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Development of an effective gene delivery system: a study of complexes composed of a peptide-based amphiphilic DNA compaction agent and phospholipid

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ABSTRACT

We recently described a basic technology to efficiently combine compacted DNA with phospholipids and hydrophobic peptides, to produce homogenous complexes that are completely resistant to nuclease. We have developed this technology further to form gene delivery complexes that transfect cells effectively *in vitro.* In addition to plasmid DNA, the complexes contained two basic components: (i) a DNA compacting peptide (-CGKKKFKLKH), either conjugated to lipid or extended to contain (WLPLPWGW-) and (ii) either phosphatidylethanolamine or phosphatidylcholine. Complexes containing a 5.5-fold charge equivalence (peptide charge/DNA charge) of WLPLPWGWCG-KKKFKLKH and 5 nmol dimyristoleoylphosphatidyl-ethanolamine/μg DNA produced the highest luciferase gene expression, exceeding 1×10^9 relative light units/s/mg protein (>3 μg luciferase per mg protein). These complexes transfected OVCAR-3, COS-7 and HeLa cells at either similar or superior levels when compared to polyethylenimine or lipofectamine complexes. With green fluorescent protein reporter gene, >50% of HeLa cells were positive 30 h after addition of these complexes. Furthermore, these optimal complexes were the least sensitive to pre-treatment of cells with chloroquine, indicating efficient endosomal escape. Our results indicated that self-assembling complexes of plasmid DNA, amphiphilic peptide and phosphatidyl-ethanolamine are highly effective non-viral gene delivery systems.

INTRODUCTION

Non-viral gene delivery systems must proceed through a series of adverse extracellular and cellular environments in order to deliver genetic material successfully into the nucleus of a eukaryotic cell. The most fundamental and essential requirement of a gene delivery system is adequate protection of the DNA throughout the delivery process, because nuclease activities are found in most extracellular environments and cellular compartments encountered. Typically, multivalent cationic molecules are used to compact DNA by neutralizing the anionic phosphates of the DNA backbone (reviewed in 1), providing protection of the DNA from nucleases.

Most non-viral gene delivery systems are broadly classified as either 'lipoplexes' or 'polyplexes' (2). Lipoplexes generally consist of liposomes containing cationic lipids such as 1,2-dioleoyl-3-(trimethylammonio)propane or dioctadecylamidoglycylspermine and helper molecules such as dioleolyphosphatidylethanol-amine or cholesterol. Cationic liposomes electrostatically interact with DNA to form complexes that effectively transfect cells both *in vitro* (3–6) and *in vivo* (7–12). Polyplexes generally consist of polymers such as polyethylenimine (PEI) or cationic peptides, which are hydrophilic and contain multiple amines that interact with and compact DNA highly effectively. Poly-plexes prepared with either PEI or cationic peptides effectively transfect cells *in vitro* (13–18) and recently PEI has been shown to be effective *in vivo* (19–21). An advantage of peptide-based polyplexes is the ability to utilize peptides known to function as receptor ligands, amphipathic helices or nuclear localization signals.

In a recently published study (22) we described a basic technology to produce compacted DNA in a manner that allows for its subsequent combination with both polymers and/or phospholipids. This technology produces complexes that feature properties of both lipoplexes and polyplexes. First, the DNA is condensed by electrostatic interactions between DNA and a multivalent cationic peptide that is conjugated to phosphatidylethanolamine (PE). Condensation is performed in the presence of the anionic detergent, sodium cholate, which attenuates the otherwise strong electrostatic interactions between the DNA and cationic peptide. As a result, compacted DNA complexes are formed that are stable in solution. Next, zwitterionic lipid is added and the cholate detergent is removed by dialysis. This second assembly step is driven by the hydrophobic interactions between the zwitterionic lipid and the peptide–PE conjugate. The entire process results in the complete combination

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of compacted DNA with natural phospholipids (which can be anionic, zwitterionic or neutral) and avoids the need for subsequent separation of complexes from unincorporated material. The complexes are homogeneous with respect to density and are fully resistant to nuclease.

In this communication, we have developed this basic assembly technology further to produce gene delivery complexes that exhibit high levels of gene expression when incubated with cells in serum-containing medium. To achieve this goal, we compared the transfection efficiency of complexes assembled with (i) different types of hydrophobic additions to the multivalent peptide used for DNA compaction and (ii) zwitterionic phospholipids with different hydrocarbon chain lengths and head groups. We found the optimum formulation for gene expression contained (i) an eight amino acid hydrophobic addition to the multivalent peptide used for DNA compaction (WLPLPWGWCGKKKFKLKH or W3–K5) and (ii) the relatively short-chain fusogenic lipid dimyristoleoylphosphatidylethanolamine (di14:1PE). In addition, this optimum formulation produced transfection levels that were the least sensitive to pre-treatment of cells with chloroquine, indicating efficient endosomal escape and delivery of genetic material into the cytoplasm. When compared to both Lipofectamine™ and 25 kDa PEI in OVCAR-3, COS-7 and HeLa cells, the W3–K5/di14:1PE complexes transfected all three cell lines at either comparable or superior levels.

MATERIALS AND METHODS

Cell culture

Both the HeLa (human, cervical adenocarcinoma) and COS-7 (monkey, SV40 transformed kidney) cell lines were cultured in Eagle minimal essential medium (MEM) (Sigma, St Louis, MO) with 10% fetal bovine serum (FBS) (Gemini Bio-Products, Calabasas, CA). The NIH:OVCAR-3 (human, ovarian adenocarcinoma) cell line was cultured in RPMI 1640 medium (Life Technologies, Inc., Rockville, MD) supplemented with 20% FBS, 1 mM sodium pyruvate, 0.01 mg/ml bovine insulin (Sigma) and 10 mM HEPES pH 7.4. All cell lines were obtained from ATCC (Manassas, VA) and incubated at 37°C in a humidified 5% CO_2 atmosphere. Cells were maintained in 100 mm tissue culture dishes and passaged when cell density reached 70–80% confluency. For transfection studies, the cells were plated into 24-well plates (Falcon #35–3047) with 25 000–35 000 cells/well and grown overnight in the appropriate medium. Before the complexes were added, the medium was replaced with medium containing FBS, antibiotic/antimycotic (Gemini Bio-Products) and gentamicin (Sigma). Complexes in 1 mM sodium phosphate buffer pH 8.0 were added to the medium containing serum and antibiotics and remained in the medium for the entirety of the experiment.

Plasmid DNA

A 4.2-kb plasmid containing a firefly luciferase reporter gene with a cytomegalovirus promoter region (pCMV-luc) was a gift from Dr Robert Debs (California Pacific Medical Center) and has been described previously (11,12). pCMV-luc was maintained in *Escherichia coli* strain JM109 and large-scale purification was performed by Jason Murphy at the University of Houston using a published method (23). A 5.8-kb plasmid containing a green fluorescent protein (GFP) reporter gene with a CMV immediate early gene promoter/enhancer region (gWIZ™) was purchased from Gene Therapy Systems (San Diego, CA). This plasmid was maintained in *E.coli* strain JM109 and purified with Qiagen Endofree™ Maxi kits (Qiagen, Valencia, CA).

