Delivery of oligonucleotides into mammalian cells by anionic peptides: comparison between monomeric and dimeric peptides

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The use of antisense oligonucleotides as putative therapeutic agents is limited by their poor delivery into the cytosol and/or the nucleus because they are not able to efficiently cross lipid bilayers. To circumvent this pitfall, anionic amphipathic peptides derived from the influenza virus fusogenic peptide have been used to destabilize membranes in an acidic environment. In this paper, we compare the ability of a monomeric and a dimeric peptide to introduce oligonucleotides into the cytosol and nuclei of several types of cultured cells. Cells incubated at pH 6.2 or at a slightly lower pH in the presence of the monomeric peptide but not the dimeric peptide were efficiently permeabilized. The location of fluorescent derivatives of peptides and of oligo-

nucleotides was assessed by confocal microscopy. Both the peptides and oligonucleotides remained entrapped in vesicular compartments at neutral pH; at acidic pH, oligonucleotides in the presence of the monomeric peptide were mainly in the nucleus, while in the presence of the dimeric peptide they colocalized with the peptide into vesicles. The data are interpreted on the basis of the spectroscopic behaviour of monomeric and dimeric peptides in relation to the environmental pH.

Key words: antisense oligonucleotide, confocal microscopy, fusogenic peptide, membrane permeabilization.

INTRODUCTION

One limitation in the use of antisense oligonucleotides (ODN) as gene-expression inhibitors is their limited ability to cross a lipid bilayer. The delivery into the cytosol is a prerequisite for reaching their target, usually an RNA, knowing that ODN diffuse freely from cytosol to nucleus [1,2]. Although ODN are taken up by cells through an endocytotic pathway [3], they remain essentially entrapped inside endocytotic vesicles. The membranes of these vesicles prevent the access of ODN to the cytosol and/or the nucleus. It is of course possible to bypass this problem by microinjection [1,2] and electroporation [4]; these approaches are suitable with cells in culture but not for clinical applications. To improve the uptake and the cytoplasmic delivery of ODN, various methods have been proposed. Synthetic carriers have been developed: cationic lipids associated or conjugated with ODN [5,6], nanoparticles [7] and dendrimers [8] as well as cationic polymers including poly-L-lysine [9] or polyethylenimine [10]. Cationic polymers require the concomitant use of helpers such as chloroquine to destabilize endosomal membrane [11].

Peptides as synthetic vectors have been proposed to bypass the endocytotic pathway, leading to a direct introduction of the ODN through the plasma membrane to the cytosol [12–16]. Peptides, derived from fusogenic segments of viral proteins [17], such as the influenza virus haemagglutinin, have demonstrated their strong membrane-destabilization effect on model membranes as well as on living cell membrane [18]. Their efficiency in gene transfer has been clearly shown [19–22] but so far this property has not been investigated in depth for the field of ODN delivery. We have previously shown that the addition of an anionic

peptide E5CA in the transiently acidified extracellular medium down to pH 5.8, a pH close to that of the endosome lumen, allows the direct introduction of ODN into the nucleus via the cytosol [15]. The elongation of the C-terminus of peptide derivatives of the fusion segment of the influenza haemagglutinin subunit HA2 with Trp-Tyr-Gly, and the C-terminal dimerization of such peptides, enable the enhancement of both erythrocyte leakage activity and the transfection efficiency of transferrin-poly-lysine-DNA complexes [20,21]. Therefore, to improve the E5CA peptide efficacy, we have synthesized two peptide derivatives, which are closer to the natural sequence of influenza virus ($\times 31$) fusion peptide: the peptide E5WYG and its dimeric counterpart (E5WYGGAA)₂KA (where A is β -alanine). The E5WYG peptide corresponds to the E5 sequence [23] to which the Trp-Tyr-Gly amino acids were added in C-terminal position; the dimer (E5WYGGAA)₂KA corresponds to a head-to-head dimerization of the E5WYG peptide by a linkage to both the amino groups of a lysyl residue. A spacer arm made of one glycyl residue and of two β -alanyl (A) residues have been introduced to evaluate its impact on peptide activity. The peptide efficiency in increasing the nuclear delivery of ODN bearing a fluorescent tag (fluorescein or rhodamine) was evaluated by confocal microscopy. The behaviour of these two peptides was studied in comparison with the E5CA peptide. In addition, the intrinsic fluorescence and their conformation as assessed by CD were studied at various pH values. The optimal conditions leading to an efficient peptide-dependent permeabilization were determined and the intracellular location of both the peptides and the ODN at acidic and neutral pHs was investigated using two human cell lines.

Abbreviations used: ODN, oligonucleotides; psODN, phosphorothioate-type ODN; F-psODN, fluorescein-labelled psODN; R-psODN, rhodamine-labelled psODN; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; Fmoc, fluorenylmethoxycarbonyl; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

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EXPERIMENTAL

Peptide synthesis and purification

Peptides were assembled by solid-phase synthesis on an Applied Biosystems 433A synthesizer (Foster City, CA, U.S.A.) with conductimetric monitoring by using a fluorenylmethoxycarbonyl (Fmoc) strategy, starting from a Rink amide 4-methylbenzhydrylamine (MBHA) resin (Novabiochem, Lufelfingen, Switzerland) as solid support. Amino acids were coupled by the HBTU/HOBt [2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/*N*-hydroxybenzotriazole] activation method [24]. Side-chain protections were as follows: Glu(*t*-butyl ester), Trp(*t*-butyloxycarbonyl), Tyr(*t*-butyl), Cys(trityl) and Lys(Fmoc) (Neosystem, Strasbourg, France). The use of *N*^{},*N*^{}-di-Fmoc-lysine allows, upon deprotection, the synthesis of a peptide head-to-head dimer with lysine as linking amino acid. The peptides were cleaved from the resin and the protecting groups were removed with a trifluoroacetic acid/water/phenol/thioanisole/ethanedithiol/[tri-isopropylsilane, in the presence of Cys(trityl)] mixture (40:2:3:2:1:1, by vol.) for 3 h at room temperature. Crude peptides were precipitated in *t*-butyl-methyl ether and were collected by centrifugation. Peptides were washed three times with ether and subsequently dried. The thiol group of the cysteinyl residue of E5CA peptide was further protected by a thiopyridyl group, as described previously [15]. E5WYGC and (E5WYGGAA)₂KAC were synthesized with a cysteine at the C-terminus for coupling of a fluorescent tag (Table 1). The free thiol group of the cysteinyl residue (1 µmol) was reacted with 5 µmol of fluorescein iodoacetamide (Molecular Probes, Eugene, OR, U.S.A.) for 4 h at 20 C in thoroughly degassed 20 mM Tris/HCl, pH 8.0. The same reaction was performed on the thiol group of the E5CA cysteinyl residue (1 µmol) after reduction of the thiopyridyl protecting group with 1 µmol of Tris(2-carboxyethyl)phosphine (Molecular Probes) [25] for 2 h at 20 C in degassed 20 mM Tris/HCl, pH 8.0. Peptides, fluoresceinylated or not, were purified by gel filtration on a Biogel P2 or P4 column (Bio-Rad, Hemel Hempstead, Herts., U.K.) eluted with 20 mM Tris/HCl, pH 8.0, for mono-

