3510–3517 *Nucleic Acids Research, 1999, Vol. 27, No. 17*

A novel potent strategy for gene delivery using a single peptide vector as a carrier

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Received May 7, 1999; Revised and Accepted July 8, 1999

ABSTRACT

We have shown previously that a peptide, MPG, derived from the hydrophobic fusion peptide of HIV-1 gp41 and the hydrophilic nuclear localisation sequence of SV40 large T antigen, can be used as a powerful tool for the delivery of oligonucleotides into cultured cells. Now we extend the potential of MPG to the delivery of nucleic acids into cultured cells. *In vitro*, MPG interacts strongly with nucleic acids, most likely forming a peptide cage around them, which stabilises and protects them from degradation in cell culture media. MPG is non-cytotoxic, insensitive to serum and efficiently delivers plasmids into several different cell lines in only 1 h. Moreover, MPG enables complete expression of the gene products encoded by the plasmids it delivers into cultured cells. Finally, we have investigated the potential of MPG as an efficient delivery agent for gene therapy, by attempting to deliver antisense nucleic acids targeting an essential cell cycle gene. MPG efficiently delivered a plasmid expressing the full-length antisense cDNA of human cdc25C, which consequently successfully reduced cdc25C expression levels and promoted a block to cell cycle progression. Based on our results, we conclude that MPG is a potent delivery agent for the generalised delivery of nucleic acids as well as of oligonucleotides into cultured cells and believe that its contribution to the development of new gene therapy strategies could be of prime interest.

INTRODUCTION

The major barrier to the development of gene/antisense therapy is the poor permeability of the cell membrane to nucleic acids (1–4). Despite the myriad of different transfection approaches available, including techniques based on the use of cationic liposomes (5–8), of polymeric DNA-binding cations (9–12) or of recombinant viral vectors (1–4), most are limited in their overall efficiency. As such, in order to improve the means of delivering sense and antisense nucleic acids into cells, research in the field of gene therapy has focused on the design of new non-viral vectors and on the development of corresponding techniques for efficient gene delivery.

Most methods developed for gene delivery involve transport of the gene of interest into cells via the endosomal pathway, which unfortunately leads to its extensive degradation in the acidic lysosomal compartments. In search of novel carriers, research in this field has focused on compounds that are able to perturb or to disrupt the lysosomal membrane and that reduce the degradation of the gene of interest in the lysosome. Several groups have already shown that lysine-rich peptides and cationic peptides, derived from viral proteins which mimic the endosomal disruptive properties of viral particles, penetrate cells and facilitate the delivery of nucleic acids (13–17). In addition, cationic peptide synthetics which adopt a structure that potentially allows crossing the liposomal bilayer membranes and then promotes the release of nucleic acids have been proposed (15–17).

We have developed a peptide vector, MPG, derived from both the nuclear localisation sequence (NLS) of SV40 large T antigen and the fusion peptide domain of HIV-1 gp41 and have shown recently that MPG could deliver short oligonucleotides into cells efficiently and independently of the endosomal pathway (18,19). In the present work we show that MPG exhibits high affinity for nucleic acids and protects them from degradation. We demonstrate that the use of MPG as an efficient agent for the delivery of short oligonucleotides can be generalised to the efficient delivery of plasmid expression vectors. Moreover, MPG was successfully used to deliver a full-length antisense cDNA encoding human protein phosphatase cdc25C, thus knocking out its mitotic function in cultured mammalian cells, and thereby supporting the potential of MPG for targeting cellular proteins efficiently in a therapeutic context.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (PBS) were from BioWhittaker (Walkersville, MD, USA). L-Glutamine, penicillin, streptomycin and trypsin were from Imperial Laboratories (London, UK). Foetal calf serum (FCS) was from Gibco BRL (Rockville, MD, USA). Lipofectamine® was purchased from Life Technologies Inc. (Rockville, MD, USA). The pRL-SV40 plasmid encoding a *Renilla reniformis* luciferase gene under control of an SV40 promoter was from Promega (Madison, WI, USA). The pJ3O expression plasmid was provided by Dr P. Jay (IGH, Montpellier, France), the pJ3O-sense and pJ3O-antisense Hucdc25C plasmids,