Synthetic peptides

Peptide synthesis, cleavage from the resin and purification were performed as described (22) with the exception of N-terminal acetylation, which was not carried out for these studies. Additionally, during peptide synthesis, all amino acids were double coupled. The peptide sequences of CGKKKFKLKH-amide (K5) and WLPLPWGWCGKKKFKLKH-amide (W3–K5) were verified by fast atom bombardment mass spectroscopy. Peptide concentrations were determined using Ellman's reagent [DTNB:5,5′-dithio-bis(2-nitrobenzoic acid), absorbance at 410 nm, $\varepsilon = 13\,600\ M^{-1}cm^{-1}$].

Preparation of peptide–lipid conjugates

The heterobifunctional cross-linking reagent MBS [3-maleimidobenzoic acid n-hydroxysuccinimide ester (Sigma)] was coupled to the primary amine of either dioleoylphosphatidylethanolamine (di18:1PE *cis*-9) or dimyristoleoylphosphatidylethanolamine (di14:1PE *cis*-9) as described previously (22). The product (di18:1PE–MBS or di14:1PE–MBS) was purified by LH-20 Sephadex chromatography and stored at –70°C in ethanol until further use.

For conjugation to peptide, 1 µmol dried residue of the peptide CGKKKFKLKH-amide was dissolved in 400 µl dimethylformamide (DMF) containing 2 µmol PE–MBS and allowed to react at room temperature. The progress of the reaction was followed by taking 5 µl aliquots and assaying for free cysteine with DTNB (150 µM DTNB, 1% SDS, 1 ml PBS, pH 7.4). If free cysteine was detected after 1 h, an additional 1 µmol PE–MBS was added. The DMF was evaporated using nitrogen gas, the residue dissolved in 50 µl dimethylsulfoxide and the product precipitated three times by the addition of 5 ml *t*-butyl methyl ether. TLC analysis of the ether supernatants indicated complete separation of product from the PE–MBS reactant (which remained dissolved in the ether) after the first precipitation step (R_f of di14:1PE–MBS = 0.85 and R_f of di18:1PE–MBS = 0.80, solvent system of chloroform/acetone/methanol/acetic acid/water 50/20/10/10/5).

Zwitterionic lipids

Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). They were stored at –70°C in chloroform and concentrations were verified by lipid phosphorus analysis (24).

Complex assembly and transfection

Plasmid DNA (20 µg) in 2 ml of 1 mM phosphate buffer pH 8.0 and 27.5 mM cholate pH 8.0, was compacted with a 2.5–7.0-fold charge equivalence (peptide positive charge/DNA negative charge) of CGKKKFKLKH-amide, WLPLPWGWCGKKKFKLKH-amide or PE-CGKKKFKLKH-amide. The compaction agent was dissolved in 100 µl DMF and added to the DNA solution slowly through a capillary pipette while stirring the solution vigorously. Subsequently, zwitterionic phospholipid (0–200 nmol) in 100 µl DMF was added slowly to the compacted DNA solution while stirring the solution vigorously.

This solution was dialyzed using dialysis tubing with molecular weight cut off of 12–14 kDa (Spectrum Laboratories, Inc., Rancho Dominguez, CA) three times against 1 l of 1 mM phosphate buffer with a minimum of 8 h between changes of buffer. This extensive dialysis procedure is necessary because residual cholate detergent is toxic to cells (data not shown).

After dialysis the samples were centrifuged at 25 000 *g* for 40 min (Beckman SW50.1 rotor). The supernatant was removed carefully and the pellet was resuspended in 100 µl of 1 mM phosphate buffer pH 8, to give a concentration of 0.2 µg DNA/µl. Complexes containing a total of 0.1–8 µg DNA were added per well of a 24-well plate for transfection studies.

Some loss of material occurred due to sticking of the W3–K5 peptide and W3–K5/di14:1PE complexes to the dialysis membrane. Loss of DNA and peptide during dialysis was estimated by solubilizing (to optical clarity) an aliquot of sample, taken both before and after dialysis, in 2% SDS and measuring the absorbance of the solutions at both 260 and 280 nm. The A_{260}/A_{280} ratio for the DNA was found to be 1.85. The A_{260}/A_{280} ratio for the peptide was 0.605. The relative amounts of DNA and peptide could then be determined by solving for the two unknowns in the following two equations:

$$A_{280}(\text{measured}) = (\text{absorbance of DNA at 280 nm}) + (\text{absorbance of peptide at 280 nm}) \quad \mathbf{1}$$

$$A_{260}\ (\text{measured}) = 1.85 \times (\text{absorbance of DNA at 280 nm}) + 0.605 \times (\text{absorbance of peptide at 280 nm}) \quad \mathbf{2}$$

By this analysis, it was found that the recovery of DNA after dialysis was 65 ± 4% (n = 6) and the recovery of W3–K5 peptide was 41 ± 5% (n = 6).

The zwitterionic phospholipids used in the experiments varied in acyl chain length from 12 to 18 carbons. To test for the possible loss of lipid during dialysis, samples of all the PEs (di12:0 to di18:1) were solubilized individually in 27.5 mM cholate and quantitated before and after dialysis by fluorescamine analysis (25) of the ethanolamine primary amine. Within experimental error, recovery of lipid after dialysis was consistently 100%, regardless of acyl chain length.

Preparation of Lipofectamine (Life Technologies, Inc.) complexes was carried out as described in the manufacturer's protocol. Complexes containing 4 µg Lipofectamine/1 µg plasmid DNA were found to be optimal. Addition of complexes containing >1 µg of DNA were highly toxic to cells in culture.

Preparation of PEI, with an approximate molecular weight of 25 kDa (Aldrich, Milwaukee, WI) was conducted as described previously (14). Complexes containing five equivalents of PEI (PEI nitrogen/DNA phosphate) were found to produce optimal transfection in cell culture.

Characterization of complexes

For analysis on sucrose density gradients, complexes were assembled as described above with the addition of 1% 1-(oleoyl)-2-(5,7-dimethylBODIPY-1-hexadecanoyl)-phosphatidylcholine (D-3821; Molecular Probes, Eugene, OR). After dialysis complexes were centrifuged at 25 000 *g* for 40 min. The supernatant was removed and the pellets were resuspended in 0.5 ml 1 mM phosphate buffer. The resuspended complexes were layered on top of linear 4 ml gradients of 0–70% (w/v) sucrose in 1 mM phosphate buffer. The gradients were centrifuged at 150 000 *g* for 3 h at 4°C. Fractions of ~150 µl were collected and diluted to 1 ml with 1 mM phosphate buffer. Hoechst 33258 dye was added to a final concentration of 200 pmol/ml. The fluorescence of the BODIPY-labeled lipid was measured at λ_{ex} = 490 nm, λ_{em} = 520 nm. The fluorescence of the Hoechst dye binding to the DNA was measured at λ_{ex} = 350 nm, λ_{em} = 460 nm.