meric and dimeric peptides respectively, in order to remove the reagent excess. Peptides were purified further by a semi-preparative-scale HPLC (Waters, St. Quentin-en-Yvelines, France) using a pH-stable reversed-phase C₈ column (5 µm, 250 × 10 mm; Vydac, Hesperia, CA, U.S.A.): solvent A, 20 mM Tris/HCl, pH 8.0, containing 5% acetonitrile; solvent B, acetonitrile containing 5% 20 mM Tris/HCl, pH 8.0; gradient elution, 0–100% solvent B in 35 min at a 1 ml/min flow rate. After precipitation in acetic acid and washings in water, the peptides were freeze-dried. The purity of the peptides (Table 1) was confirmed by analytical HPLC on a pH-stable reversed-phase C₈ analytical column (5 µm, 250 × 4.6 mm, Vydac) and by negative ion electrospray MS on a Platform quadrupole mass spectrometer VG Biotech (Fisons Instruments, Altrincham, Cheshire, U.K.). The samples were dissolved in a 0.4% ammonia/acetonitrile mixture (50:50, v/v), pH 9.0, at a concentration of 20–50 pmol/µl. For the biological experiments, each peptide was dissolved in 20 mM Tris/HCl, pH 8.0, at 1 mg/ml, and aliquots were stored at –20 C. The peptide concentration was determined by using ₂₈₀ values of 12350 M^{–1}·cm^{–1} for E5WYG, which contains one tyrosyl and two tryptophanyl residues, 24700 M^{–1}·cm^{–1} for (E5WYGGAA)₂KA which contains two tyrosyl and four tryptophanyl residues and 10480 M^{–1}·cm^{–1} for E5CA, protected by a thiopyridyl group. The concentrations of the fluorescein-labelled peptides [E5CA-*F*, E5WYGC-*F* and (E5WYGGAA)₂KAC-*F*] were determined by using ₄₉₂ = 80000 M^{–1}·cm^{–1} for the molar absorbance of fluorescein at pH 8.0.

Antisense ODN and their fluorescent derivatives

A phosphorothioate-type pentacosanucleotide (psODN) complementary to the AUG initiation site of the gag HIV-1 gene, 5'-CTCTCGCACCCATCTCTCTCCTTCT-3' (GEM91), kindly provided by Dr S. Agrawal (Hybridon, Worcester, MA, U.S.A.), was fluorescein-labelled (to produce *F*-psODN) on the phosphorothioate backbone as described by Stewart et al. [26]. Briefly, psODN (0.2 µmol) in 0.1 M sodium phosphate, pH 8.0, was

Table 1 Sequences and masses of the synthetic peptides and natural fusion peptide of influenza virus haemagglutinin subunit HA2 (PF-HA2)

A indicates a β-alanyl residue. The cysteine side chain of the peptides was substituted either with a thiopyridyl group (SPy) or with a fluorescein group (F).

Peptide	Sequence	Calculated mass	
		(monoisotopic) (Da)	Measured mass (Da)
PF-HA2	N _t 1 10 20 C _t GLFGAIAAGFIENGWEGMIDGWY ^C		
E5CA	H-GLFEAIAEFIEGGWEGLIEGC(SPy)A-OH	2420.12	2420.1
E5WYG	H-GLFEAIAEFIEGGWEGLIEGWYG-NH ₂	2542.83	2542.1
E5WYGC- <i>F</i>	H-GLFEAIAEFIEGGWEGLIEGWYGC(<i>F</i>)-NH ₂	2754.60	2754.4
(E5WYGGAA) ₂ KA	H-GLFEAIAEFIEGGWEGLIEGWYGGAA	5664.93	5664.7
	KA-NH ₂		
(E5WYGGAA) ₂ KAC- <i>F</i>	H-GLFEAIAEFIEGGWEGLIEGWYGGAA	6154.74	6155.1
	KAC(<i>F</i>)-NH ₂		

incubated with 1 μ mol of fluorescein iodoacetamide (Molecular Probes) for 20 h at 50 °C. The reaction was followed by HPLC. The *F*-psODN was precipitated with ethanol and purified by chromatography on a Biogel P2 column (Bio-Rad) equilibrated in 5% butanol/95% water. The *F*-psODN purity was confirmed by HPLC by using a Waters system equipped with Millennium Software on a LiChrospher 100 RP-18 analytical column (5 μ m, 250 $\times$ 4 mm; Merck, Darmstadt, Germany) with a linear gradient from 0 to 30% solvent C over 30 min (flow rate of 1 ml/min). Solvent C was composed of 95% 0.1 M triethylamine acetate, pH 7.0/5% acetonitrile, and solvent D was 95% acetonitrile/5% 0.1 M triethylamine acetate, pH 7.0.

