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Design, Synthesis, and Characterization of a Cationic Peptide That Binds to Nucleic Acids and Permeabilizes Bilayers[†]

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ABSTRACT: We have designed a cationic amphipathic peptide, KALA (WEAKLAKALAKALA-KHLAKALAKALKACEA), that binds to DNA, destabilizes membranes, and mediates DNA transfection. KALA undergoes a pH-dependent random coil to amphipathic α-helical conformational change as the pH is increased from 5.0 to 7.5. One face displays hydrophobic leucine residues, and the opposite face displays hydrophilic lysine residues. KALA-mediated release of entrapped aqueous contents from neutral and negatively charged liposomes increases with increasing helical content. KALA binds to oligonucleotides or plasmid DNA and retards their migration in gel electrophoresis. It displaces 50% of ethidium bromide from DNA at a charge ratio (+/−) of 0.9/1. In cultured cells, KALA assists oligonucleotide nuclear delivery when complexes are prepared at a 10/1 (+/−) charge ratio. KALA/DNA (10/1) (+/−) complexes mediate transfection of a variety of cell lines. The KALA sequence provides a starting point for a family of peptides that incorporate other functions to improve DNA delivery systems.

Viruses have evolved sophisticated and specific mechanisms for cell attachment, penetration, and genome replication. The relationship between structure and function of proteins responsible for these activities has been advanced by studies with synthetic peptides that mimic recognition, binding, or membrane disruption aspects of the viral proteins (Epand, 1993). The lessons learned from these biochemical studies are now being applied to create nonviral DNA delivery systems that mimic the advantageous aspects of viral transfection with greatly simplified components. Gene expression has been shown for nonviral delivery systems where complexes are formed between DNA and cationic liposomes (Gao & Huang, 1995), peptides such as polylysine (Wu & Wu, 1987), gramicidin S/lipid complexes (Legendre & Szoka, 1993), dendritic and linear/branched polymers (Haensler & Szoka, 1993; Boussif et al., 1995), as well as multimolecular DNA/cation complexes incorporating ligands capable of receptor-mediated endocytosis (Wu & Wu, 1988a,b; Wagner et al., 1991).

The ideal nonviral delivery systems should protect the DNA from degradation, target the cell of choice, introduce the DNA into the cytoplasm, and direct DNA to the host cell nucleus. The components must display a delicate balance between associating with the DNA to form a soluble complex and retaining the ability to interact with membranes and dissociate from the DNA in the target cell (Xu & Szoka, 1996; Zelphati & Szoka, 1996). Most approaches to cytoplasmic delivery use the endosomal pathway and then disrupt this acidic compartment and introduce DNA into the cytoplasm prior to fusion of the endosome with the lysosome (Wagner et al., 1992a,b; Behr, 1994; Gao & Huang, 1995). One multicomponent approach employs polylysine to protect DNA by compacting it, transferrin as a receptor ligand to attach and introduce the complex into the cell via receptor-mediated endocytosis, and adenoviral particles to disrupt the endosome (Wagner et al., 1992a,b). Peptides which mimic the endosomal disruptive properties of viral particles have been substituted for the adenovirus; for example, the influenza hemagglutinin peptide undergoes a change in structure upon acidification and causes membrane disruption. When the influenza peptide is included with complexes containing transferrin/polylysine/DNA, gene delivery is significantly enhanced (Wagner et al., 1992a,b; Plank et al., 1994; Gottschalk et al., 1996). Haensler and Szoka showed that, when GALA, a synthetic peptide designed to undergo a pH-dependent conformational change which creates a structure that is capable of penetrating liposomal bilayer membranes causing release of entrapped contents (Parente et al., 1988, 1990a,b), is coupled to a dendrimer, a 10−100-fold increase in gene delivery over the dendrimer alone was observed (Haensler & Szoka, 1993). Thus, no single-component system using a designed synthetic peptide has been reported which can promote efficient transfection.

We have designed a low-molecular weight cationic peptide, designated KALA, which by itself mediates nucleic acid delivery and transfection. KALA is an amphipathic peptide when in the α-helical conformation; one face displays hydrophobic leucine residues, while the opposite face displays hydrophilic lysine residues. Herein, we evaluate the ability of KALA to bind and compact DNA, cause membrane disruption, and mediate transfection of a variety of cell lines. KALA provides a low-molecular weight alternative, on the basis of membrane-disrupting peptides, to the high-molecular weight polymer complexes currently employed for DNA transfection.

EXPERIMENTAL PROCEDURES

Solvents for the HPLC were obtained from Fisher Scientific (Springfield, NJ). Lipids were obtained from Avanti

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Polar Lipids Inc. (Birmingham, AL). 8-Aminonaphthalene-1,2,3-trisulfonic acid (ANTS),[1] *p*-xylenebis(pyridinium) bromide (DPX), and SYBR Green I were obtained from Molecular Probes (Eugene, OR). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), (hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes), and polylysine-100 were obtained from Sigma. The sixth-generation fractured poly(amidoamine) dendrimer was provided by Carl Redeman and Mary Tang from Frank Szoka's lab. Phosphate-buffered saline (PBS) containing 0.1 g/L $CaCl_2$, 0.1 g/L $MgCl_2 \cdot 6H_2O$, 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 8 g/L NaCl, and 2.16 g/L $Na_2HPO_4 \cdot 7H_2O$ was provided by the University of California, San Francisco (UCSF), cell culture facility. The protected amino acids and resins for the synthesis of the peptide were obtained from Applied Biosystems (ABI) (Foster City, CA) or Bachem (La Jolla, CA).

Peptide Synthesis and Purification

The KALA peptide (WEAKLAKALAKALAKHLAKALAKALKACEA) was synthesized using an Applied Biosystems 430A peptide synthesizer utilizing Fast-moc chemistry. The peptide was cleaved from the resin with trifluoroacetic acid (TFA) in the presence of phenol, ethanedithiol (EDT), and thioanisole. The free peptide was separated from the resin by filtration followed by precipitation with ether. The KALA white solid was taken up in 20 mg/mL water and chromatographed on a Sephadex G-10 column in 0.1% TFA in HPLC grade water. The resulting lyophilized white powder was again dissolved in water (10 mg/mL) and purified by reverse-phase HPLC on a Rainin Dynamax C_{18} column using the following conditions: solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile; gradient, 0 to 40% B in 10 min and 40 to 60% B in 20 min at a flow rate 9 mL/min; and eluant monitored at 215 and 280 nm. KALA eluted at 52% B. Mass spectroscopy of the purified KALA on a Platform 2 V. G. Co. electrospray mass spectrometer yielded the molecular ion of the peptide at mass unit 3132 which corresponds to the protonated peptide (mass unit = 3131).