Dynamic light scattering measurements were performed as previously described (22). The average hydrodynamic diameter for complexes containing a 5.5-fold charge equivalence of W3–K5 and 5 nmol of di14:1PE/µg of DNA was 194 nm. Eighty percent of the complexes were between 158 and 253 nm in diameter.

Assay for luciferase expression

Luciferase activity was measured with the Promega luciferase assay system (Promega, Madison, WI). Transfected cells in 24-well plates were rinsed twice with PBS and lysed with 250 µl supplied lysis buffer for 10 min. The cell lysate was diluted by a factor of 50 with lysis buffer. Next, 20 µl diluted sample was mixed with 50 µl supplied assay buffer and analyzed for 10 s on a model 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). Standard curves for determination of nanograms of luciferase were obtained using firefly luciferase enzyme (Sigma). One nanogram of luciferase produced 8×10^5 relative light units (RLU) per second. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) as described in the manufacturer's protocol.

Transfection in the presence of chloroquine

HeLa cells were grown in 24-well plates for 24 h. At 3 h prior to addition of the complexes, the medium was changed to MEM + 10% FBS and antibiotics, containing 0–100 µM chloroquine. Complexes were prepared and added to the cells as described above. At 6 h following the addition of the complexes, the medium was removed and replaced with the same medium without chloroquine. Luciferase activity was measured 24 h after the addition of the complexes.

GFP time course

Complexes containing the plasmid with the GFP reporter gene were assembled as described above. The complexes contained a 5.5-fold charge equivalence of W3–K5 and 5 nmol di14:1PE/µg of DNA. Complexes containing a total of 4 µg DNA were added to HeLa cells. At the desired time points (0, 10, 16, 24, 36, 48 and 72 h), the cells were rinsed three times with PBS and scraped in PBS. Formaldehyde was added to the cell suspension to give a final concentration of 2% formaldehyde and the samples were stored at 4°C until they were analyzed. GFP fluorescence (λ_{ex} = 480 nm, λ_{em} = 510 nm) in the cell suspensions was analyzed on an SLM-Aminco 8100 fluorometer, 450 W Xenon lamp, 4 nm slit widths, with all measurements relative to the fluorescence of a rhodamine dye reference sample. Photomultiplier tube (PMT) voltages were established by setting the fluorescence of the 36 h time point equal to 60% of full scale, using the auto-range feature of the instrument.

For photography, GFP positive cells were observed at 10× magnification with a Nikon TE-300 epi-fluorescence microscope using a FITC filter set. Digital pictures were taken with a Nikon Coolpix 990 digital camera and transferred to a computer in TIFF format.

Table 1. Description of the gene delivery system

Components of the system	Function
Plasmid DNA with CMV-luciferase or CMV-GFP	Reporter gene expression
A cationic peptide (CGKKKFKLKH) conjugated to PE or extended to include a hydrophobic amino acid sequence (Table 2A)	DNA compaction
PE or PC (Table 2B)	Endosomal/lysosomal escape

RESULTS

Transfection efficiencies of gene delivery complexes containing hydrophobic additions to the DNA compacting peptide CGKKKFKLKH

As described in Table 1, the gene delivery complexes consisted of three components: (i) the plasmid DNA containing a reporter gene, (ii) a DNA compacting peptide, CGKKKFKLKH-amide (abbreviated K5), conjugated to a hydrophobic region (phospholipid or extension of the peptide sequence) and (iii) a zwitterionic phospholipid. The compaction agent has two functions: (i) the cationic sequence, K5, compacts the DNA and (ii) the hydrophobic region interacts with the phospholipid to enable formation of the complex upon dialysis of the cholate detergent. Table 2A lists the three different hydrophobic additions to the compacting peptide, K5. Two of the additions were (i) dimyristoleoylphosphatidylethanolamine or (ii) dioleoylphosphatidylethanolamine conjugated to K5 (di14:1PE–K5 and di18:1PE–K5). The third addition was an extension of the peptide sequence to add an eight amino acid hydrophobic domain to give the peptide WLPLPWGWCGKKKFKLKH-amide (W3–K5). All assemblies contained a 5.5-fold charge equivalence (peptide positive charge/DNA negative charge) of compaction agent, unless otherwise specified.

Table 2B lists the types of zwitterionic phospholipids that were studied. These were four different hydrocarbon chain lengths (di12:0, di14:1, di16:1 and di18:1) of either phosphatidylcholine (PC) or PE. These phospholipids were added to increase the membrane destabilization capability of the delivery system within the endosomes and lysosomes of the cell. All complexes contained 5.0 nmol lipid/μg DNA, unless otherwise specified.

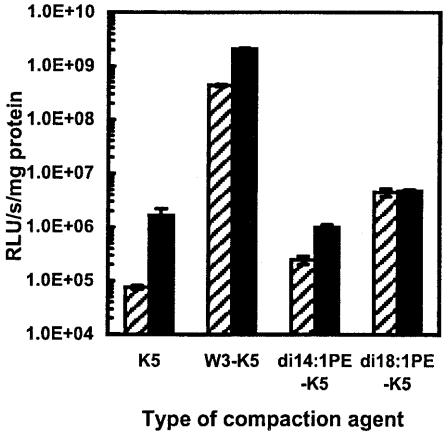


Figure 1. Luciferase gene expression of HeLa cells transfected with complexes containing different hydrophobic regions conjugated to the DNA compacting peptide, K5. Approximately 25 000 HeLa cells/well of a 24-well plate were cultured in MEM + 10% FBS overnight. Next, complexes containing a total of 5 μg DNA were added to the cells for a period of 24 h and the luciferase activity was measured in the cell lysates. Complexes contained a 5.5-fold charge equivalence (peptide positive charge/DNA phosphate negative charge) of the compaction agent. Abbreviations of the different compaction agents are described in Table 2. The solid bars indicate complexes containing 5 nmol/μg DNA of di14:1PE and the striped bars indicate complexes that do not contain di14:1PE. The values represent means (±SEM) of a triplicate experiment.