Another antisense psODN (21-mer) complementary to the env HIV-1 gene, 5'-TCCCTCATATCTCCTCCTCCA-3', bearing 5'-alkyl dithiopyridine and 3'-alkylamine groups, was also used. This psODN (0.2 μ mol), synthesized by Y. Aubert (of Dr U. Asseline's laboratory, Centre de Biophysique Moléculaire, Orléans, France), was rhodamine-labelled (*R*-psODN) on its 3' NH₂ group in 50 mM sodium phosphate buffer containing 2 M sodium acetate, pH 8.0, by reaction with 6 μ mol of Rhodamine Red[®]-X succinimidyl ester (R-6160, Molecular Probes) at 20 °C for 24 h. The *R*-psODN was precipitated in ethanol to remove the excess of rhodamine and was directly purified by HPLC on a LiChrospher 100 RP-18E column (10 μ m, 10 $\times$ 250 mm; Merck) eluted with solvent C with a linear gradient from 5 to 40% solvent D over 30 min at 3 ml/min. Detection was achieved either by UV absorbance using a Waters photodiode array detector or by fluorescence using a Gilson 121 filter fluorimeter.

Spectrofluorimetry

Fluorescein fluorescence is very sensitive to the pH. The fluorescence of the fluorescein-labelled peptides at different pH values was measured at 20 °C, using a Shimadzu RF-5000 spectrofluorimeter (Roucaire, Courtaboeuf, France) with a 5 $\times$ 5 mm quartz cuvette. The fluorescence intensity was recorded at 520 nm emission wavelength under a 490 nm excitation wavelength. Fluoresceinylated peptides were diluted to 2 μ M with the following buffers: from pH 4.6 to 6.7, 50 mM citrate/phosphate buffer/0.15 M NaCl; at pH 7.4, 50 mM phosphate buffer/0.15 M NaCl.

The tryptophan fluorescence intensity of the E5WYG-type peptides was measured by spectrofluorimetry under the same conditions as described above, from pH 5.6 to 7.4 with 10 μ M peptide concentration. The fluorescence intensity was recorded at 300–450 nm emission wavelength range under a 280 nm excitation wavelength.

CD

CD spectra were recorded on a Jobin-Yvon Mark IV dichrograph with a quartz cell of 1 mm path length using 44 μ M E5WYG and 22 μ M (E5WYGGA)₂KA in order to have the same quantity of anionic peptide in the solution. Under these conditions, the peptide concentrations, expressed in terms of the monomer moiety, were the same. The temperature of the cell was maintained at 25 °C with a circulating-water bath. All the peptides precipitated below pH 5.4, the spectra were measured between pH 5.6 and 6.6 in 10 mM citrate/phosphate/0.15 M NaCl, and at pH 7.4 in 10 mM sodium phosphate/0.15 M NaCl. Spectra were signal-averaged with a minimum of three repeats and presented with a similarly signal-averaged buffer baseline withdrawn.

Cell culture

A549 human non-small lung carcinoma cells (ATCC CCL 185, ATCC, Rockville, MD, U.S.A.) and the HeLa human cervix epithelioid carcinoma cell line (ATCC CCL 201, ATCC) were grown in complete Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, Renfrewshire, U.K.) containing 2 mM L-glutamine (Merck), antibiotics (100 units/ml penicillin and 100 μ g/ml streptomycin; Eurobio, Paris, France) and 10% heat-inactivated foetal bovine serum (FBS; Gibco). Cells were cultured at 37 °C in a humidified atmosphere (95% air/5% CO₂). Cells were harvested after treatment at 37 °C for 5 min in PBS, pH 7.4 containing 0.2 mg/ml EDTA (Sigma, St. Louis, MO, U.S.A.) and 2.5 μ g/ml trypsin (PET solution) and were plated at 10⁵ cells/well directly on to plastic or on glass coverslips put into 4- or 24-well plates (well area, 2 cm²) 1 day before the experiments.

Cellular uptake and intracellular location of ODN and peptides

The uptake of the psODN and the intracellular distribution of permeabilizing peptides in A549 and HeLa cells were analysed by confocal microscopy. Cells were incubated with solutions 1 or 2, which were prepared just before adding to cells.

Solution 1 was used for *F*-psODN + unlabelled peptide: to follow fluorescent *F*-psODN, cells (10⁵ cells/well) were incubated with 0.1 μ M *F*-psODN and 40 μ M E5WYG or 20 μ M (E5WYGGA)₂KA peptides in serum-free DMEM (200 μ l). Solution 2 was used for *R*-psODN + peptide-*F*: to study both the psODN and the peptide's intracellular location, cells (10⁵ cells/well) were co-incubated with a mixture of 0.4 μ M *R*-psODN and 4 or 2 μ M fluoresceinylated peptide [E5WYGC-*F* or (E5WYGGA)₂KA-*F*, respectively] in serum-free DMEM (200 μ l). For optimal visualization of the two fluorescent probes with the appropriate concentration of peptide, the solution contained 10% fluoresceinylated peptide and 90% unlabelled peptide. The mixture used at a 40 μ M final concentration of E5WYG peptide corresponds to 4 μ M E5WYGC-*F* and 36 μ M E5WYG; a 20 μ M final concentration of (E5WYGGA)₂KA peptide corresponds to 2 μ M (E5WYGGA)₂KA-*F* and 18 μ M (E5WYGGA)₂KA.

Plasma-membrane permeabilization by transient acidification in the presence of peptides

After two washes with serum-free DMEM, 200 μ l of either solution 1 or solution 2 were added to the plated cells (well area, 2 cm²). The extracellular medium was immediately acidified to the required pH, between pH 6.5 and 5.5, with a 1 M stock solution of Mes buffer (Sigma) and cells were incubated at 37 °C for 30 min. At the end of the incubation, the supernatant was discarded and its pH was measured. Cells were washed twice with complete medium containing 10% FBS, and either fixed in paraformaldehyde or further incubated at 37 °C in culture medium to check their viability after 24 or 48 h.

Uptake of ODN at neutral pH

After two washes in serum-free DMEM, cells were incubated at 37 °C for 2 h at pH 7.4 with solution 1 or solution 2. After two washes with complete medium, cells were further incubated at 37 °C with 1 ml of complete DMEM for 2 or 12 h. Finally, cells were fixed and mounted for confocal microscopy analysis.