Circular Dichroic Spectra

Circular dichroic spectra were recorded on a Jasco-J500A spectropolarimeter which was routinely calibrated using 10-camphorsulfonic acid-*d*. The sample temperature was controlled by an external circulating water bath. Spectra recorded at a scan rate of 20 nm/min with a time constant of 1 s were signal averaged over four scans. Molar ellipticities (per residue) were calculated using the equation $[\Theta] = 100(\Theta)/(lcN)$, where $[\Theta]$ is the molar ellipticity per residue, (Θ) is the observed ellipticity in degrees, l is the optical path length in centimeters, c is the molar concentration of the peptide, and N is the number of residues in the peptide. The α-helical content of the samples at various pHs was determined using the equation $[\Theta]_{222} = (f_H - ik/N)[\Theta]_{H\infty}$, where $[\Theta]_{222}$ is the molar ellipticity at 222 nm, f_H is the fraction of the peptide in the helical conformation, i is the number of helical segments per peptide (taken as 1 in this case), k is a wavelength-dependent constant (2.57 at 222 nm), N is the number of residues in the peptide, and $[\Theta]_{H\infty}$ is the molar ellipticity at 222 nm for a helix of infinite length (-39500 deg cm^2 dmol^{-1}) (Chen et al., 1974; Chang et al., 1978; McLeish et al., 1994; Fan & Mayo, 1995). The spectra were recorded at 20 °C using a cell with an optical path length of 2 mm, and all the samples contained 150 mM NaCl.

Leakage Assays

Reverse-phase evaporation vesicles (REVs) were prepared as described previously (Szoka & Papahadjopoulos, 1978; Subbarao et al., 1987; Parente et al., 1988, 1990a,b) in 5 mM TES, 12.5 mM ANTS, 45 mM DPX, and 20 mM KCl at pH 7.0 and extruded through a 0.1 mm polycarbonate membrane (Nucleopore Corp., Pleasanton, CA) through a hand-held extrusion device (Avestin, Ottawa, CA). A Sephadex G-75 column (1 × 20 cm) was used to separate vesicles from unencapsulated material with an elution buffer of 5 mM TES and 100 mM KCl at pH 7.0. Lipid phosphorus was determined by a modification of the Bartlett (1959) method. The ANTS/DPX assay (Ellens et al., 1984) was used to monitor KALA-induced leakage of encapsulated ANTS from liposomes. The fluorescence signal resulting from the dequenching of ANTS released into the medium was observed through a Schott GG 435 nm cutoff filter (50% transmittance at $\lambda = 435$ nm) while samples were irradiated at $\lambda = 360$ nm. Fluorescence intensity from intact vesicles in buffer, F_0, was set to 0% leakage. To begin an experiment, the peptide KALA in 5 mM TES at pH 7.5 was added to the stirred vesicle suspensions (0.1 mM lipid) at 20 °C in the appropriate buffer. Reaction buffers consisted of 10 mM sodium acetate, 100 mM KCl (pH 4.5 or 5.0), 10 mM MES, 100 mM KCl (pH 5.5, 6.0, or 6.5), 10 mM TES, 100 mM KCl (pH 7.0 or 7.5) or 10 mM TRIS, and 100 mM KCl (pH 8.5). Data points were recorded at 1 s intervals. The plateau in fluorescence intensity, F, was obtained in less than 30 min. Then, vesicles were lysed with the detergent dodecyloctaethylene glycol monoether ($C_{12}E_8$) (Calbochiem, La Jolla, CA) to obtain the maximal fluorescence intensity, F_{100}, corresponding to 100% ANTS leakage. The leakage extent of ANTS due to the interaction of KALA with liposomes was determined by the following formula: percent leakage $= (F - F_0)/(F_{100} - F_0)$. In the KALA/oligonucleotide complex leakage investigations, a constant amount of peptide was mixed with various amounts of the 27-mer oligonucleotide in HBS at pH 7.4 30 min prior to addition to the liposomal suspension. Corresponding charge ratios of KALA/oligonucleotide (+/−) were determined with 5 net positive charges for KALA and 26 negative charges for the 27-mer oligonucleotide. All the leakage experiments were performed in triplicate. λ_{max}, the wavelength that corresponds to the maximum intensity of the tryptophan fluorescence emission spectrum, was monitored when the sample was excited at $\lambda = 280$ nm. We used a constant peptide concentration of 2.5 μM and increasing amounts of lipids.

Expression Vectors and Oligonucleotides

The plasmid pCMVLuc coding for firefly luciferase was generously provided by K. Hong. Oligonucleotides used in

[1] Abbreviations: ODN, oligonucleotide; F-ODN, fluorescein-labeled oligonucleotide; CD, circular dichroism; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ANTS, 8-aminonaphthalene-1,2,3-trisulfonic acid; DPX, *p*-xylenebis(pyridinium) bromide; Hepes, (hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid; HBS, hepes-buffered saline.

these studies were provided by G. Zon of Lynx Therapeutics Inc. (Foster City, CA). A phosphorothioate antisense anti-rev 28-mer (5′-TCGTCGCTGTCTCCGCTTCTTCCTGCCA-3′) and an anti-murine β-actin 25-mer (5′-TCTGGGT-CATCTTTTCACGGTTGGC-3′) labeled at the 5′ end with fluorescein (F-ODN) were synthesized and purified by a method previously reported (Zon & Geiser, 1991). A mixed phophodiester (underlined in sequence) and phosphorothioate 27-mer (5′-CTCCTTGGGTCCTGCTCAACAGTATGA-3′) used in the leakage experiments was prepared by C. Huang at Glaxo.

Gel Retardation Assay

Agarose Gel. The preparation of the DNA/KALA peptide complexes was carried out as follows. Plasmid DNA (pCMVLuc) was diluted in HBS to 30 μg/mL; 20 μL of this solution was mixed with 20 μL of each of the peptide dilutions in HBS to result in the desired +/− charge ratios (1−16-fold). After 30 min of incubation at room temperature, 20 μL of the samples (corresponding to 0.3 μg of DNA) was electrophoresed through a 1% agarose gel containing 0.5 μg/mL ethidium bromide to visualize DNA using Tris acetate/EDTA buffer (pH 8.0).

PAGE. The preparation of the ODN/KALA peptide complexes in water was carried out in 96-well plates as follows. ODN (1 μg) was added to increasing amounts of peptide to result in +/− charge ratios ranging from 0.3 to 20. The mixture was allowed to stand at room temperature for 30 min; then 30% glycerol (10 μL) was added and the mixture loaded on a 20% nondenaturing polyacrylamide gel which was run at 150 V for 4 h. ODNs were detected by staining the gel with SYBR Green I.

Ethidium Bromide Displacement Assay

One and one-half milliliters of 20 mM HBS buffer (pH 7.4) containing 15 μg of plasmid DNA and ethidium bromide at a 1/29 molar ratio (ethidium bromide/DNA phosphates) was mixed with 1.5 mL of HBS solution containing various amounts of KALA, corresponding to charge ratios (+/−) ranging from 0 to 5. The fluorescence (excitation, 510 nm; emission, 595 nm) was monitored at 20 °C as the ethidium bromide was displaced from the DNA by KALA. The fluorescence for free ethidium bromide was subtracted from all measurements to give the values for the decrease of the fluorescence of the ethidium bromide/DNA complex alone. The fluorescence values are calculated as a percentage of the maximum (fluorescence of the ethidium/DNA complex alone).