Figure 1 compares the luciferase activities of HeLa cells transfected with complexes containing the three different hydrophobic additions to the K5 peptide. Complexes containing the compaction agent with the hydrophobic peptide sequence (W3–K5) produced much greater luciferase activity than the complexes containing compaction agents with either no hydrophobic addition (K5) or a lipid as the hydrophobic addition (di14:1PE–K5 or di18:1PE–K5) (Fig. 1). Luciferase activity for cells transfected with complexes containing di14:1PE and W3–K5 was 2.1×10^9 RLU/s/mg protein. This value was 1245-, 2096- and 432-fold greater than the luciferase activity of cells transfected with complexes containing

Table 2. Experimental variations in the composition of the gene delivery system

A	Hydrophobic additions to the DNA compacting peptide CGKKKFKLKH (K5)	
	1	None (K5)
	2	di14:1PE–K5
	3	di18:1PE–K5
	4	H2N-Trp-Leu-Pro-Leu-Pro-Trp-Gly-Trp- (W3–K5)
	Complexes containing W3–K5 produced the highest level of reporter gene expression (Fig. 1)	
B	Zwitterionic phospholipids	
	1	None
	2	di12:0, di14:1, di16:1, di18:1 PE
	3	di12:0, di14:1, di16:1, di18:1 PC
	Complexes containing the di14:1PE produced the highest level of reporter gene expression (Fig. 2)	

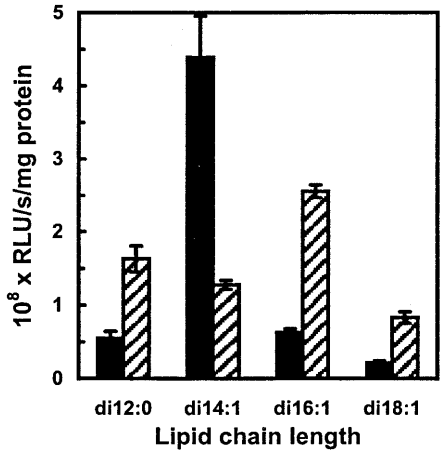


Figure 2. Luciferase gene expression of HeLa cells transfected with W3–K5 complexes containing different hydrocarbon chain lengths of PE or PC. Complexes were assembled with a 5.5-fold charge equivalence of W3–K5 and 5 nmol of the indicated lipid/μg DNA. Complexes containing a total of 5 μg DNA were added per well of a 24-well plate. Cell culture and transfections were performed as described in the Materials and Methods. Solid bars denote complexes containing PE and striped bars denote complexes containing PC. The values represent means (±SEM) of a triplicate experiment.

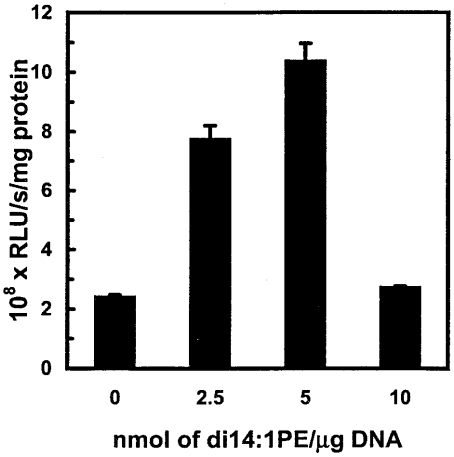


Figure 3. Optimization of the amount of dimyristoleoylphosphatidylethanolamine (di14:1PE) in the W3–K5 complexes. Complexes contained a 5.5-fold charge equivalence of W3–K5 and 0–10 nmol di14:1PE/μg DNA. Luciferase activity of HeLa cells transfected with complexes containing a total of 5 μg DNA was measured. Cell culture and transfections were performed as described in the Materials and Methods. The values represent means (±SEM) of a triplicate experiment.

di14:1PE and either K5, di14:1PE–K5 or di18:1PE–K5, respectively.

Figure 1 also shows the effect of the zwitterionic phospholipid di14:1PE on luciferase gene expression. When di14:1PE was excluded from the formulations (striped bars in Fig. 1) lower luciferase activities were observed for each type of compaction agent studied. In particular, the W3–K5 complexes containing di14:1PE produced a 4.9-fold higher luciferase activity compared to W3–K5 complexes not containing di14:1PE. We conclude that inclusion of the di14:1PE produced an additive effect in transfection efficiency by promoting membrane destabilization within the endosomes and lysosomes of the cell and allowing the complexes to escape into the cytoplasm.

Transfection efficiency of complexes prepared with different hydrocarbon chain lengths of PE or PC

Changing the type of phospholipid head group and the hydrocarbon chain length produced significant effects on transfection levels as measured by luciferase activity. Figure 2 shows the luciferase activity of HeLa cells transfected with complexes containing (i) W3–K5 as the compaction agent and (ii) different hydrocarbon chain lengths (di12:0, di14:1, di16:1, di18:1) of PE or PC. Complexes containing di14:1PE produced the highest transfection levels of all the phospholipids studied. The di14:1PE complexes produced a luciferase activity of 4.4×10^8 RLU/s/mg protein, which was 8.0-, 7.0- and 20.9-fold higher than di12:0, di16:1 and di18:1PE complexes, respectively. Among the PCs tested, di16:1PC produced the greatest amount of luciferase activity; 2.6×10^8 RLU/s/mg protein, relative to di12:0, di14:1 and di18:1PC which were 1.6-, 2.0- and 3.1-fold lower, respectively. These observations led to further optimization studies of the W3–K5 complexes containing di14:1PE.

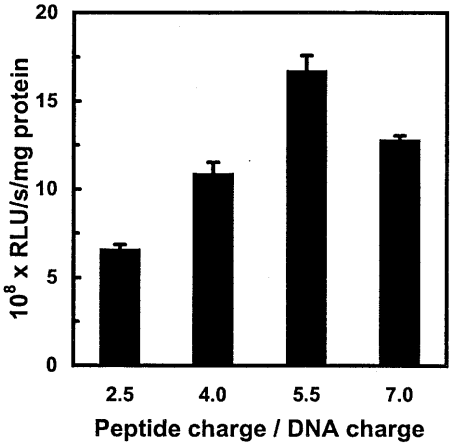


Figure 4. Optimization of the amount of W3–K5 compaction agent in the complexes. Complexes contained between 2.5- and 7.0-fold charge equivalence of W3–K5 and 5 nmol of di14:1PE/μg DNA. Luciferase activity of HeLa cells transfected with complexes containing a total of 5 μg DNA was measured. Cell culture and transfections were performed as described in the Materials and Methods. The values represent means (±SEM) of a triplicate experiment.

Optimization of complexes containing di14:1PE and W3–K5

Complexes containing W3–K5 were assembled with di14:1PE ranging from 0 to 10 nmol/μg DNA. HeLa cells were transfected with these formulations and the results are shown in Figure 3. Complexes assembled with 5 nmol of di14:1PE/μg DNA produced the highest luciferase activity (1.0×10^9 RLU/s/mg protein). Complexes assembled with 0, 2.5 and 10 nmol of di14:1PE/μg DNA resulted in luciferase activities that were 4.3-, 1.3- and 3.8-fold lower, respectively.