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carrying the full-length sense and antisense cDNA encoding human cdc25C, respectively, were constructed by Dr M. C. Morris (20). pcDNA3.1NT-GFP expression plasmid encoding GFP was from Invitrogen (Carlsbad, CA, USA). Polyclonal rabbit antibody against human cdk2 (#sc-163) was purchased from Tebu, Santa Cruz. An affinity-purified polyclonal antibody against human cdc25C was kindly provided by Dr P. Russell (TSRI, La Jolla, CA, USA). The Oligotex Direct mRNA kit was obtained from Qiagen (Hilden, Germany). The digoxigenin (DIG) labelling/detection kit was purchased from Boehringer Mannheim (Mannheim, Germany). cDNA encoding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was a gift from Dr P. Jay.

Peptide synthesis and analysis

MPG was synthesised by solid-phase peptide synthesis (21,22) using AEDI-expensin resin with a 9050 Pepsynthetiser (Millipore, Watford, UK) according to the Fmoc/tBuc method, as described previously (22). MPG was purified by semi-preparative HPLC and identified by electrospray mass spectrometry and amino acid analysis (22). For cellular localisation MPG was coupled with lucifer yellow iodoacetamide dipotassium salt (Molecular Probes, Eugene, OR, USA) as described previously (22).

Fluorescence titrations

Fluorescence experiments were performed on a Spex II Jobin Yvon spectrofluorimeter. The intrinsic tryptophan fluorescence of MPG was routinely excited at 290 nm in order to minimise the substrate inner-filter effect and the emission, spectrum was recorded between 310 and 380 nm, with a spectral bandpass of 2 and 8 nm for excitation and emission, respectively. A fixed concentration of MPG (1×10^{-8} to 1×10^{-6} M) was titrated by increasing the concentration of each plasmid (from 0 to 10 nM) at 25°C in a buffer containing 17 mM KH_2PO_4, 5 mM Na_2HPO_4, 150 mM NaCl, pH 7.4. All measurements were corrected for the equipment and the dilution and curve fitting was performed with the Grafit program (Erithacus Software Ltd (Middlesex, UK) using a quadratic equation which allowed the determination of the MPG/plasmid ratio (18,19).

Stability and DNase I protection assays

An aliquot of 0.5 µg of the pRL-SV40 plasmid encoding the reporter *R.reniformis* luciferase was incubated for 20 min at 25°C in PBS buffer, with an MPG concentration corresponding to a peptide/DNA charge ratio ranging from 0:1 to 15:1.

Gel retardation assays. MPG/plasmid preparations (corresponding to 100 ng of DNA) were analysed by electrophoresis on a 1% agarose gel in TBE buffer, followed by staining with ethidium bromide.

DNase I protection assays. Preformed MPG/plasmid complexes were treated with DNase I (0.5 µg/ml) in 50 µl of reaction buffer, containing 21 mM HEPES–NaOH, pH 7.5, 135 mM NaCl, 5.0 mM KCl, 0.76 mM Na_2HPO_4 10 mM $MgCl_2$ and 10 mM $CaCl_2$. After 30 min at 37°C, reactions were stopped by addition of 4 M ammonium acetate and 20 mM EDTA and immediate chilling on ice.

Serum protection assays. Preformed MPG/plasmid complexes were incubated for 5 h in the presence of cell culture medium containing 10% FCS. For both DNase I and serum protection assays, plasmids were extracted with phenol–chloroform, precipitated with ethanol, and then analyzed by agarose gel electrophoresis (1% w/v).

Cell culture, cytotoxicity assays, MPG-mediated transfection

Adherent fibroblastic HS-68 and NIH 3T3 cell lines, C2C12 myoblasts and COS-7 cells, as well as human CEM-SS lymphoblasts in suspension, were cultured in DMEM supplemented with 1% 200 mM glutamine, 1% antibiotics (streptomycin, 10 000 µg/ml; penicillin, 10 000 IU/ml) and 10% (w/v) FCS, at 37°C in a humidified atmosphere containing 5% CO_2 as described previously (23,24).