Laser-scanning confocal microscopy analysis

After incubation, cells, plated on glass coverslips, were fixed for 30 min at room temperature in PBS containing 20 mg/ml para-

formaldehyde and further mounted on slides in a PBS/glycerol (Merck) mixture (1:1, v/v) containing 10 mg/ml 1,4-diazadicyclo-(2,2,2)-octane (DABCO) to minimize photobleaching [27]. Intracellular *F*- or *R*-psODN and peptide-*F* location was analysed with a confocal imaging system (MRC-1024, Bio-Rad) equipped with a Nikon Optiphot epifluorescence microscope (Nikon, Tokyo, Japan) and a 60 $\times$ oil immersion Planapo objective (numerical aperture, 1.4). A krypton/argon laser, tuned to produce both 488 nm fluorescein excitation and 565 nm rhodamine-wavelength beams, allowed simultaneous excitation of fluorescein and rhodamine. The diaphragm and fluorescence detection levels were adjusted to minimize interference between fluorescein and rhodamine channels. Pictures were recorded with a Kalman filter (average of ten images). Photos were obtained using Adobe Photoshop software.

Uptake of peptides: flow cytometry analysis

To study the fluorescein-labelled peptide uptake in the absence and presence of psODN, plated A549 and HeLa cells (3×10^5 cells) were incubated at 37 °C with 10 μ M fluoresceinylated peptide with or without 0.1 μ M psODN in serum-free DMEM (200 μ l) for 2 h (solution 3). After 2 h of incubation, cells were washed twice with PBS and further incubated in 1 ml of fresh medium at 37 °C for 1 and 2 h. After washings with PBS, cells were harvested with PET solution, centrifugated (1000 *g* for 5 min) and resuspended in sheath fluid (134 mM NaCl/3.75 mM KCl/15.2 mM NaF/1.9 mM KH_2PO_4 /16.5 mM NaHPO_4 /0.2% 2-phenoxyethanol, pH 7.4). The cell fluorescence intensity was measured before and after a post-incubation period of 30 min at 4 °C in the presence of 50 μ M monensin (Calbiochem, La Jolla, CA, U.S.A.). This ionophore neutralizes endocytotic compartments, allowing the retrieval of the fluorescence quenched in an acidic environment [28] and provides information regarding the intracellular location of the endocytosed material [29]. The cell fluorescence was recorded at 520 nm under excitation at 488 nm using a FACSsort flow cytometer (Becton Dickinson, Le Pont de Claix, France). Data shown represent the mean values of 5000 cells calculated by using Cell Quest Software (Becton Dickinson).

Cell viability

After permeabilization, cells were cultured for 24 or 48 h in culture medium containing 10% FBS. Cell viability was assessed by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) [30].

RESULTS

Design and characterization of peptides

The N-terminal peptide of the influenza virus haemagglutinin subunit HA2 is known to adopt an amphipathic helical conformation and to destabilize lipid bilayers in an acidic environment. Based on this concept, synthetic derivatives have been proposed to deliver nucleic acids in association with polylysine conjugates used in gene transfer [19,20,22]. Such peptides have demonstrated their efficiency for a gene-transfer system but have not been investigated in depth for ODN delivery. Previously, we have shown that upon transient acidification of the extracellular medium in the presence of the anionic peptide E5CA derived from the HA2 viral fusion domain, antisense ODN are introduced directly through the plasma membrane into the cytosol, from where they spontaneously migrate to the nucleus of various cells [15]. To improve the peptide-permeabilization efficiency and to enhance the internalization of antisense ODN into the cytosol and the nucleus where their targets are located,

Figure 1 pH dependence of the molar ellipticity at 222 nm of the amphipathic anionic peptides

The CD spectra of the peptide E5WYG (44 μ M; ■) and of the dimer (E5WYGGAA)₂KA (22 μ M; ◇) were run from pH 5.6 to 6.6 in 10 mM citrate/phosphate/0.15 M NaCl buffer and at pH 7.4 in 10 mM sodium phosphate/0.15 M NaCl. Inset: CD spectra of E5WYG peptide at pH 7.4 (●), 6.0 (◆) and 5.6 (△).

two different anionic peptides, one monomer and one dimer, deriving from the N-terminal segment of the HA2 subunit of the influenza virus haemagglutinin (PF-HA2), were synthesized and characterized (Table 1). E5WYG, a 23-residue peptide, corresponds to the E5 sequence, a highly soluble peptide [18,23] in which five residues are replaced: Gly-4, Gly-8 and Asp-19 by Glu, Asn-12 by Gly and Met-17 by Leu. Furthermore, this peptide contains in the C-terminal position a tripeptide, Trp-21, Tyr-22 and Gly-23, to improve the interaction with membranes. Indeed, the elongation of the C-terminus with Trp-Tyr-Gly residues was shown to increase the transfection efficiency of plasmid–polycation complexes [20,21]. The (E5WYGGAA)₂KA dimeric peptide corresponds to the dimerized E5WYG sequence using a C-terminal lysine as linking residue plus Gly and two β -Ala (A) residues, as short linkers (Table 1). The dimeric peptide was designed as a first step towards the influenza virus fusion protein, which is trimeric. All peptides, including E5CA peptide used as a reference, were tagged with a fluorescein molecule on an added cysteine in the C-terminal position to investigate their intracellular routing by fluorescence microscopy (Table 1).