Using this optimal lipid concentration of 5 nmol of di14:1PE/μg DNA, the amount of W3–K5 was varied in the formulations. Figure 4 shows luciferase expression as a function of increasing charge equivalence of W3–K5 in the

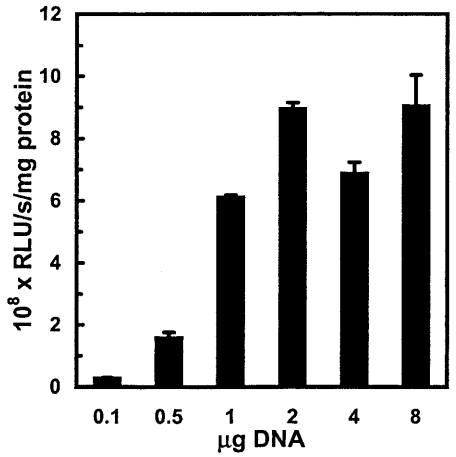


Figure 5. Optimization of the amount of W3–K5 complexes added to HeLa cells. Complexes contained the optimal formulation: a 5.5-fold charge equivalence of W3–K5 and 5 nmol di14:1PE/µg DNA. Luciferase activity of HeLa cells transfected with complexes containing a total of 0.1–8 µg DNA was measured. Cell culture and transfections were performed as described in the Materials and Methods. The values represent means (±SEM) of a triplicate experiment.

formulations. Complexes assembled with a 5.5-fold charge equivalence of W3–K5 positive charge exhibited the greatest luciferase activity, 1.7×10^9 RLU/s/mg protein, compared to complexes with 2.5-, 4.0- and 7.0-fold equivalencies which were 2.6-, 1.5- and 1.3-fold lower, respectively. Complexes with a 5.5-fold charge equivalence of W3–K5 were used for all further studies.

After establishing the optimal formulation (5.5-fold charge equivalence of W3–K5 and 5 nmol of di14:1PE/µg DNA), we added increasing amounts of the complexes (0.1–8 µg DNA) to HeLa cells and measured the luciferase activity (Fig. 5). Adding complexes containing 2.0 µg of DNA per well of a 24-well plate resulted in the highest amount of luciferase activity, 9.0×10^8 RLU/s/mg protein, relative to complexes containing 0.1, 0.5, 1.0, 4.0 and 8.0 µg of DNA, that were 30.0-, 5.6-, 1.5-, 1.3- and 1.0-fold lower, respectively.

Total protein did not differ significantly among the samples analyzed in Figures 3–5. This result indicated that the decline in luciferase activity at the higher lipid, peptide or DNA concentrations was not the result of cytotoxicity that lead to the loss of adherent cells.

Characterization of the optimal W3–K5/di14:1PE complexes

Sucrose density gradient centrifugation was used to determine the extent of di14:1PE association with the compacted plasmid DNA (Fig. 6A). To measure the phospholipid content in each fraction, 1 nmol of a BODIPY-labeled lipid was incorporated into the complexes during assembly. To measure the DNA content, Hoechst 33258, a DNA binding dye, was added to each fraction to give a final concentration of 200 pmol/ml. The optimal transfection complexes (5.5-fold charge equivalence of W3–K5 and 5 nmol of di14:1PE/mg of DNA) demonstrated complete association of fluorescent lipid (open circles) with the DNA (closed squares) in high density fractions (19–22) (Fig. 6A). Similar complexes differing only by the replacement of the W3–K5 peptide with one of the four different compaction agents (K5, di14:1PE–K5 and di18:1PE–K5) studied in Figure 1,

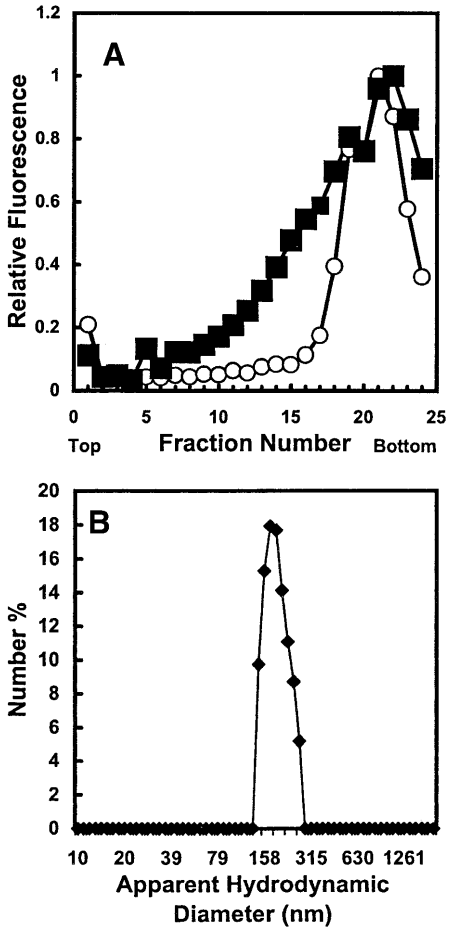


Figure 6. Characterization of the optimal formulation. (**A**) Sucrose density gradient analysis of complexes containing a 5.5-fold charge equivalence of W3–K5 and 5 nmol of di14:1PE/µg DNA. Sucrose gradient centrifugation was performed as described in the Materials and Methods. Lipid content (circles) was measured using a BODIPY-labeled lipid (λ_{ex} 490 nm, λ_{em} 520 nm) that was included during complex assembly. DNA content (squares) was measured by the addition of Hoechst 33258 (λ_{ex} 350 nm, λ_{em} 460 nm) to each fraction. (**B**) Dynamic light scattering was performed on the optimal formulation described above. A histogram of the apparent hydrodynamic diameters of the complexes is shown. The average diameter was determined to be 194 nm. Details of the instrument and analysis are given in the Materials and Methods.

also demonstrated association of fluorescent lipid with the DNA in high density fractions (data not shown). Free lipid was not detected in significant quantities in the lower density fractions for any of the complexes studied.

After dialysis, similar W3–K5 complexes containing di14:1PE were measured using dynamic light scattering as described in the Materials and Methods. The mean hydrodynamic diameter of these complexes was 194 nm and the histogram is shown in Figure 6B. Previous attempts to produce smaller complexes with mean hydrodynamic diameters between 50 and 100 nm resulted in a tremendous decline in transfection efficiency *in vitro* (data not shown).