Cells and Cell Culture Reagents

The adherent cell lines CV-1 (monkey kidney fibroblast), Hep G2 (human hepatoma), and C2C12 (murine muscle), the suspension cell line K562 (human erythroleukemia) provided by the UCSF cell culture facility, and the CaCo2 (colon cancer) cells obtained from American Type Culture Collection (Rockville, MD) were cultivated at 37 °C in a humidified atmosphere containing 5% CO_2. CV-1 cells and C2C12 myoblasts were plated at a density of 2×10^4 cells/well 24 h prior to transfection and Hep G2 and K562 at 5×10^4 cells/well in a 96-well plate. CV-1 cells were cultivated in DME-H21 and Hep G2 cells in MEM both supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units/mL penicillin and 100 units/mL streptomycin). Hep G2 cells were grown to confluency in ∼10 days. C2C12 myoblasts were cultivated in DME-H21 supplemented with 20% FBS and antibiotics. C2C12 Myotubes were grown (∼10 days) from differentiated myoblasts in 5% horse serum. CaCo2 cells were grown on collagen-coated 12 mm transwell polycarbonate cell inserts plated at a density of 15×10^4 cells/well, fed every other day for the first week and then every day thereafter with DMEM media with high glucose supplemented with 10 mM Hepes, glutamine (2 mM), nonessential amino acids (1X's), antibiotics (100 units/mL penicillin G and 100 μg/mL streptomycin), and 10% heat-inactivated fetal calf serum. Experiments were performed between 24 and 30 days postseeding. The K562 cells were cultivated in RPMI 1640 containing 10% FBS and antibiotics. One day prior to plating K562 cells for transfection, deferoxamine mesylate was added to a final concentration of 50 μM to up-regulate the transferrin receptor and cells were cultured for 24 h prior to transfection. Primary bovine airway epithelial cells were obtained from J. Widdicome (UCSF), plated at a density of 30×10^4 cells per 12 mm transwell culture plate, and cultivated for 14 days in DME-H21/F12 (50/50) containing antibiotics, fungizone, gentamicin, and growth factors [insulin (10 μg/mL), transferrin (5 μg/mL), triiodothyronine (20 ng/mL), hydrocortisone (0.36 μg/mL), endothelial cell growth supplement (7.5 μg/mL), and epidermal growth factor (25 ng/mL)].

KALA Peptide/Oligonucleotide Complex Trafficking and Toxicity Assays

For the preparation of complexes in water, 0.2 μg of fluorescently labeled oligonucleotides was added to different quantities of peptide as a function of +/− charge ratio (0.2−20-fold) in a 50 μL total volume in 96-well plates. The charge ratio was calculated as 25-mer ODN having 24 negative charges and KALA peptide having 5 positive charges per molecule. The mixtures were allowed to stand at room temperature for 30 min, and then 150 μL of serum free DME-H21 medium was added to the complexes which were then transferred to the CV-1 cells. This resulted in a 160 nM ODN concentration, and the KALA concentration, which varied as a function of the charge ratio, resulted in concentrations between 0.15 and 15.5 μM.

For the confocal microscopy experiments, F-ODN alone or complexed with KALA was incubated in DME-H21 medium without serum on CV-1 cells growing on coverslips. Cells were then incubated at 37 °C for 3 or 24 h. Then, CV-1 cells present on coverslips were washed three or four times in PBS, and coverslips were immediately mounted on hanging drop slides (Fisher Scientific) with a drop of PBS. Finally, the cells were directly observed by confocal microscopy.

In the cytotoxicity assay, CV-1 cells were plated in 96-well plates at 5×10^4 cells/well. The complexes were prepared in water as follows; the ODN and KALA were 2-fold serially diluted with water into two separate plates. Equal volumes of the serially diluted ODN and KALA were mixed to form complexes (total volume of 50 μL). Then, 150 μL of serum free medium was added to each well, and complexes were transferred onto the cells. After 3 h, serum was added to each well (10% final). The resulting varying

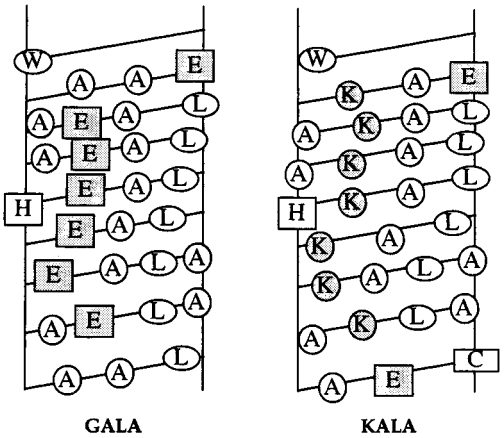

FIGURE 1: Helical grid representation of KALA and GALA.

concentrations of ODN (28-mer) alone (54−865 nM) or complexed with KALA (at +/− charge ratios ranging from 1.25 to 40, 0.36−186 μM KALA) were incubated with cultured cells for 2 days at 37 °C. The viability of cells was tested by a colorimetric MTT assay as described previously (Mosmann, 1983).

Transfection Protocol

In a typical 96-well plate experiment, cells were transfected in 150 μL of medium containing serum (serum free for CV-1, opti-MEM I for C2C12) by addition of 50 μL of HBS containing 1.2 μg of plasmid DNA encoded for the firefly luciferase reporter gene complexed with the desired amount of KALA. The KALA peptide 2-fold serial dilution was prepared in water in one 96-well plate (total volume of 25 μL) and then added to the 96-well plate containing 1.2 μg of DNA in 25 μL of HBS per well. The DNA/KALA complexes were incubated for 15 min at room temperature followed by addition to cells. After 5 h of incubation, the medium was removed and replaced by fresh medium containing serum. The cells were cultured for an additional 48 h and assayed for luciferase expression. Total protein per well was measured using the Pierce BCA protein assay. For CaCo2 and airway epithelial cells, 2.4 μg of plasmid DNA complexed to KALA (100 μL of HBS) was used in a total volume of 500 μL.

Confocal Laser Scanning Microscopy

A MRC-600 (Biorad) confocal laser scanning imaging system with a krypton/argon mixed gas laser and equipped with an upright microscope (Nikon, NY) was used for these experiments. A 60× oil immersion objective (Nikon) was used to observe cells and take photographs. COMOS confocal software program was employed to control the confocal module's functions. Images were stored on a Panasonic 7010-1 1GB rewritable optical disk (CDW Electronics).