The amino acid residues in these peptides are positioned according to an amphipathic helical structure, in which the five glutamic acid residues in E5WYG are located on the same hydrophilic face. The peptides carrying an overall negative charge at neutral pH are able to adopt an α -helical conformation, characterized by two minima at 208 and 222 nm (Figure 1, inset), in slightly acidic medium upon protonation of the γ -carboxy group of Glu side chains, as shown by CD. In contrast, the dimeric peptide (E5WYGGAA)₂KA already elicits an α -helix conformation at pH 7.4 which increases upon decreasing the pH down to 6.0 (Figure 1), whereas the monomeric peptide E5WYG started to exhibit a helical conformation below pH 6.0 (Figure 1

Figure 2 Fluorescein-labelled ODN internalization in the presence of peptide

A549 cells (10^5 cells/well) were incubated in 200 μ l of serum-free medium containing 0.1 μ M *F*-psODN in the presence of (A, C) 40 μ M E5WYG peptide or (B) 20 μ M (E5WYGGAA)₂KA peptide. After lowering the extracellular medium pH to pH 5.8 by adding 1 M Mes, cells were kept at 37 °C for 30 min (A, B). As a control (C), the extracellular medium pH was kept at pH 7.4 and cells were incubated at 37 °C for 2 h. After washings with medium containing 10% serum, cells were incubated at 37 °C with 1 ml of fresh complete medium for 2 h. Cells were further washed twice with medium without serum, fixed with paraformaldehyde and mounted for confocal microscopy analysis. Scale bar, 10 μ m.

and inset). Similar conformational changes were obtained with the E5CA peptide.

The effect of the peptide concentration on the CD spectra of both the monomeric and dimeric peptides was studied at pH 5.9, where the monomer is still poorly helical and the dimer is mainly helical. No significant change of ellipticity was observed, neither for the monomer in the 21–115 μ M range, nor for the dimer in the 14–42 μ M range.

The dimeric peptide aggregates were shown by gel filtration: on average, 20% of the dimer formed aggregates with a measured molecular mass found to be 100 times larger than expected for the peptide (results not shown). Such aggregation could partially explain its inefficiency in plasma-membrane permeabilization in the *F*-psODN delivery into cells. The tryptophan fluorescence intensity was measured for each peptide: no shift in the fluorescence intensity maximum could be observed; it was always centred at 350 nm at any pH with any peptide (results not shown). The fluorescence intensities are very similar when taking into account that E5CA, E5WYG and the dimer contain one, two and four tryptophan residues, respectively. These results indicate that Trp residue(s) were in all cases in contact with the aqueous solvent.

Uptake of ODN and permeabilizing peptides upon acidification of the extracellular medium

We have shown previously that E5CA peptide allowed a direct introduction of *F*-psODN into the nucleus of different cells upon

Table 2 Influence of pH on the nuclear location of *F*-psODN in the presence of E5CA or E5WYG peptides

A549 cells, plated on glass coverslips (10^5 cells), were incubated with 0.1 μ M *F*-psODN and 40 μ M E5WYG or E5CA peptide at 37 °C for 30 min at various pHs. The medium was acidified by adding 1 M Mes buffer to the medium. After incubation, cells were washed twice in complete medium, fixed, mounted and observed using confocal microscopy. Permeabilized cells showed a bright fluorescent nucleus. The percentage of nuclear-labelled cells, representing the number of permeabilized cells, was determined by counting an average of 100 cells.

pH	Nuclear-labelled cells (%)	
	E5CA	E5WYG
7.4	0	0
6.5	0	0
6.2	0	20 $\pm$ 10
6.0	20 $\pm$ 10	50 $\pm$ 10
5.8	90 $\pm$ 10	100
5.5	100	100

a transient acidification (down to pH 5.6 ± 0.2) of the extracellular medium [15]. In order to evaluate the ability of the monomeric E5WYG and the dimeric (E5WYGGAA)₂KA peptides, with respect to that of the monomeric E5CA peptide, to deliver ODN into the cytosol of A549 cells upon a short acidification exposure, we followed the intracellular location of *F*-psODN by confocal microscopy.

pH dependence

Solution 1, containing 0.1 μ M *F*-psODN and 40 μ M monomeric peptide or 20 μ M dimeric peptide in serum-free medium, was added to the A549 plated cells; the pH was lowered to between 6.5 and 5.5 by addition of a 1 M Mes buffer (Mes was used instead of D-gluconic acid used previously [15] because it is more efficient in this pH range). Cells were incubated with this solution for 30 min at 37 °C. As a control, A549 cells were incubated with *F*-psODN either at pH 7.4 in the presence of the peptide, or at acidic pH in the absence of the peptide. Whatever the pH, *F*-psODN remained in vesicular compartments when cells were incubated in the presence of the dimeric peptide (E5WYGGAA)₂KA (Figure 2B). In contrast, when cells were incubated with E5CA or E5WYG (Figure 2A) monomeric peptides at pH 5.8, *F*-psODN accumulated in their nuclei. At pH 6.0–6.2, the E5WYG peptide was more efficient than E5CA peptide in permeabilizing either A549 cells (Table 2) or HeLa cell lines (results not shown) at pH 6.0 or above. Under optimal conditions, the E5WYG peptide (40 μ M, pH 5.8, 30 min, 37 °C) permeabilized 100% of the cells, as shown by the fluorescence of the nucleus. Cell viability, assessed by an MTT assay after either 24 or 48 h in culture medium containing 10% FBS, was 75 and 85% respectively, indicating that the treatment had a low toxicity.

To test the capacity of the peptide to destabilize cell surface and/or intracellular membranes, A549 cells were incubated for 2 h at 37 °C in serum-free medium, at pH 7.4, with 0.1 μ M *F*-psODN and 40 μ M E5WYG (Figure 2C) or 20 μ M (E5WYGGAA)₂KA (solution 1) followed by a 2 h (or 12 h) chase in medium containing 10% serum. *F*-psODN were found distributed in vesicles throughout the cytoplasm. Whatever the peptide used (monomers or dimers) and the chase duration, endocytosed *F*-psODN remained entrapped inside endocytotic vesicles and neither nucleus nor cytosol was labelled.

Figure 3 Intracellular location of both fluorescein-labelled peptide and rhodamine-labelled psODN

HeLa cells (10^5 cells/well) were incubated for 30 min at 37 °C with a mixture of 0.4 μ M *R*-psODN and 40 μ M E5WYG (**A, B**) or 20 μ M (E5WYGGAA)₂KA (**C**) in 200 μ l of serum-free medium at pH 7.4 (**A**) or pH 5.8 (**B, C**). A 40 μ M concentration of E5WYG peptide includes 4 μ M E5WYGC-*F* peptide and a 20 μ M concentration of (E5WYGGAA)₂KA peptide includes 2 μ M (E5WYGGAA)₂KAC-*F* peptide (solution 2; see the Experimental section). After incubation, the medium was discarded. Cells were washed twice with complete medium, fixed with paraformaldehyde and mounted for confocal microscopy. Fluorescence from fluorescein-labelled peptides (left-hand panels) and from rhodamine-labelled psODN (right-hand panels) were collected simultaneously. Scale bar, 10 μ m.