Transfection efficiency in the presence of chloroquine

Chloroquine, a weak base that accumulates in acidic organelles such as late endosomes and lysosomes (defined as lysosomotropic), is known to enhance the transfection efficiency of

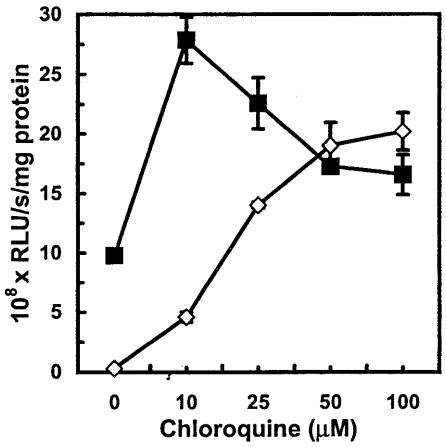


Figure 7. Luciferase gene expression of HeLa cells transfected with W3–K5 complexes containing either di14:1PE or di18:1PE in the presence of increasing amounts of chloroquine (0–100 μM). Complexes contained a 5.5-fold charge equivalence of W3–K5 and 5 nmol of lipid/μg DNA. HeLa cells were transfected with complexes containing a total of 2.5 μg DNA. Cell culture and transfection in the presence of chloroquine were performed as described in the Materials and Methods. Squares denote complexes containing di14:1PE and open diamonds denote complexes containing di18:1PE. The values represent means (±SEM) of a triplicate experiment.

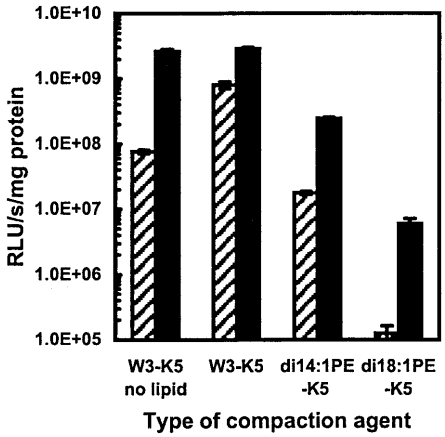


Figure 8. The effect of chloroquine on transfection efficiency for complexes containing the different compaction agents. HeLa cells were transfected with complexes containing a total of 2.5 μg DNA. W3–K5, di14:1PE–K5 and di18:1PE–K5 complexes contained a 5.5-fold charge equivalence of compaction agent and 5 nmol di14:1PE/μg DNA. The (W3–K5 no lipid) complexes also contained a 5.5-fold charge equivalence of W3–K5, but did not contain di14:1PE. Cell culture and transfection in the presence and absence of chloroquine were performed as described in the Materials and Methods. Striped bars denote transfection in the absence of chloroquine and solid bars denote transfection in the presence of 50 μM chloroquine. The values represent means (±SEM) of a triplicate experiment.

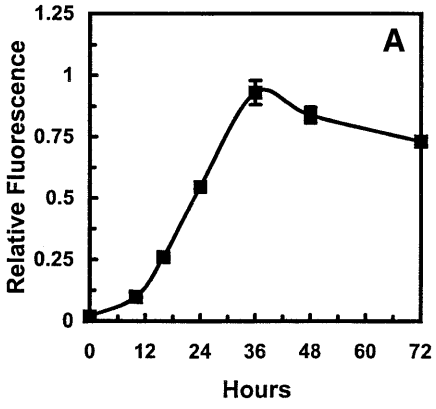
non-viral gene delivery systems. Figure 7 shows the luciferase activity of HeLa cells transfected with complexes containing (i) a 5.5-fold charge equivalence of W3–K5 and (ii) 5 nmol/μg DNA of either di14:1PE or di18:1PE, in the presence of increasing concentrations of chloroquine (0–100 μM). In the absence of chloroquine, complexes prepared with di14:1PE produced luciferase activities (9.8×10^8 RLU/s/mg protein) that were 33-fold higher than complexes prepared with di18:1PE (3.0×10^7 RLU/s/mg protein). These results indicated that in the absence of chloroquine, transfection efficiency was highly dependent upon phospholipid hydrocarbon chain length, with shorter chains providing much more fusogenic activity. In the presence of chloroquine (50 and 100 μM) the hydrocarbon chain length dependence was eliminated. Complexes containing either di14:1PE or di18:1PE produced luciferase activities in the range of $15–20 \times 10^8$ RLU/s/mg protein. These results suggested that in the presence of chloroquine, the uptake and cytoplasmic delivery of the complexes containing either di14:1PE or di18:1PE were similar. Whereas, in the absence of chloroquine, complexes containing di14:1PE produced superior gene delivery, apparently because of their ability to achieve more rapid endosomal escape.

Figure 8 shows a comparison of HeLa cell transfection in the presence and absence of 50 μM chloroquine using complexes composed of either W3–K5, di14:1PE–K5 or di18:1PE–K5. In the absence of chloroquine (striped bars), complexes containing 5.0 nmol of di14:1PE/μg DNA and a 5.5-fold charge equivalence of W3–K5, di14:1PE–K5 or di18:1PE–K5 produced luciferase activities of 8.0×10^8, 1.8×10^7 and 1.3×10^5 RLU/s/mg protein, respectively. In the presence of 50 μM chloroquine (solid bars), the same formulations produced luciferase activities of 28.7×10^8, 2.4×10^8 and 5.9×10^6 RLU/s/mg protein, respectively. The complexes containing W3–K5 and di14:1PE were more effective in the absence of chloroquine compared to complexes containing di14:1PE and either di14:1PE–K5 or di18:1PE–K5 even in the presence of 50 μM chloroquine. This result confirms that the combination of the di14:1PE and W3–K5 is the most favorable for gene delivery and that remarkably high transfection activity can be obtained without any need for agents that reduce lysosomal degradation.

Figure 8 also shows, in the absence of chloroquine, the complexes containing W3–K5 and di14:1PE (shown as W3–K5) produced a 10.5-fold increase in luciferase activity relative to complexes containing W3–K5 without di14:1PE (shown as W3–K5 no lipid). However, in the presence of 50 μM chloroquine, the complexes containing W3–K5 and di14:1PE produced only a 1.1-fold increase in luciferase activity relative to complexes containing W3–K5 without di14:1PE. These results show that di14:1PE is an effective endosomal/lysosomal membrane destabilizing agent when combined with the W3–K5 compaction agent.

Time course of gene expression

Cells were transfected with complexes containing GFP reporter gene and the total cellular GFP fluorescence was measured at time points between 0 and 72 h after the addition of complexes (Fig. 9A). The maximum GFP fluorescence was measured 36 h after the addition of the complexes composed of a 5.5-fold charge equivalence of W3–K5 and 5.0 nmol/μg DNA of di14:1PE. GFP expression was apparent after 3 h and a considerable number of lightly fluorescent cells were observed at 10 h (data not shown). Between 10 and 36 h there was a large increase in fluorescence followed by a slight decrease and plateau in fluorescence between 36 and 72 h. The percentage of transfected cells appeared similar between 16 and 36 h, but the intensity of fluorescence increased with time. Figure 9B shows a representative picture of the cells 30 h after the addition of the complexes. In Figure 9B, 50.3% of the cells