The cytotoxicity of both MPG and MPG/plasmid (pRL-SV40 plasmid encoding reporter *R.reniformis* luciferase) complexes were investigated in the cell lines mentioned above. Cells grown in 35 mm diameter dishes to 75% confluency ($0.5–1 \times 10^6$ cells per dish) were incubated with 0.1 µM to 1 mM MPG alone or complexed with DNA in a 5:1 ratio, in 1 ml DMEM for 3 h, after which 10% serum was added. Cell culture medium with MPG or MPG/plasmid was not changed, and cell proliferation was measured over 4 days. Cytotoxicity was evaluated by the MTT colorimetric assay, after removing cell culture medium and replacing it with PBS containing 5 mg/ml of MTT (25). Results shown correspond to the averages of four separate experiments.

Lipofectamine™-mediated transfections were performed as described by the manufacturer (Gibco BRL, Cergy Pontoise, France), on cells grown to 75% confluency. For MPG-dependent transfections, cells were grown to 75% confluency and overlaid with preformed MPG/pRL-SV40 plasmid complexes in DMEM (500 µl of DMEM containing 0.5 µg of plasmid, and a variable peptide/DNA charge ratio from 0:1 to 20:1, per 35 mm diameter dish or $0.5–1 \times 10^6$ cells). After 1 h incubation with MPG/plasmid at 37°C, 2 ml of fresh DMEM supplemented with 10% FCS were added to the cells, without removing the overlay of DMEM/MPG/plasmid, and cells were incubated at 37°C for another 24 h. For transfections in the presence of serum, MPG and DNA were preincubated for 15 min in DMEM, and 10% FCS was then added to this solution before overlaying onto cultured cells. Twenty-four hours after transfection, cells were scraped from their dishes and the luciferase activity of the cell extracts was determined by the luminometric method described in the commercial protocol (26). The protein concentration of cell extracts was routinely determined using the bicinchoninic acid protein assay.

For MPG-mediated delivery of pcDNA3.1NT-GFP expression plasmid encoding GFP, HS68 fibroblasts were grown in 35 mm diameter dishes, at 37°C in DMEM supplemented with 10% FCS until they reached 75% confluence ($0.6–1.2 \times 10^5$ cells per coverslip). Transfections were performed as described for the pRL-SV40 plasmid with preformed complexes of 1 µM MPG and 0.5 µg ADN in a 500 µl overlay of DMEM supplemented with 10% serum (corresponding to a charge ratio of 5:1 MPG/plasmid). After 24 h, GFP expression was monitored by fluorescence miscroscopy.

For MPG-mediated delivery of antisense nucleic acids directed against human cdc25C, HS68 fibroblasts were grown in 35 mm diameter dishes, at 37°C in DMEM supplemented with 10% FCS until they reached 75% confluence ($0.6–1.2 \times 10^6$ cells),

synchronised for 48 h by serum deprivation, then restimulated to enter the cycle and grown into mid–late G_1 for 12–14 h by addition of fresh DMEM supplemented with 10% FCS. Transfections were performed as described above, with preformed complexes of 1 μM MPG and 0.5 and 1 μg DNA in a 500 μl overlay of DMEM supplemented with 10% serum (corresponding respectively to a charge ratio of 5:1 and 10:1 MPG/plasmid). Forteen hours after transfection, cells were scraped from their dishes and cdc25C and cdk2 protein levels were evaluated by western blotting. Protein samples were separated on 12.5% acrylamide electrophoresis gel, electrotransferred onto polyvinylidene difluoride membrane (Schleicher & Schuell, Northein, Germany) and probed with either polyclonal rabbit anti-human cdc25C or polyclonal rabbit anti-human cdk2, followed by horseradish peroxidase-conjugated goat anti-rabbit secondary antibody. Signals were revealed with the western blotting detection kit (Amersham, pharmacia Biotech, Upsalla, Sweden) and membranes were exposed to X-Omat AR film (Kodak, Eastman Kodak, Cochester, NY, USA).