RESULTS

Peptide Synthesis and Purification. KALA, its sequence shown in Figure 1, has lysine-alanine-leucine-alanine as the repeating motif designed to create a hydrophobic and a hydrophilic face. The hydrophilic lysine residues were chosen to bind DNA, while the glutamic acid residues were positioned at either end of the peptide to provide increased water solubility at physiological pH or when the peptide was

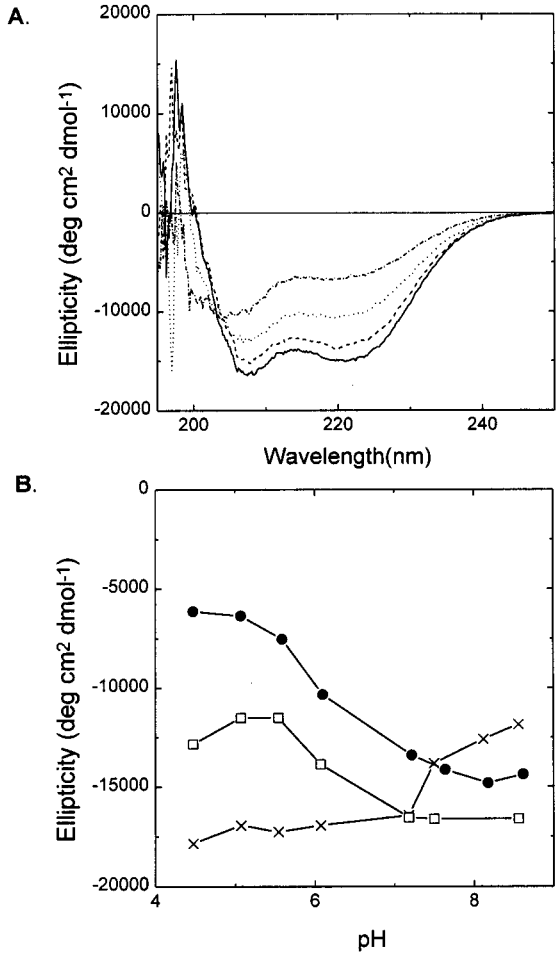

FIGURE 2: CD spectra of KALA in the presence of 150 mM NaCl at various pHs. (A) CD Spectra of KALA at pH 8.18 (solid line), 7.22 (dashed line), 6.10 (dotted line), and 5.10 (dotted and dashed line). (B) Variation of molar ellipticity of KALA at 222 nm with pH (closed circle), KALA in the presence of POPC liposomes (open squares, lipid/peptide ratio = 200), and KALA in the presence of 3/1 POPC/POPG liposomes (times signs, lipid/peptide ratio = 200).

associated with the nucleic acid but would protonate in the endosome. Following cleavage from the resin and separation on a Sephadex G-10 column, one main peak was observed on a reverse-phase C_{18} preparative HPLC column at 52% acetonitrile/0.1% TFA in water when monitored at 215 and 280 nm. Tryptophan (absorbance maximum of 280 nm), which is a sensitive indicator of the polarity of the peptide's environment responding with emission shifts or quantum yield changes, was included at the peptide's N terminus as a spectroscopic probe. When a small amount of this material was injected on an analytical column, only one peak was observed. The mass spectral data agree with the expected molecular weight of 3131.

α-Helicity and Membrane Destabilization. The solution conformation of KALA in the absence and presence of POPC or 3/1 POPC/POPG liposomes at various pHs was studied by CD spectroscopy. Figure 2A shows the CD spectra of the peptide alone at different pHs in 150 mM NaCl. The CD spectra of KALA in solution at a pH of greater than 6 show features characteristic of α-helical conformation with double minima centered around 222 and 207 nm followed by a positive band below 200 nm (Chang et al., 1978). The ϵ-amino groups of lysine residues are charged under these conditions. Figure 2B shows the variation of the ellipticity at 222 nm with pH. In the pH range of 7−9, the peptide

shows substantial α-helical content (43−45%). Lowering the pH below 7 resulted in the gradual decrease in the α-helical content up to pH 5 (24%), and reducing the pH further did not alter the spectrum. It is interesting to note that, in the physiologically relevant pH range, this peptide exists in the α-helical conformation in spite of the number of positive charges from the protonated lysine side chains.

The pH dependence of this peptide can be based on the ionization characteristics of the ϵ-amino groups of seven lysines, the imidazole of histidine, and γ-carboxyl groups of two glutamic acid residues. As the pH is lowered from 8, the α-helicity of the peptide is decreased (pH 8 to 4.5, 45 to 24%). With five net positive charges (+7 from lysine residues and −2 from glutamic acid residues), the peptide exists in the α-helical conformation. A decrease in the pH below 7 results in protonation of the histidine, charge neutralization of the glutamic acid side chains, and hence an increase in the net positive charge in the molecule which appears to disrupt the α-helix.

In the presence of lipid vesicles, the situation is different due to the binding of KALA to the liposomes and the aggregation of peptides in the membrane to form pores that cause leakage of encapsulated compounds, as described later. A comparison of the peptide conformation in the presence of POPC and POPC/POPG liposomes shows that the peptide behaves very differently depending on the liposome composition. With neutral POPC liposomes, the pH dependence of the conformation is similar to that observed in the absence of liposomes (Figure 2B), although an increase in the α-helical content compared to that for KALA alone is observed over the entire pH range studied. Previous reports have shown an increase in the α-helical content for peptides when in a more hydrophobic environment (Li & Deber, 1993, 1994; McLeish et al., 1994) which may be the explanation for the increased α-helical content seen with KALA in the presence of POPC liposomes. In the presence of POPC liposomes, KALA binding to the amphiphilic surface of the vesicle may favor the amphipathic α-helical conformation. In the presence of 3/1 POPC/POPG, where 25% of the lipids are negatively charged, the peptide has a relatively high α-helical content in the low pH range (pH 4.5 to 7.0, 54 to 50%). As the pH is increased above 7.0, the α-helical content is decreased (pH 8.5, 39%). In contrast, in the presence of neutral POPC liposomes, KALA has a high α-helical content at pH 8.6 (51%) and a low α-helical content at pH 4.5 (41%). In the case of negatively charged vesicles, below pH 6.0, the CD reflects interactions between the cationic peptide and the negatively charged POPG lipid which promotes helical content. Above pH 7.0, the negatively charged glutamates near the terminus impede peptide−POPG lipid interactions and the peptide becomes less helical.

The pH dependence of KALA-induced leakage of the fluorophore ANTS from neutral POPC and negatively charged 3/1 POPC/POPG liposomes is shown in Figure 3A,B. KALA-induced leakage from POPC neutral liposomes increases as the pH is raised from pH 4.5 to a maximum leakage at pH 7.5 (Figure 3A). This indicates that KALA destabilizes neutral membranes more efficiently when the pH is increased. These results can be correlated to the increase in the percentage of peptides in an α-helical conformation when the pH is raised. In contrast, with 3/1 POPC/POPG liposomes, the extent of leakage slightly decreases when the pH is increased from 4.5 to 8.5 (Figure 3B), following the same evolution as that of the α-helical content, as determined previously. At pH 7.5, the leakage efficiency of KALA with 3/1 POPC/POPG liposomes is slightly higher than that with POPC liposomes.

