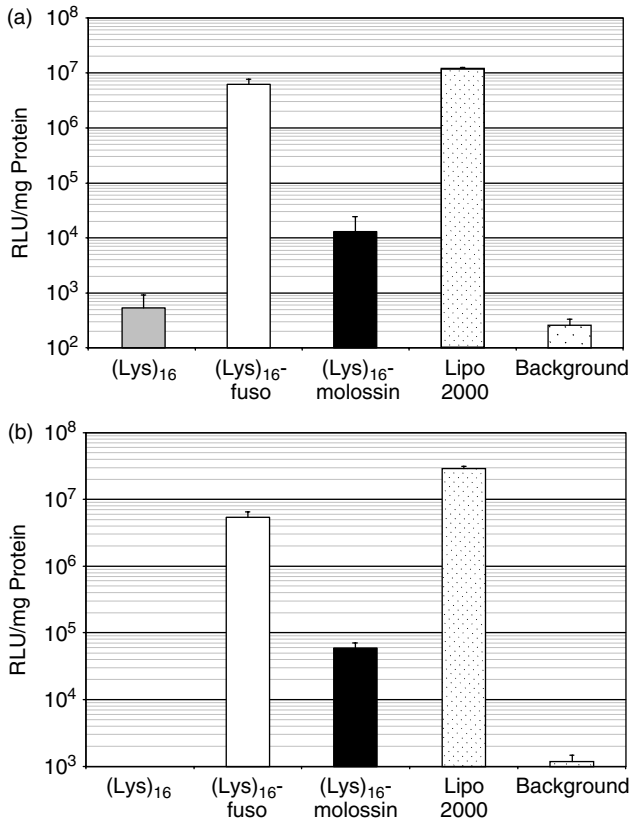

Figure 3. Gene delivery to cell lines. Peptide/DNA complexes as indicated and Lipofectamine 2000™/DNA complexes (Lipo 2000) were used with the pGL3 plasmid at a concentration of 5 μg/ml in culture medium for *in vitro* gene delivery to the T-24 (a) and the HepG2 (b) cell lines. Peptide/DNA complexes were used at a + to − charge ratio of ~2.8 : 1, which is optimal for gene delivery. Lipofectamine 2000™ was used as recommended by the manufacturer. Background refers to cells with DNA only. Results are means ± SE of triplicates. RLU refers to relative light units

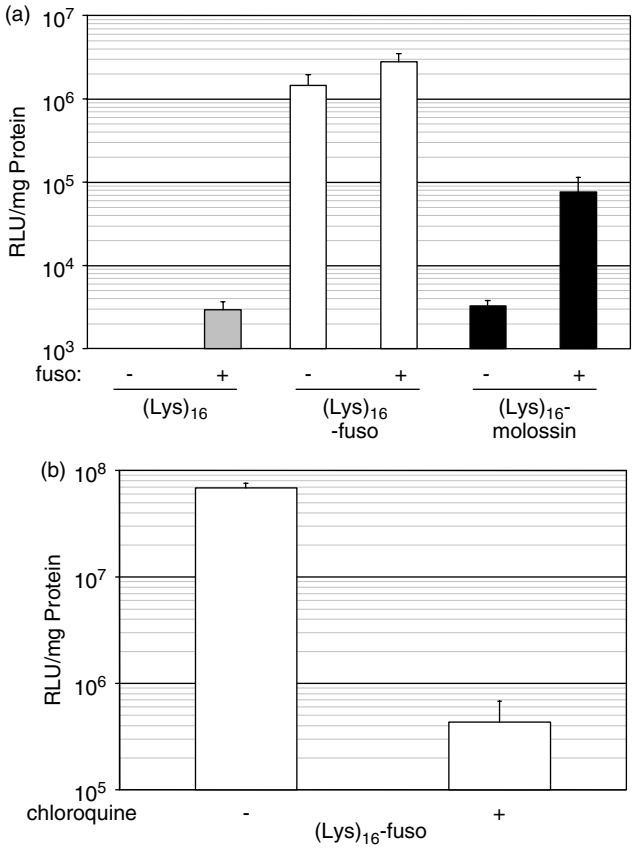

Figure 4. Endocytic escape agents. Peptide/DNA complexes as indicated at a + to − charge ratio of ~2.8 : 1 and the pGL3 plasmid at a concentration of 5 μg/ml were used in culture medium for transfection of the T-24 cell line. This charge ratio is optimal for these particles. (a) Fusogenic peptide was added at 10 μg/ml where indicated. This corresponds to a w/w ratio of 2.1 : 1 : 2, 3 : 1 : 2 and 6 : 1 : 2 for $(Lys)_{16}$/DNA/fuso, $(Lys)_{16}$-molossin/DNA/fuso and $(Lys)_{16}$-fuso/DNA/fuso, respectively. (b) Chloroquine was added at 100 μM where indicated. Results are mean ± SE of triplicates. RLU refers to relative light units