Dose and time effect

To determine the optimal conditions of permeabilization by using E5WYG peptide, A549 cells were incubated for 30 min at 37 °C at pH 5.8 in the presence of the peptides: 20 μ M of E5WYG and E5CA led to $60 \pm 10\%$ and $50 \pm 10\%$ nuclear labelling, respectively; below 10 μ M peptide, less than 5 % of the cells were permeabilized by either monomeric peptide.

Using 40 μ M of the E5WYG and the E5CA peptides, the percentages of labelled nuclei were $50 \pm 10\%$ and $30 \pm 10\%$, respectively, after 5 min of incubation at pH 5.8; $70 \pm 10\%$ and $60 \pm 10\%$ after 15 min incubation; $100 \pm 10\%$ and $90 \pm 10\%$ after 30 min at 37 °C. Therefore, the effect of E5WYG on *F*-psODN uptake and nuclear location was dependent on the peptide concentration as well as on the incubation time.

Intracellular location of the peptides and ODN at acidic pH

As shown above, in slightly acidic medium, E5WYG peptide was an efficient permeabilizing agent leading to an efficient accumulation of *F*-psODN in the cell nucleus, whereas under the same conditions, the dimeric (E5WYGGAA)₂KA peptide was

totally inefficient. In addition, E5WYG had no permeabilizing effect when the extracellular medium was maintained at neutral pH. In order to shed light on the mechanism, the location of each fluoresceinylated peptide in the presence of *R*-psODN was investigated by confocal microscopy. HeLa cells were incubated for 30 min at 37 °C in the presence of 0.4 μ M *R*-psODN and 40 μ M E5WYG peptide, including 4 μ M fluoresceinylated E5WYGC-*F* peptide (solution 2). After acidification of the medium down to pH 5.8, the fluorescent peptide was found mainly in the cytoplasm (Figure 3B, left-hand panel) and was almost absent from the nucleus, while *R*-psODN was localized in the nucleus, with traces in the cytoplasm (Figure 3B, right-hand panel). The nuclear distribution of ODN was not dependent on the nature of the fluorophore (fluorescein or rhodamine), on the ODN backbone type (phosphorothioate or phosphodiester; results not shown) or on the cell line used (A549, HeLa or HepG2). When the extracellular medium was maintained at pH 7.4 or pH 5.8 for 30 min in the absence of peptides or in the presence of a low peptide concentration (results not shown), conditions under which the peptides are unable to induce cell permeabilization, both the E5WYGC-*F* peptide (Figure 3A, left-hand panel) and *R*-psODN (Figure 3A, right-hand panel) were localized and sequestered in the same vesicles. The dimeric (E5WYGGAA)₂KAC-*F* peptide, which did not induce cell permeabilization at both neutral (results not shown) and acidic (Figure 2B) pH values, was located near the surface of the cells (Figure 3C, left-hand panel) and was concentrated inside vesicles with the *R*-psODN (Figure 3C, right-hand panel) whatever the incubation pH (down to pH 5.0) and the peptide concentration (up to 40 μ M).

Uptake of ODN and permeabilizing peptides upon endocytosis at neutral pH

Confocal analysis

We have seen that the fluorescent ODN was not delivered into the cytosol when cells were incubated at neutral pH with either peptide (Figure 2C), indicating that endocytotic vesicles were not permeabilized. To determine whether the peptide inefficiency was related to its poor cell uptake and to a lack of internalization of the ODN with them, both peptides and psODN location were analysed by confocal microscopy. HeLa cells were incubated at 37 °C for 2 h in the presence of 0.4 μ M *R*-psODN and either 40 μ M E5WYG peptide (including 4 μ M fluorescent E5WYGC-*F* peptide) or 20 μ M (E5WYGGAA)₂KA peptide [including 2 μ M fluorescent (E5WYGGAA)₂KAC-*F* peptide] (solution 2) and further incubated for 2 h (or 12 h) at 37 °C in fresh complete medium. Under these conditions, peptides were internalized and co-localized with the rhodamine-labelled psODN into endocytic vesicles throughout the cytoplasm (results not shown). After 12 h of chase, no difference was observed with the fluoresceinylated E5WYGC-*F* or (E5WYGGAA)₂KAC-*F* peptide. Apparently, the two peptides were internalized in compartments also containing the ODN. The absence of ODN delivery to the cytosol may have been due to too low a concentration of the peptides in the vesicles and/or a lack of acidification of the vesicles where the peptides are located.

Flow cytometry analysis

In order to answer these questions and to understand why the dimeric peptide was inefficient, fluorescent peptide uptake was followed by flow cytometry analysis. To determine if the peptides were internalized in acidic compartments, cell fluorescence was measured before and after a post-treatment with monensin, a

Table 3 Uptake of fluorescein-labelled peptides upon incubation in the absence or presence of psODN: intracellular amount estimated as the cell-associated fluorescence intensity and estimated pH of vesicular compartments in which they were concentrated

A549 and HeLa cells (3×10^5 cells) were incubated at 37 °C for 2 h in 200 μ l of DMEM containing 10 μ M of fluorescent peptides E5CA-F, E5WYGC-F or (E5WYGGAA)₂KAC-F in the absence (—) or presence (+) of 0.1 μ M psODN. After two washings in complete medium, cells were incubated at 37 °C for 2 h with 1 ml of complete DMEM at neutral pH, harvested and resuspended. Cell fluorescence intensity (*I*, expressed as relative fluorescence units) was measured by flow cytometry before and after post-incubation with 50 μ M monensin at 4 °C for 30 min. The ratio *r* (post-monensin cell fluorescence intensity/pre-monensin cell fluorescence intensity) was used to determine the pH of endosomal vesicles with a calibration curve representing the fluorescence intensity of fluorescein-labelled peptide at different pH values (see Figure 4).