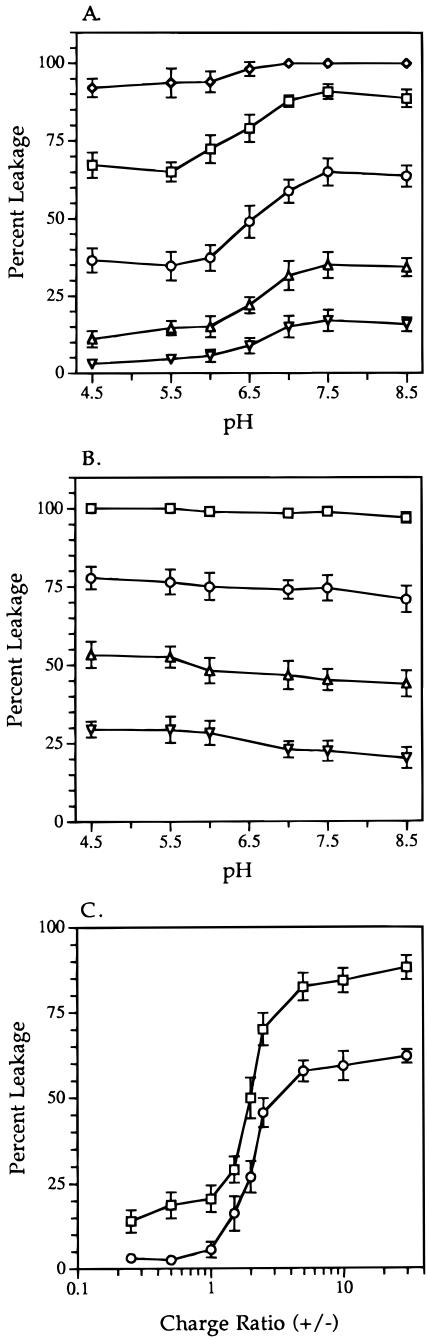

FIGURE 3: KALA induced leakage from liposomes as a function of pH. KALA induced ANTS/DPX leakage from POPC neutral liposomes (A) and from 3/1 POPC/POPG negatively charged liposomes (B) at the following lipid/peptide molar ratios: diamonds, 50/1; squares, 200/1; circles and 800/1; triangles, 2000/1; and inverted triangles, 3000/1. A constant lipid concentration of 0.1 mM was used. (C.) KALA induced ANTS/DPX leakage from POPC neutral liposomes at pH 7.5 (squares) and 5.5 (circles) when the peptide was preincubated with ODN at various charge ratios (+/−) before addition to the liposomal suspension. Constant peptide and lipid concentrations of 0.5 μM and 0.1 mM, respectively, were used, (lipid/peptide molar ratio = 200/1).

The ability of KALA to destabilize POPC membranes when it is complexed to negatively charged oligonucleotides has also been investigated (Figure 3C). The peptide KALA was mixed with an oligonucleotide (27-mer) at various

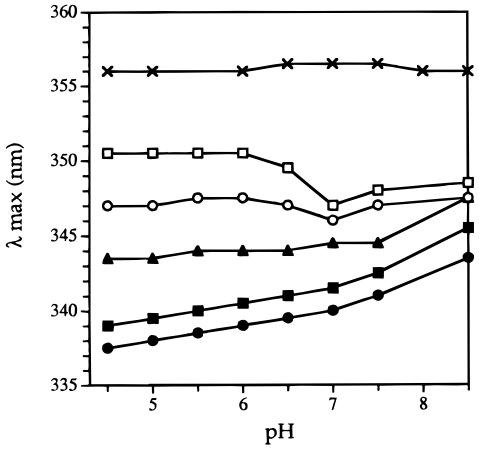

FIGURE 4: KALA tryptophan emission spectrum as a function of pH. λ_{max}, the wavelength at which maximum tryptophan fluorescence is observed, is monitored under the following conditions: times signs, KALA alone; open squares, POPC (0.1 mM) + KALA (lipid/peptide molar ratio = 40/1); open circles, POPC (0.4 mM) + KALA (160/1); closed triangles, 5/1 POPC/POPG (0.025 mM) + KALA (10/1); closed squares, 5/1 POPC/POPG (0.1 mM) + KALA (40/1); and closed circles, 5/1 POPC/POPG (0.4 mM) + KALA (160/1). A constant KALA concentration of 2.5 μM was used.

charge ratios (+/−) prior to addition to the liposomal suspension. The final extent of ANTS leakage was determined as previously described at pH 5.5 and 7.5 as a function of the charge ratio. Results indicate that the ability of the peptide to induce leakage is retained down to a charge ratio of 2.5/1 (+/−). A further increase of oligonucleotide concentration induces a sharp decrease of the leakage extent. Nevertheless, at pH 7.5, a residual leakage is observed, even at a charge ratio of 1/4.

To correlate interaction of the peptide with the bilayer and the leakage, we examined the wavelength of maximum intensity for the tryptophan emission spectrum (λ_{max}) as a function of increasing pH (Figure 4). A lower λ_{max} indicates a less polar environment for the tryptophan. In the absence of liposomes, λ_{max} remains fairly constant and high (356 nm) over the pH range of 4.5−8.5, suggesting that the peptide does not aggregate under these conditions. In the presence of neutral POPC liposomes, we notice a blue shift of λ_{max} (from 356 to 350.5 nm at pH 4.5 with 0.1 mM lipid) that indicates that a fraction of the peptide is bound to the liposomes, with the tryptophan in a more hydrophobic environment than when the peptide is in solution. This phenomenon is even more pronounced with negatively charged 5/1 POPC/POPG liposomes; the λ_{max} increases from 339 nm at pH 4.5 to 345 nm at pH 8.5. The increase of the shift observed for both liposome compositions when the lipid concentration is raised indicates an increase in the membrane-bound fraction of the peptide. The shift for a particular lipid concentration does not appreciably change in the pH range studied with POPC liposomes, whereas there is a noticeable decrease of the shift when the pH is raised with 5/1 POPC/POPG liposomes, which suggests a decrease of the membrane-bound fraction of the peptide.

We have shown that KALA exists in an α-helical conformation over a wide pH range and is able to cause leakage of entrapped dyes from lipid vesicles in the presence and absence of oligonucleotides, and the leakage is correlated with interaction of the peptide with the bilayer. The next step in the biophysical characterization of KALA is the interaction with DNA, i.e., gel retardation for charge neutral complexes, nuclear delivery of oligonucleotides, and ultimately transfection capabilities.