Extraction of mRNAs and northern blotting

mRNA samples were directly prepared using the Oligotex Direct mRNA kit, as described by the manufacturer, from 6×10^6 cells either mock transfected with MPG/pJ3O vector, or transfected with MPG/pJ3O-antisense Hucdc25C. mRNA samples were diluted 1:1 in sample buffer containing 50% formamide, 10% bromophenol blue, 20% formaldehyde, 5 mM sodium acetate, 1 mM EDTA in 20 mM MOPS, pH 7.0, heated for 5 min at 65°C, and chilled on ice before loading onto a 1.25% agarose gel in MOPS/formaldehyde buffer (5 mM sodium acetate, 1 mM EDTA, 20 mM MOPS, pH 7.0, 3% formaldehyde, 1 μl ethidium bromide; 10 mg/ml per 100 ml gel). Samples were separated on the gel run at 5 V/cm in MOPS/formaldehyde buffer. Standard mRNA transfer from the gel onto an N+ Hybond membrane was performed overnight in 20× SSC (3 M NaCl, 0.3 M sodium citrate) 2% formaldehyde. mRNAs were fixed onto the membrane by heating for 2 h at 80°C. DIG-labelled control GAPDH probe and Hu-cdc25C probe were prepared as described by the manufacturer from 50 ng GAPDH and 200 ng Hu-cdc25C PCR product amplified through 40 cycles of 1 min at 94°C, 2 min at 56°C and 2 min at 72°C, with oligonucleotides: 5'-CGGGATCCCGATGTCTACGGAACTCTTCTCATCC-3' and 5'-CCCCATGGGGTCATGGGCTCATGTCCTTCACCAG-3'. Pre-hybridation, hydridisation, washes and detection were performed as described in the DIG labelling/detection protocol.

Fluorescence techniques, photography and image processing

Cells expressing GFP were directly observed on a LEICA DM IRB using a 40× 1.4 NA lens. Fluorescent images were shot using a Hamamatsu CCD camera directly connected to a PC, and acquired in Adobe Photoshop version 4.0. Images were transferred to a Silicon graphics O2 workstation and converted to SGI raster format using 'convertfile'. Figures were assembled completely and prepared for printing under SGI Showcase 3.2.

RESULTS AND DISCUSSION

Peptide vector design and characterisation

The peptide vector MPG was designed so as to satisfy the major requirements for efficient gene delivery, including the ability to cross the cell membrane, the high affinity and specificity for nucleic acids, and a particular selectivity for the nuclear versus cytoplasmic compartment (18,19). As such, the 27-residue MPG peptide 'GALFLGFLGAAGSTMGAWSQPKSKRKV' was derived from two independent domains: a hydrophobic domain (residues 1–16) issued from the fusion sequence of HIV-1 gp41 (27,28) required for efficient crossing of the cell membrane, and a hydrophilic domain (residues 20–27) derived from the NLS of SV40 large T antigen, (29), required for the nuclear addressing of the peptide. MPG is stable and highly soluble in physiological conditions and presents a very versatile structure, an unusual feature which is most likely key for its cellular uptake (30,31). In standard cell culture conditions, MPG localises rapidly to the nucleus of human HS68, murine NIH 3T3 fibroblasts and simian kidney SV40 transformed cos-7 (18).

Formation of MPG/DNA complexes

We have investigated to what extent MPG could interact with nucleic acids using the pRL-SV40 plasmid (3.2 kb), which expresses *R.reniformis* luciferase under control of the SV40 enhancer/promoter region (26), and the pJ3O-antisense-Hucdc25C plasmid (4.9 kb), which encodes the antisense full-length cDNA sequence of human cdc25C under control of an SV40 promoter (20). Three different approaches were used to monitor peptide/DNA interactions: (i) quenching of intrinsic tryptophan fluorescence; (ii) agarose gel-shift assay; (iii) DNase I or serum nuclease protection assays.