Peptide	— psODN			+ psODN		
	<i>I</i>	<i>r</i>	Estimated pH	<i>I</i>	<i>r</i>	Estimated pH
A549 cells						
E5CA-F	18	1.2	6.7	12	1.2	6.7
E5WGC-F	33	1.4	6.35	21	1.4	6.4
(E5WYGGAA) ₂ KAC-F	158	1.3	6.8	117	1.4	6.7
HeLa cells						
E5CA-F	56	1.8	6.4	41	2.0	6.3
E5WGC-F	92	1.7	6.2	79	1.5	6.3
(E5WYGGAA) ₂ KAC-F	197	1.9	6.4	164	1.6	6.55

Figure 4 pH dependence of the fluorescence of anionic peptides

Stock solution of both fluorescein-labelled peptides E5WYGC-F (■) and (E5WYGGAA)₂KAC-F (◇) was diluted to 2 μ M with the following buffer: at pH 7.4 in 50 mM phosphate buffer/0.15 M NaCl, and at pH 4.6–6.7 in 50 mM citrate/phosphate buffer/0.15 M NaCl. The fluorescence intensity was recorded at 520 nm (emission wavelength) under an excitation at 490 nm.

sodium-proton ionophore that neutralizes acidic vesicles [28]. A549 and HeLa cells were incubated 2 h at 37 °C with a mixture of 10 μ M peptide-F (final concentration) and 0.1 μ M unlabelled psODN (solution 3). Cell-associated fluorescence was measured after a 2 h chase at 37 °C in fresh complete medium. As shown in Table 3, the cell-associated fluorescence intensity was higher after monensin post-treatment in both cell types, indicating that the peptides accumulated in slightly acidic compartments. The estimated pH (Table 3), using a calibration curve of peptide fluorescence intensity, measured at 490 nm, versus the pH (Figure 4), were between pH 6.2 and 6.7; these pH values are at or above

the limit for efficient permeabilization by the E5CA or E5WYGC peptides (Table 2). The estimated pHs of vesicles reached by the monomeric or dimeric peptides incubated with the cells alone or in the presence of psODN were quite similar, whatever the nature of the peptide and the cell type (Table 3). Surprisingly, the uptake of the dimeric peptide appeared to be higher than that of the monomeric peptides. In addition, as shown by cell-associated fluorescence intensity, the intracellular concentration of fluorescein-labelled peptide was lower when cells were incubated in the presence of psODN than when the cells were incubated with the peptides alone (Table 3). However, the intracellular concentration of the monomeric peptide was probably too low to induce a vesicular permeabilization. Indeed, as shown above, when the extracellular medium was lowered at acidic pH, the efficiency of the peptide decreased rapidly when its extracellular concentration decreased below 20 μ M. According to these data, the lack of permeabilization of intracellular vesicles when cells were incubated in the presence of either the monomeric or the dimeric peptides at neutral pH can be partly attributed to the relatively high pH of these compartments, and/or to too low a peptide concentration inside these compartments.

DISCUSSION

The limited cell uptake of ODN and the sequestration of a large proportion of ODN in irrelevant cell compartments are two main impairments in their use as therapeutic drugs. To overcome these limitations, various peptides derived from the fusion peptide sequence of the haemagglutinin HA2 subunit of influenza virus have been designed and tested. This viral peptide involved in inducing the fusion between the viral and the endosomal membranes allows the viral DNA to be delivered into the cytosol of the host cell. An anionic peptide E5CA, derived from this viral peptide, was shown to help the transfer of plasmids in various transfection assays [19], and to induce a rapid cell uptake of both *F*-psODN and fluorescein-labelled phosphodiester-type ODN upon transient acidification of the extracellular medium [15]. Dimeric peptides, obtained by linking the C-terminal amino acid to a simple stem peptide, were found to be more efficient than their monomers in transferring genes with plasmidic DNA–glycosylated poly-lysine complexes [22] or DNA–poly-lysine–transferrin complexes [20,21]. Furthermore, Freulon et al. [31] showed that the transfection efficiency of cells was higher in the presence of the dimer (E5WYGGAA)₂KA (8×10^5 relative light units/ 10^6 cells) than in the presence of the corresponding monomer E5WYG (1.5×10^5 relative light units/ 10^6 cells) or in absence of any anionic peptide (3×10^3 relative light units/ 10^6 cells), and found that the dimer efficiency was dependent on the length of the spacer which links the two monomeric stretches.

In order to shed light on the mechanism leading to a facilitated uptake of ODN, and to design an optimized system allowing a direct entry of the ODN into the cytosol, the efficiencies of the monomeric peptide E5WYG and the dimeric peptide (E5WYGGAA)₂KA were compared. By confocal microscopy, we showed that the E5WYG peptide was more efficient in opening the way for ODN to reach the nucleus. E5WYG permeabilization started at pH 6.2 (20 % of cells were permeabilized within 30 min) and increased when the pH of the medium was lowered to pH 6.0 (50 % of cells permeabilized).

Under these conditions, the fluorescein-labelled monomeric peptide (E5WYGC-F) was mainly retained in the cytoplasm whereas R-psODN was in the nucleus. It should be noted that the nature of the fluorophore tag (fluorescein or rhodamine) did not affect the localization of the ODN, as previously reported by others [32]. The E5WYG-induced permeabilization increases

with the three following parameters: an increase in the peptide concentration, an increase in the incubation time and a decrease in the surrounding medium pH. When the surrounding pH was too high, no permeabilization occurred, and both the *R*-psODN and the fluorescein-labelled peptide were sequestered into the same vesicular compartments. Surprisingly, whatever the concentrations used, the dimeric (E5WYGGAA)₂KA peptide allowed neither a cytosolic nor a nuclear delivery of *F*-psODN; both dimeric peptide and ODN remained entrapped in slightly acidic compartments. At neutral pH, after 2 h of incubation at 37 °C, the psODN in the presence of either the monomeric or the dimeric peptides was delivered into neither the cytosol nor the nucleus; the psODN always appeared concentrated in endocytotic vesicles. This lack of delivery was still observed after a further 2 or 12 h of chase. By flow cytometry analysis, it was shown that monomeric and dimeric peptides, incubated in the absence or presence of psODN, reached, upon endocytosis, similar acidic compartments and that the dimeric peptide was more efficiently taken up into cells than the monomeric peptide in any of the cell types used.