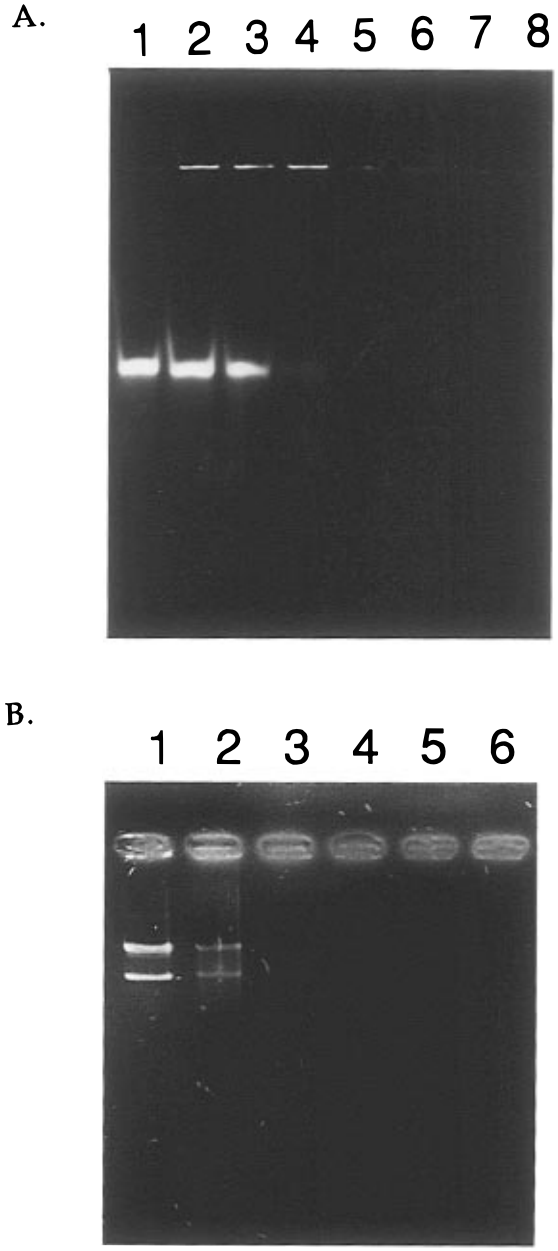

FIGURE 5: Interaction of KALA with oligonucleotides. (A) Polyacrylamide gel electrophoresis: lane 1, ODN alone; and lanes 2−8, ODN/KALA peptide complexes at different charge ratios relative to positive charges of KALA per negative charge of ODN. Lane 2, 0.31× (+/−); lane 3, 0.62×; lane 4, 1.25×; lane 5, 2.5×; lane 6, 5×; lane 7, 10×; and lane 8, 20×. (B) Agarose gel electrophoresis: lane 1, plasmid DNA alone; and lanes 2−8, DNA/KALA peptide complexes at different charge ratios relative to positive charges of KALA per negative charge of DNA. Lane 2, 1× (+/−); lane 3, 2×; lane 4, 4×; lane 5, 8×; and lane 6, 16×.

Interaction of KALA Peptides with DNA. To demonstrate the formation of complexes between negatively charged nucleic acids and positively charged KALA peptides, we have used gel retardation assays (Figure 5). KALA and oligonucleotides (ODNs) form complexes between a 1.25/ and 2.5/1 (+/−) charge ratio as shown by retention of the complex at the site of application on a 20% polyacrylamide electrophoresis gel (Figure 5A). Each lane contains 1 μg of

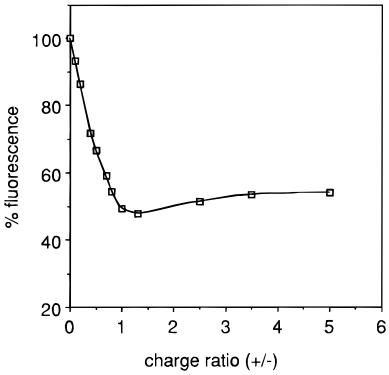

FIGURE 6: Ethidium bromide displacement assay. Complexes at various charge ratios were prepared by adding a solution of peptide in HBS to an equal volume of a mixture of plasmid DNA and ethidium bromide (10 μg/mL DNA; 0.4 μg/mL ethidium bromide in HBS).

oligonucleotide (28-mer), and lanes 2−8 contain increasing amounts of KALA resulting in charge ratios (+/−) of 0.31, 0.62, 1.25, 2.5, 5, 10, and 20, respectively. When complexes were formed with an excess of positive charge (lanes 4−8), the ODN did not enter the gel; hence, no band was visible. However, ODN alone or complexed at an excess of negative charge (lanes 2 and 3) migrates into the gel (Figure 5A). On the contrary, when ODN was mixed with a negatively charged peptide (GALA), no retention of ODN was observed (data not shown). In a 1% agarose gel retardation assay, plasmid DNA/KALA complexes were retained at the origin at charge ratios between 1/1 and 2/1 (+/−) (Figure 5B). Since the GALA peptide has a similar hydrophobic face but a negative charge and did not cause retention, the complexes formed between plasmid DNA or ODN and KALA are dependent upon electrostatic and not hydrophobic interactions.

Further, interaction of KALA with DNA was demonstrated by an ethidium bromide (EtBr) displacement assay (Figure 6). On basis of the distinct fluorescence enhancement of EtBr when it is intercalated into DNA, spectrofluorometric quantitation of displacement from its intercalation site was used to quantitate the charge ratio at which KALA competitively displaces ethidium bromide from DNA. We found that, with plasmid DNA, 50% of the ethidium bromide was displaced from the DNA at a charge ratio of 0.9/1 (+/−). While this assay demonstrates that KALA efficiently binds to DNA, it also indicates that KALA is not a highly efficient DNA-compacting agent as the curve of fluorescence versus charge ratio flattens out at charge ratios above 1/1 where approximately 50% of the ethidium bromide is still associated with DNA.

Intracellular Delivery of Oligonucleotides with or without KALA. We have evaluated the intracellular localization of fluorescein-labeled oligonucleotides (F-ODN) incubated alone or complexed with KALA. When F-ODNs (25-mer) were added alone in culture media, they were poorly internalized and localized into punctate cytoplasmic regions, corresponding to endosomes and/or lysosomes as previously published (Loke et al., 1989). Independent of the concentration (up to 2.5 μM), the time tested (24 or 48 h), and the fluorescent probe, fluorescein or rhodamine, ODNs were never detected in the nucleus of viable cells. In contrast, after their association with KALA at a charge ratio of 10/1 (+/−, 7.75 μM KALA and 160 nM oligonucleotides), the