consequence of factors other than lack of mitosis in the corneal endothelial cells. Transfection experiments were therefore performed in HeLa cells which were either freely dividing or had been cell cycle arrested at G_1/S by double thymidine block. Cell cycle arrest did not diminish the effectiveness of $(Lys)_{16}$/DNA complexes with fusogenic peptide: in fact they gave marginally *increased* levels of gene expression in the growth arrested cells (Figure 6). By contrast, $(Lys)_{16}$-fuso/DNA complexes were only 5–10% as effective in growth arrested cells. $(Lys)_{16}$-molossin/DNA complexes plus fusogenic peptide were ~60% as effective in growth-arrested cells. PEI and Lipofectamine 2000™ were sensitive to cell cycle status, with efficiencies reduced to 15–20% in growth-arrested cells.

Discussion

The use of $(Lys)_{16}$-containing synthetic peptides for non-viral gene delivery is an attractive option. The cationic nature of these peptides resides in the epsilon amino group ($pK_a \sim 10.5$) of the natural amino acid L-lysine, and polymerisation is based on normal peptide bonds, thus reducing potential for toxicity. The fact that $(Lys)_{16}$-containing peptides potentially have both DNA-binding and nuclear-translocating capabilities is a very valuable and initially unanticipated property, which has enabled gene delivery to non-dividing cells. This is critical for *in vivo* gene therapy for many cell types, including hepatocytes, skeletal muscle cells and vascular endothelium.

Early studies established cell cycle dependence of gene delivery by cationic lipids (e.g. [29]), high molecular weight polylysines and branched polyethylinimines [30], suggesting a poor ability for nuclear translocation of DNA. The precise structural requirements for nuclear translocation by oligolysines are not clear. Our studies on freely dividing and growth-arrested HeLa cells suggest that the free $(Lys)_{16}$ peptide is the most effective peptide for nuclear translocation, as it is equally effective on dividing and non-dividing cells. The

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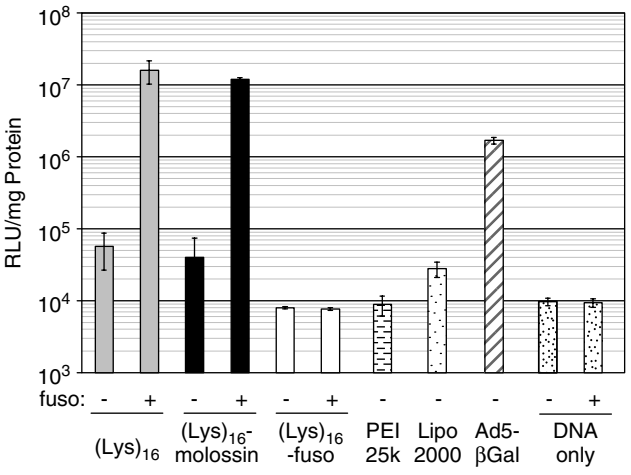

Figure 5. Gene delivery to post-mitotic corneal endothelial cells. Vector/DNA complexes in dextrose/Tris were prepared with peptides as indicated at a + to − charge ratio of ∼2.8 : 1 and with PEI and Lipofectamine 2000™ as recommended by the manufacturer. The pCMV*β* plasmid was used at a DNA concentration of 10 μg/ml. The adenovirus vector carrying the *β*-galactosidase gene from the pCMV*β* plasmid was used at 10^9 plaque-forming units (pfu)/ml, which corresponds to a multiplicity of infection of ∼200 with regard to the corneal endothelial cells. Rabbit corneas were exposed to the above DNA vectors *in vitro*, and harvested 48 h later. Results are means ± SE of triplicates. RLU refers to relative light units

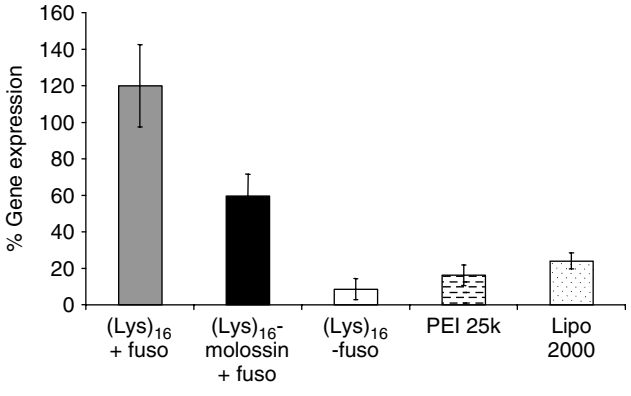

Figure 6. Gene delivery to cell cycle arrested HeLa cells. Vector/DNA complexes were prepared with peptides as indicated at a + to − charge ratio of ∼2.8 : 1 and with PEI and Lipofectamine 2000™ as recommended by the manufacturer. The complexes were used to transfect HeLa cells that were either freely dividing or cell cycle arrested. The efficiency of gene expression in G_1/S-arrested cells compared to freely dividing cells is shown