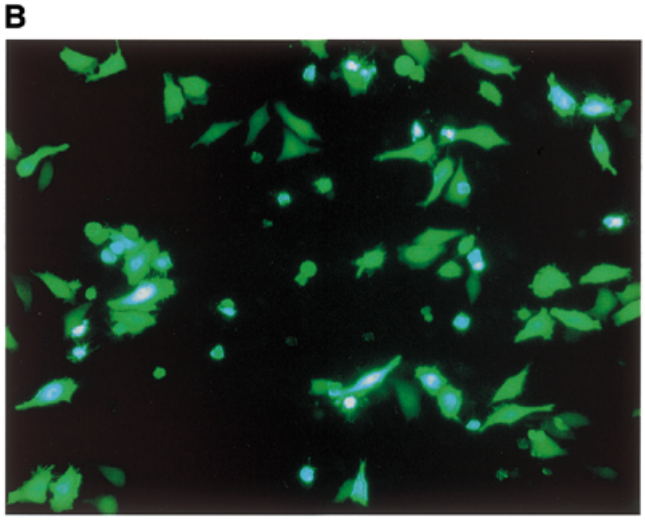


Figure 9. Time course of GFP expression in HeLa cells. HeLa cells were transfected with complexes containing a total of 4 µg DNA for between 0 and 72 h. Complexes contained a 5.5-fold charge equivalence of W3–K5 and 5 nmol of di14:1PE/µg DNA. Cell culture and transfection were performed as described in the Materials and Methods. (**A**) At the indicated time point, HeLa cells were scraped in PBS and fixed in 2% formaldehyde and the GFP fluorescence was measured using a fluorimeter as described in the Materials and Methods. The values represent means (±SEM) of a triplicate experiment. (**B**) Photograph of the GFP fluorescence in HeLa cells 30 h after the addition of the complexes [taken during the time course in (A)]. The photograph shown depicts the average transfection in a well of a 24-well plate. After comparing the fluorescence image shown with the same phase contrast image, 50.3% of the cells were calculated to be GFP positive.

were transfected as determined by comparing a picture of the cells in Figure 9B using phase contrast microscopy to the GFP fluorescence shown. The photograph in Figure 9B shows a region that represents the average transfection throughout the well. Regions close to the edge of the well transfected at higher efficiencies while regions in the center of the well produced slightly lower efficiencies.

Comparison of W3–K5 complexes with polyethylenimine and Lipofectamine™ complexes

In Figure 10, OVCAR-3, COS-7 and HeLa cells were transfected with complexes consisting of either (i) a 5-fold charge equivalence of PEI (hatched bars), (ii) 4 µg of lipofectamine/µg DNA (striped bars) or (iii) a 5.5-fold charge equivalence of W3–K5 and 5 nmol of di14:1PE/µg DNA (solid bars). Figure 10 demonstrates that the W3–K5/di14:1PE complexes transfect multiple cell lines effectively, at levels either comparable or superior to both PEI and Lipofectamine in the three cell lines studied.

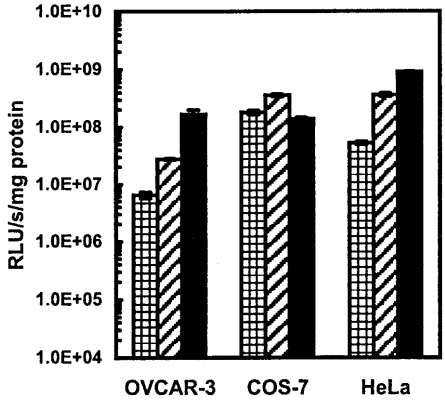


Figure 10. A comparison of transfection with the optimal formulation to transfection with PEI and Lipofectamine in multiple cell lines. W3–K5 complexes contained a 5.5-fold charge equivalence of W3–K5 and 5 nmol di14:1PE/µg DNA (solid bars). Luciferase activity of OVCAR-3, COS-7 and HeLa cells transfected with complexes containing a total of 2 µg DNA was measured. The same three cell lines were transfected simultaneously with complexes consisting of DNA and either a 5-fold charge equivalence of PEI (hatched bars) or 4 µg Lipofectamine/µg of DNA (striped bars). Luciferase activity of the three cell lines transfected with either PEI complexes containing 2 µg DNA or Lipofectamine complexes containing 1 µg DNA was measured. The values represent means (±SEM) of a triplicate experiment.

DISCUSSION

A substantial barrier to gene delivery is the degradation of complexes within the lysosomes of the cell. Non-viral gene delivery systems must effectively escape from either endosomes or lysosomes by disrupting the membranes of these compartments. In this work, the main goal was to develop a gene delivery system that was highly effective in escaping from endosomes or lysosomes. Such a system would produce high transfection efficiency and would be minimally sensitive to pre-treatment of cells with chloroquine.

For this study, the two components present in every formulation were (i) the plasmid DNA and (ii) the cationic peptide sequence CWKKKFKLKH-amide (K5). When added to HeLa cells, this combination alone produced very low gene expression, $<1 \times 10^5$ RLU/s/mg protein after 24 h. Slightly higher levels of expression, in the order of 10^6 RLU/s/mg protein, were achieved when the cationic peptide sequence was conjugated to either di14:1PE or di18:1PE. However, these transfection efficiencies were quite low and therefore disappointing.

The highest levels of gene expression were achieved when K5 was conjugated to the hydrophobic amino acid sequence WLPLPWGW (W3). In our experiments, the presence of this hydrophobic sequence was the single most effective component that increased gene expression to high levels. Without these eight amino acids, levels of luciferase gene expression were in the range of 10^6 RLU/s/mg protein. With them, levels of gene expression exceeded 10^9 RLU/s/mg protein (>3 µg luciferase/mg protein).

One important function of the W3 hydrophobic region is to interact with the zwitterionic lipid and drive complex formation via hydrophobic interactions. The W3 sequence was designed to minimize aggregation in solution while providing enough hydrophobicity for assembly of the complex. Based on the study by Wimley *et al*. (26), every amino acid in the W3 sequence, with the exception of glycine, has a positive solvation free energy when partitioning from *n*-octanol to buffer at pH 9. Furthermore, using the scale described in Wimley *et al*. (26), tryptophan has the largest solvation free energy and leucine has the third largest solvation free energy relative to the other 20 amino acids. Five of the eight amino acids in the W3 sequence are either leucine or tryptophan, providing considerable hydrophobicity within such a short sequence. The two prolines in the interior of the W3 sequence were included to minimize hydrogen bonding and deter formation of secondary structure. In preliminary studies, we found that adding six leucines to K5 instead of the W3 sequence addition resulted in peptide aggregation and extremely low transfection efficiency (E.A.Murphy and K.J.Longmuir, unpublished results). Such a large difference in transfection efficiency between polyleucine-K5 and W3–K5 demonstrates that the W3 sequence has a second function, in addition to interacting with phospholipid to promote complex formation.