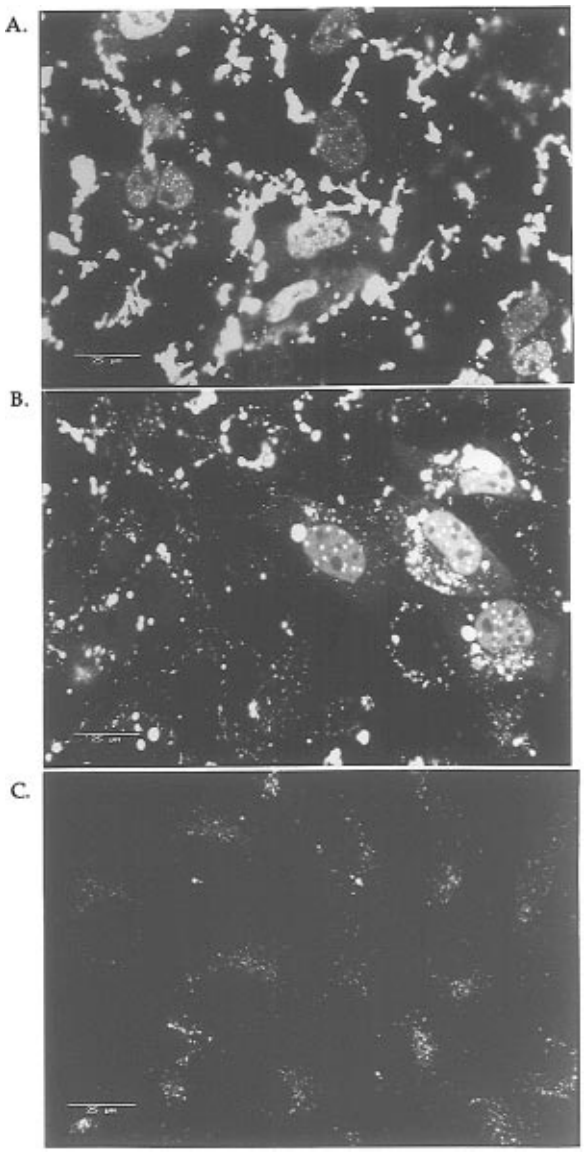

FIGURE 7: Cellular uptake of F-ODN associated with KALA or GALA peptides. F-ODN complexed to KALA at a 10/1 +/− charge ratio was incubated for 3 or 24 h at 37 °C on CV-1 cells. Finally, after washing, cells were observed by confocal microscopy with a 60× oil immersion objective. Images were taken at identical settings. F-ODNs were used at a concentration of 160 nM, and KALA was used at 7.75 μM. The percentage of cells having a fluorescent nucleus was determined; the mean of two separate experiments is given, and the standard deviation (SD) did not exceed 10%. The scale bar indicates 25 μm: (A) F-ODN/ KALA at a 10/1 charge ratio (+/−) after 3 h of incubation and (B) after 24 h of incubation and (C) F-ODN/GALA at a 10/1 charge ratio (−/−) after 24 h of incubation.

cellular fluorescence increased and some cells exhibited a fluorescent nucleus (Figure 7A). Thus, KALA modifies the intracellular distribution and increases nuclear accumulation of ODNs, causing their redistribution from punctate cytoplasmic regions into the nucleus. The efficiency of ODN delivery was charge ratio-dependent (relative to positive charge present on KALA per negative charge present on ODN). A positively charged complex was required for the delivery, and the highest efficiency was obtained at 10/1 (+/−), where 34 ± 5% of cells exhibit fluorescent nucleus after either 3 or 24 h of incubation (Figure 7A,B). In contrast, when ODNs were mixed with negatively charged GALA peptides, their intracellular distribution was not modified (Figure 7C).

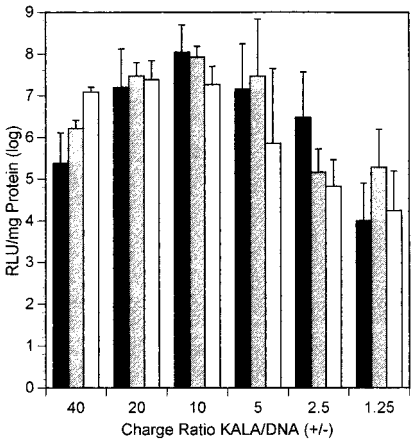

FIGURE 8: Transfection efficiency in CV-1 cells of DNA/KALA as a function of the charge ratio and the amount of DNA. Plasmid DNA was complexed to KALA at varying +/− charge ratios ranging from 1.25 to 40×. DNA was used in quantities of 0.6 μg (white bar), 1.2 μg (hatched bar), and 2.4 μg (solid bar) per well.

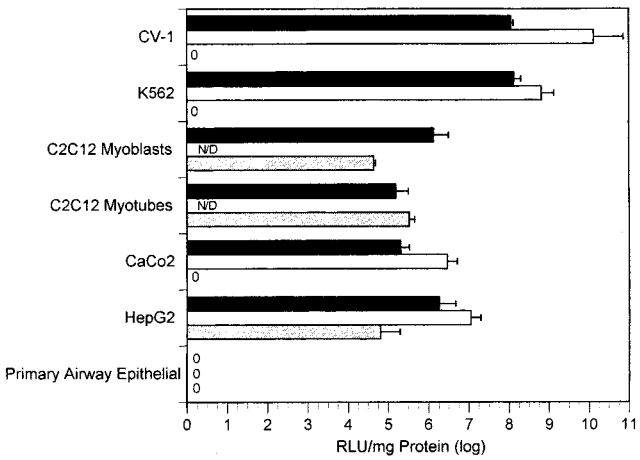

FIGURE 9: KALA-mediated transfection of mammalian cells. Cells were transfected in triplicate as described in Experimental Procedures with 1.2 μg of plasmid DNA complexed to KALA at a 10/1 charge ratio (+/−) (2.4 μg of plasmid DNA in CaCo2 and primary airway epithelial cells). The transfection efficiency of the optimized complex (solid bar) was compared to that for 6/1 sixth generation fractured dendrimer/DNA (+/−) (open bar) or naked DNA (hatched bar); N/D means not determined. Luciferase activity was measured 48 h post-transfection.

The cytotoxicity of the 28-mer ODN, KALA, and ODN/KALA peptide complexes was monitored by an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. Toxicity was seen when KALA was added at concentrations of ≥25 μM with or without ODN present (data not shown). Phosphorothioate oligonucleotides alone were not cytotoxic up to 865 nM (data not shown).

From the data above, we have shown that KALA is able to cause delivery of single-stranded oligonucleotides to the nucleus, but for transfection, double-stranded DNA must be employed.

Transfection of Various Cells with KALA. CV-1 cells plated in a 96-well plate assay at 20 000 cells/well were transfected with pCMVLuc/KALA complexes at various charge ratios (40/1 to 1.25/1, +/−) and varying amounts of plasmid DNA/well (2.4, 1.2, or 0.6 μg/well) in order to optimize the complex for transfection (Figure 8). KALA/DNA complexes showed maximum luciferase activity [10^8 RLU/(mg of cell protein)] at a 10/1 (+/−) charge ratio. No luciferase activity was observed for cells incubated with DNA alone. Using KALA at a 10/1 charge ratio, luciferase activity was about 100-fold greater than that found for the optimal polylysine/DNA complexes [10^6 RLU/(mg of cell protein)]. Therefore, KALA was used at a 10/1 charge ratio with 1.2 μg of DNA for K562, Hep G2, and C2C12 cell lines in 96-well plate assays and 2.4 μg of DNA for CaCo2 and primary airway epithelial cells in 12 mm transwell assays (Figure 9).

KALA is also quite efficient in mediating transfection of K562 cells [10^8 RLU/(mg of protein)]. In previous studies, transferrin has been shown to increase the transfection capabilities of polylysine/DNA complexes in K562 cells which have had the transferrin receptor up-regulated by the addition of deferoxamine mesylate 1 day prior to transfection (Wagner et al., 1991, 1992a,b). In our studies, the addition of transferrin made no difference in the ability of KALA to cause transfection in K562 cells (data not shown). In other cell lines, C2C12 myoblasts and myotubes, and Hep G2 and CaCo2 cells, KALA mediated lower transfection efficiencies, 10^6-10^7 RLU/(mg of protein).