A putative mechanism to account for the dimer and monomer inefficiency upon endocytosis at neutral pH is related to the limited peptide uptake leading to a concentration below the threshold required for eliciting a permeabilizing activity. Furthermore, according to a fluorescein-based estimation, the mean pH of the compartments, where the peptides were located upon endocytosis in the presence of psODN, was equal to or above 6.3. The differential efficiency with regards to psODN entry into the cytosol and the nucleus must be related to differential physico-chemical behaviour. The fluorescence quenching of the fluorescein-labelled dimeric peptide was less sensitive to a decrease in pH than that of a monomeric peptide (between 6.0 and 7.0, respectively), as if the fluorescein in the dimeric form was hidden with regards to the environment. However, the tryptophan fluorescence intensity was constant whatever the pH used and the peptide analysed. This result shows that with both the monomeric and dimeric peptides, the tryptophan residues were highly solvated and were not, even at pH as low as 5.6, in a hydrophobic environment, as it should have been if non-solvated aggregates occurred. However, by gel-filtration chromatography it was found that the dimeric peptide behaves as an oligomer at slightly acidic pH (already at pH 6.8). Those aggregates contained roughly 100 molecules. Such aggregates are obviously fully solvated because neither the tryptophan fluorescence intensity nor the maximum wavelength (350 nm) of the emitted light was altered. In contrast, the ellipticity of the dimeric peptide (E5WYGGAA)₂KA increased more readily upon lowering the pH than that of monomeric peptides. Indeed, the transition from a random form at neutral pH to a helical form at low pH, started below pH 6.0 for the monomeric form, suggesting that the aggregation of the dimeric form accompanies the helical conformation.

The anionic peptides used are readily soluble in aqueous solvent at neutral pH and exhibit a more or less random coil structure. However if they are able to reach a bilayer, they may interact with the bilayer and adopt an α -helical conformation more easily, as has been observed for melittin [33], E5 peptide [18], magainin [34] and other amphipathic polypeptides [35] as well as in the case of hydrophobic peptides flanked with di-lysine units [36]. Similarly, histidine-rich peptides, such as H5WYG [37] or LAH(4) [38], had, at pH 5.0, a low helical content. LAH(4), at pH 5.5, was 25% helical and reached a 70% helix content in the presence of membranes made of 1-palmitoyl 2-oleyl *n*-glycerol-3-phosphoglycerol. E5 exhibited a transition from a random-coil state at neutral pH to an α -helix or β -structure in solution at

acidic pH; it interacted with membranes, made of egg phosphatidylcholine, only in helical conformation at both neutral and acidic pH, causing the fusion of vesicles [18,23]. The presence of one (or more) tryptophan residue(s) may help the interaction with a bilayer as in the case of the Trp³¹ in human group V phospholipase A₂ [39], which plays an important role in the binding of the enzyme to the outer layer of the plasma membrane.

On this basis, our data suggest that the lack of permeabilizing activity of the dimeric peptides in the case of ODN is linked to an aggregation phenomenon leading to an impaired accessibility of the peptide to the bilayer; the presence of ODN, which are also polyanionic, does not help. In contrast, as in the case of gene delivery, the dimeric peptide may interact with polyplexes that contain the polycationic poly-lysine. A dimeric anionic peptide may interact with polycationic stretches more efficiently than a monomeric peptide because a putative co-operative effect is expected to increase with the length of the peptide.

In addition, the efficiency of both monomeric and dimeric peptides used with polyplexes may be related to the fact that polyplexes are large particles (from several tens to hundreds of nanometres in diameter), which can push the polyplex-associated peptides close to the endosome membranes: increasing the chance for a peptide to reach and destabilize the membrane rather than to self-aggregate as in the case of ODN and free peptides. A further parameter that should be taken into consideration is that the amount of ODN which may be taken up by a cell through endocytosis is much lower than the amount which can enter a cell upon plasma-membrane permeabilization. Indeed, ODN, even in a cellular experiment, are efficient when they are used in concentrations close to 1 μ M. According to the size of the nucleus ($\approx$ 5 μ m in diameter) and accepting that all the ODN will enter the nucleus, about 300 000 molecules of ODN are required to elicit activity in a cell. Considering the small volume of the endosomal vesicles, the dilution of the luminal material, when vesicles fuse with large endosomes, induces a decrease in the concentration of both the ODN and the peptides; consequently the level of permeabilization of the endosomal membranes is impaired and there is a little chance that the delivery of ODN to the nucleus be high enough to reach an efficient concentration. Indeed, based on the use of fluorescein-labelled ODN, both the monomeric and dimeric peptides failed to lead enough ODN to see them in the nucleus by confocal microscopy.

Finally, the lack of activity of both the dimeric and the monomeric forms of the peptides taken up inside endosomes (keeping the medium surrounding the cells at pH 7.4) is related to a pH that is not low enough, and to the fact that neither the ODN nor the peptides, which are both polyanionic, are able to induce a swelling effect, as in the case of the use of polyethylene imine [10], histidylated poly-lysine [40] or chloroquine for gene transfer [41] as well as for ODN transfer [11].

In summary, we have shown that a monomeric anionic peptide such as E5WYG is quite efficient for delivering ODN into the cell nucleus upon a simple transient acidification of the extracellular medium. Based on the short-term action of a slightly acidic medium, cells retain their viability and their capacity to grow. This procedure may be applied to study various parameters associated with the activity of ODN at a cellular level, such as the interaction with their targets, the role of various substituents, the dependence on the nature of the backbone or the minimal ODN concentration required to inhibit a given gene's expression.

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