We suggest that this second important function of the W3 sequence is to increase the membrane destabilizing capability of the gene delivery complex, allowing it to escape from endosomal/lysosomal compartments. There are numerous examples of peptides that lead to membrane destabilization and fusion. Fusogenic peptides used in gene delivery systems are generally 15–30 residues long and contain hydrophobic and hydrophilic amino acids that, when arranged as an α-helix, exhibit a hydrophobic moment characteristic of an amphipathic helix (reviewed in 27). In comparison, the eight amino acid sequence we have designed is remarkably short, consists entirely of hydrophobic amino acids and with the inclusion of two prolines, would not be expected to form a helical structure.

Inclusion of PE rather than PC resulted in complexes that gave the highest levels of gene expression. PE is considered a more fusogenic lipid for several reasons (reviewed in 28). The PE polar head group is more hydrophobic and less hydrated, allowing for two opposing lipid surfaces to closely approach one another, a necessary step in membrane fusion. PE can form lipid surfaces with high negative curvature, a configuration believed to be important for facilitating membrane fusion. PE can also proceed through the transition from the liquid-crystalline lamellar (L_α) phase to the hexagonal (H_{II}) phase in excess water and at physiologic pH and temperature, a process associated with fusogenicity. In this regard, it is surprising that di14:1PE gave the highest levels of gene expression and is apparently the most fusogenic. The temperatures of the L_α to H_{II} transition for di18:1(*cis*-9)PE and di16:1(*cis*-9)PE are 10 and 42°C, respectively (29). The L_α to H_{II} transition temperature of 14:1(*cis*-9)PE has not been reported, but should be greater than 42°C, as this transition temperature increases with decreasing acyl chain length. However, the H_{II} phase itself is a stable configuration and not considered fusogenic (28). Instead, di14:1PE may transiently sample non-bilayer intermediate states between L_α and H_{II} which lead to fusion.

It is also possible that the hydrophobic sequence of the compacting peptide (WLPLPWGW) destabilizes a stable (L_α) arrangement of di14:1PE more than it can destabilize assemblies of longer chain PEs in either the L_α or the H_{II} phase. If so, di14:1PE can be the most fusion-promoting of the PEs only when it functions together with the hydrophobic peptide. At present, we have no direct measurements of the extent of destabilization of PEs of various chain lengths by the peptide. However, given the remarkably high levels of gene expression achieved with this synergistic combination of hydrophobic peptide sequence and a relatively short-chain PE, biophysical studies of this peptide/phospholipid fusogenic system will be of great interest for the further development and improvement of non-viral gene delivery systems.

Several studies of non-viral gene delivery systems which rely on receptor-mediated endocytosis have included chloroquine during transfection to greatly improve gene delivery (17,30,31). Chloroquine is a weak base that has been shown to accumulate in lysosomes and to raise the intralysosomal pH (32–34). The accumulation of chloroquine within lysosomes also reduces degradation of macromolecules by decreasing the activity of hydrolytic enzymes (35). Addition of chloroquine produces a substantial increase in the transfection efficiency of DNA/lactosylated polylysine complexes (36). Surprisingly, the weak bases ammonium chloride and methylamine and the Na^+/H^+ ionophore monensin, which are known to raise the intralysosomal pH, do not produce an increase in the transfection efficiency of these complexes (36). Therefore, chloroquine must have another function in addition to raising the intralysosomal pH.

Two other possible functions of chloroquine are (i) inhibition of endosome/lysosome fusion and (ii) destabilization of the endosomal membrane. The endosome/lysosome fusion process might require the low pH normally found in endocytic vesicles. Two molecules that lower the pH of the endosomal/lysosomal lumen, chloroquine and the Na^+/H^+ ionophore monensin, were found to inhibit the transfer of immunoglobulin-coated colloidal gold particles or ferritin from endosomes to lysosomes in cultured mouse peritoneal macrophages (37,38). The endosomes within these macrophages were still intact suggesting that chloroquine inhibited endosome/lysosome fusion. The inhibition of endosomal/lysosomal fusion would allow for gene delivery complexes to reside in the endosomes for longer periods of time, and likely increase the probability of endosomal membrane destabilization. A study by Zhou and Huang (39) analyzed electron micrographs of L929 cells transfected with lipopoly(L-lysine)/dioleoylphosphatidylethanolamine complexes and found that chloroquine treatment caused 57% of the observed endosomes to become destabilized, while only 15% of the observed endosomes were destabilized in the absence of chloroquine (39). This work showed that chloroquine increases the rate of endosomal escape by enabling membrane destabilization within endosomes. Therefore, the addition of chloroquine would greatly improve the transfection efficiency of gene delivery systems that are not fusogenic.

However, if a gene delivery system has the ability to effectively escape from endosomes, the effect of chloroquine on transfection efficiency should be minimal. For complexes containing W3–K5 and di14:1PE, the maximum chloroquine effect produced a luciferase activity only 2.8-fold higher than the same complexes in the absence of chloroquine (Fig. 7). Whereas, for complexes containing W3–K5 and di18:1PE instead of di14:1PE, the

maximum chloroquine effect produced a luciferase activity that was 66-fold higher than the same complexes in the absence of chloroquine. Additionally, the complexes containing W3–K5 and di14:1PE showed only a slight increase in transfection efficiency in the presence of chloroquine when compared to the large increases in transfection efficiency when complexes containing either di14:1PE–K5 or di18:1PE–K5 and di14:1PE were studied in the presence of chloroquine (Fig. 8). These results show that the complexes containing W3–K5 and di14:1PE produced the lowest chloroquine effect and therefore must be effective at escaping from endosomes.

Another possible contribution to the high transfection efficiency is the potential of the K5 sequence to act as a nuclear localization signal (NLS). The K5 sequence, used for compacting the DNA, was based on the putative NLS of the HIV-1 matrix protein (GKKKYKLKH) (40). However, experiments validating the role of this sequence as an NLS are controversial. Several studies have demonstrated that this sequence cannot enable nuclear import when conjugated to macromolecules which do not passively diffuse through nuclear pores (41,42). Other studies have found that the matrix NLS requires the HIV-1 viral protein R NLS for nuclear import of the HIV-1 pre-integration complex (43). At present, we have no data that indicate the K5 sequence enhances nuclear import of the gene delivery complexes. Further studies comparing K5 with other known classical NLS motifs should be performed to determine the role of K5 in nuclear import.

Although the gene delivery system reported here effectively transfects cells *in vitro*, further work is necessary to better understand and overcome the cellular and non-cellular barriers to gene delivery. This gene delivery system allows for the potential inclusion of other components designed to overcome a specific barrier, provided that they have a hydrophobic domain that allows for assembly into the complex. For instance, these components might include polyethyleneglycol for steric stabilization in the extracellular environment, targeting ligands for improved cellular uptake and NLS peptides for improved nuclear import. Clearly, non-viral gene delivery systems remain challenged at the cellular level and must be further developed to increase the efficiency of the entire delivery process within cells.

ACKNOWLEDGEMENTS

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