In all of the cell lines studied, the transfection capability of KALA was compared to that of the sixth-generation fractured dendrimer or naked DNA as controls. These controls were chosen because it has been shown that the sixth generation dendrimer has high transfection efficiency in CV-1 [10^{10} RLU/(mg of protein)], K562 [10^8-10^9 RLU/(mg of protein)], and Hep G2 cells [10^8-10^9 RLU/(mg of protein)] (Haensler & Szoka, 1993). Naked DNA is efficient at transfection of myotubes [10^6 RLU/(mg of protein)] (Wolff et al., 1992). KALA, as well as naked DNA and dendrimer, showed no transfection capability on primary airway epithelial cells.

DISCUSSION

To achieve efficient gene transfer in a water soluble nucleic acid complex, the components must be able to associate with the nucleic acid during the delivery phase, transfer across the cell membrane, and then dissociate from the nucleic acid in the target cell (Xu & Szoka, 1996). Thus, there is a delicate balance in the biochemical properties of the components and their interaction with DNA. We have combined the positive charge required for DNA binding (Mascotti & Lohman, 1993) and the amphipathic membrane-destabilizing characteristics of the GALA peptide (Subbarao et al., 1987) in a low-molecular weight peptide, KALA. To our knowledge, KALA is the first designed peptide which binds DNA, destabilizes membranes, and mediates significant gene delivery.

To design a peptide with sufficient binding to DNA, we used the binding free energies provided by Mascotti and Lohman (1993), who studied a series of polylysine peptides. We selected a sequence with a strong propensity for an α-helical conformation that positions seven lysines on one face to mediate a reversible association with DNA. This is slightly fewer positive charges than that used by Gottschalk and colleagues, who have developed a peptide, $YKAK_8WK$, with 10 lysines as the DNA binding portion of a two-component gene delivery complex (Gottschalk et al., 1996). The use of low-molecular weight components as shown by Gottschalk and colleagues and in this paper is an alternative to the use of cationic polymers. In the past, positively charged polylysine and dendritic polymers, which possess

amine termini, have been studied to that end (Wagner et al., 1991; Haensler & Szoka, 1993; Wu et al., 1994).

Once the DNA is compacted, it must be able to cross the cell membrane to enter the cytoplasm. Polylysine alone has a marginal ability to transfect cells but has been successfully used in conjunction with receptor ligands such as transferrin (Wagner et al., 1991), AsOR (Wu & Wu, 1987), or antibodies (Ferkol et al., 1995). Although polylysine possesses some endosomal-destabilizing ability, the membrane-destabilizing capability of polycations has been increased using either inactive adenoviral particles (Curiel et al., 1991; Wagner et al., 1992a,b; Wu et al., 1994; Kremer & Perricaudet, 1995), influenza fusion peptides (Plank et al., 1994; Wagner et al., 1992a,b), or GALA (Haensler & Szoka, 1993). These high-molecular weight cationic polymers are effective at mediating transfection of reporter genes to cells in culture and *in vivo* but are polydisperse, require several components, and induce an immune response (Wu & Wu, 1988a,b; Perales et al., 1994; Ferkol et al., 1995).

We have demonstrated the ability of KALA to complex DNA through gel retardation and ethidium bromide displacement assays. Oligonucleotides, as well as plasmid DNA, were retained at the point of application when the charge ratio exceeded 1/1. At a charge ratio of 0.9/1, we observed 50% ethidium bromide displacement.

For efficient transfection, escape from the endosome and delivery to the cytoplasm are required. KALA, like pore-forming GALA, is amphipathic when in an α-helical conformation. In CD studies, we found that KALA undergoes a conformational change from an α-helical conformation to a mixture of α-helix and random coil as the pH is lowered. KALA, unlike GALA, possesses α-helical character over the entire pH range studied. Even at a pH as low as 4.5, substantial α-helical content is shown by the ellipticity at the 222 nm band. At physiological pH, KALA exists in the α-helical conformation in spite of the number of positive charges from the protonated lysine side chains.

KALA is efficient at causing membrane leakage from neutral and negatively charged liposomes. At physiological pH, KALA induced 100% leakage of entrapped dyes from neutral POPC liposomes (lipid/peptide molar ratio of 50/1), and in a more accurate model of cell membranes (3/1 POPC/POPG negatively charged liposomes, lipid/peptide ratio of 200/1), KALA caused nearly 100% leakage of entrapped contents over the pH range of 4−8.5. This may be explained by the attractive electrostatic interactions between the positively charged peptide and the negatively charged liposomes. This attractive interaction may increase the membrane-bound fraction of the peptide and/or modify the interaction of the peptide with the membrane thereby increasing the leakage. From these results, we can conclude that KALA is able to cause membrane leakage in low-pH environments similar to that of the endosome. KALA is also able to induce membrane leakage in the presence of oligonucleotides (Figure 3C), and this ability may account for its capacity to deliver oligonucleotides into cultured cells.

When tested for oligonucleotide nuclear delivery, the nuclear uptake was found for approximately 35% of the cells with KALA/ODN at a 10/1 charge ratio (+/−). GALA on the other hand did not enhance ODN nuclear uptake under the same conditions. This demonstrated that the positively charged amine residues on KALA are required for DNA trafficking into cells.

In transfection studies, the complex composed of plasmid DNA and KALA at a ratio of 10 positively charged lysylamines per nucleotide was also found to be optimal in CV-1 cells [10^8 RLU/(mg of protein)]. For the suspension cells, K562 cells, the transfection efficiency is relatively good [10^8 RLU/(mg of protein)]. Previous studies have shown a requirement for transferrin for efficient transfection (Wagner et al., 1991). In our studies, the transfection efficiency is unaffected by the addition of transferrin receptor ligand. KALA is capable of mediating transfection in a variety of cells, including polarized CaCo2 cells grown on transwells, C2C12 myoblasts and myotubes, and Hep G2 cells [10^5, 10^6, 10^6, and 10^7 RLU/(mg of protein), respectively]. KALA was unable to mediate transfection in primary airway epithelial cells.

In the optimal *in vitro* studies using a 10/1 charge ratio, there is a large excess of KALA molecules compared to the number of plasmids; therefore, some of the KALA molecules may not be in complexes with DNA but rather may act as membrane disruptive agents. Above this ratio, the complex is less efficient. This may be due to either toxicity or saturable binding to putative DNA/KALA binding sights on the cell surface.

We have designed and synthesized a novel peptide capable of condensing DNA and causing membrane leakage, nuclear delivery of oligonucleotides, and transfection of plasmid DNA in various cell lines. This peptide has a number of advantages over previously reported gene delivery systems. First, it is convenient to synthesize; second, it is efficient at mediating transfection in cells without the need for other agents such as chloroquine or adenoviral endosomal disruption agents. It provides a starting point for additional improvements in the sequence that might provide ligands for cell surface or cytoplasmic receptors to improve DNA trafficking into and through the target cell. Finally, it can serve as a platform to help unravel the roles of DNA binding/dissociation and membrane destabilization in the transfection process.

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