incorporation of the 15 amino-acid molossin peptide with $(Lys)_{16}$ to form polylysine-molossin appears to diminish the effectiveness of the $(Lys)_{16}$ motif for nuclear translocation. Incorporating the 20 amino-acid fusogenic peptide to form the $(Lys)_{16}$-fusogenic peptide totally abolishes nuclear translocation, and thereby limits gene delivery to dividing cells. The peptide evaluated by Wilke *et al*. [9] (SPKRSPKRSPKR) had no nuclear translocating ability, but whether this was inherent in the sequence, or was a consequence of the addition of a palmitic ester to the amino terminal amine, is not known.

Some clinical applications, such as the delivery of genes to activate potentially toxic prodrugs in cancer and vascular disease, ideally require a DNA vector able to distinguish between dividing and non-dividing cells. This has been the basis for the use of retroviruses for gene delivery in the treatment of cancers with prodrugs (e.g. [31]). The $(Lys)_{16}$-fusogenic peptide might represent a simpler and safer gene delivery system for such applications. There are also circumstances where delivery of nucleic acids to the cytosol is *sufficient*, e.g. for the genomes of viruses which replicate their DNA in the cytosol, for synthetic mRNA or RNAi oligonucleotides (e.g. [32]), or for cytosolic gene expression systems such as those based on T7 polymerase [33].

Although it is known that cationic vector/DNA particles can be disrupted by polyanions [34], the ability of free fusogenic peptide (net charge -5) to compete with and dislodge DNA from $(Lys)_{16}$-molossin/DNA particles at very low concentrations was unexpected. However, sufficient DNA remained in the $(Lys)_{16}$/DNA and $(Lys)_{16}$-molossin/DNA particles to provide reagents with excellent gene delivery properties for both cell lines and post-mitotic corneal endothelial cells. The even greater capacity of the $(Lys)_{16}$-fuso/DNA particles for gene delivery to dividing cell lines might be a consequence of a higher DNA content of these particles, as there is no opportunity for DNA displacement from these particles.

With regard to the physical structure of vector/DNA particles, the dramatic change in particle size and shape, and particle number, in $(Lys)_{16}$/DNA particles by the addition of free fusogenic peptide was of particular interest. We have previously reported that $(Lys)_{16}$-molossin/DNA particles are much superior to $(Lys)_{16}$/DNA particles for gene delivery, using chloroquine for endocytic escape [12,14]. This is probably a consequence of the large, loose structure of $(Lys)_{16}$/DNA particles [23]. However, when using the fusogenic peptide instead of chloroquine, $(Lys)_{16}$/DNA and $(Lys)_{16}$-molossin DNA are approximately equally effective [13]. This could be a consequence of the dramatic change in $(Lys)_{16}$/DNA particle structure on addition of the fusogenic peptide. This emphasises the critical importance of evaluating the physical properties of non-viral DNA vectors.

The use of the influenza fusogenic peptide motif for promoting escape from endocytic vesicles is an attractive option. These fusogenic peptides are membrane-active only at the acidic pHs achieved within endocytic vesicles. In the neutral extracellular environment, where toxicity consequent on plasma membrane disruption is potentially a problem, they are hydrophilic and do not interact with membranes. These properties are confirmed for our bifunctional $(Lys)_{16}$-fusogenic peptide by its inability to lyse erythrocytes at neutral pH, and by the marked suppression of its gene delivery properties by chloroquine.

We were interested to note that the fusogenic peptide assumes an alpha-helical conformation at neutral pH. This was confirmed on several occasions in two laboratories.

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It is often assumed that the formation of an amphipathic alpha helix by anionic fusogenic peptides requires acidic pH, but this is not the case. The acid-dependent conformational change in the haemagglutinin molecule consists of relocating the fusogenic peptide into a position more favourable for membrane insertion [35]. The acid-dependent change in the fusogenic peptide itself consists of increasing hydrophobicity.

Low or absent immunogenicity of small peptides is a potentially important advantage of this system. This is a generally accepted principle in immunology, and rests on the low probability of any particular peptide binding to the peptide-binding groove of an individual's MHC class II molecules. We have previously demonstrated this in studies unrelated to gene therapy, using peptide immunisations with Freunds complete adjuvant [36]. Because the highly cationic $(Lys)_{16}$ moiety might impart unusual antigenic properties to our peptide vectors, we immunised four rat strains of different MHC type with $(Lys)_{16}$-molossin peptides in Freunds complete adjuvant. None showed any antibody or T cell proliferative responses (unpublished data).

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