MPG contains a single Trp-residue at position 18, located between the NLS and the fusion peptide domains, which constitutes a sensitive probe for monitoring interactions between MPG and nucleic acids (7,8). As shown in Figure 1A, the binding of nucleic acids to MPG induced a marked quenching of the intrinsic fluorescence of MPG with a saturating value of 23%, without modifying the fluorescence maximum emission wavelength (340 nm), which indicates that the Trp residue interacts directly with either the phosphate or the nucleoside moieties of the nucleic acid. It should be noted that in the absence of nucleic acids, the intrinsic fluorescence of MPG varied linearly with its concentration, suggesting that MPG molecules do not self-associate in such conditions (data not shown). Fitting of the titration binding curves revealed that MPG presented high affinity for both pRL-SV40 and pJ3O-antisense-Hucdc25C, with respective dissociation constant (K_d) values of 0.5×10^{-8} M^{-1} and 1.8×10^{-8} M^{-1}, affinity thus correlating inversely with the size of the plasmid. In both cases, saturation occurred at a concentration of DNA 1000-fold lower that of MPG (1.0×10^{-5} M), i.e. at a ratio of peptide per nucleic acid at least 10–20-fold higher than that predicted for achieving charge neutralisation between the NLS moiety of MPG (three Lys and one Arg) and the phosphate groups in nucleic acids.

Similarly to the nature of interactions proposed to occur upon association of lysine-rich peptides with DNA (4), association of MPG with DNA most likely mainly involves electrostatic interactions dependent on the cationic residues in the NLS of MPG. However, as a larger number of MPG peptides than that theoretically required for charge neutralisation of bound DNA molecules was actually involved in the formation of the MPG/DNA complex, we hypothesised that additional interactions

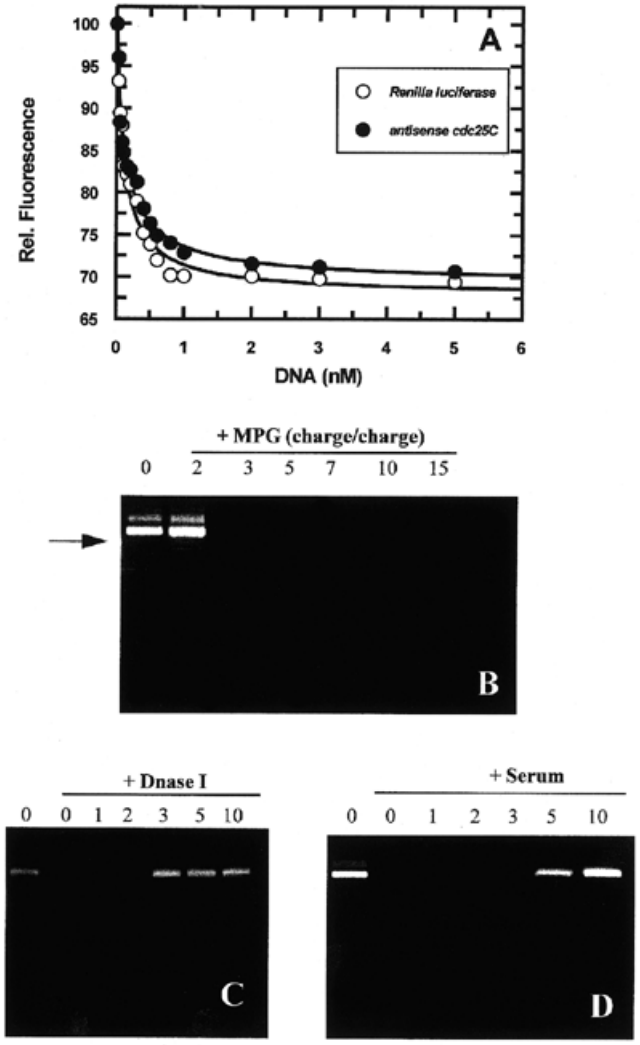

Figure 1. Formation of MPG/DNA complexes. (**A**) Binding of MPG to DNA monitored by intrinsic fluorescence quenching. The intrinsic fluorescence of MPG was routinely excited at 290 nm and the fluorescence emission was recorded at 340 nm. A fixed concentration of MPG (10 μM) was titrated by increasing the concentration of pJ3O-antisense-Hucdc25C plasmid (filled circles) and pRL-SV40 (open circles) (from 0 to 10 nM) at 25°C in PBS. The curves were fitted according to a quadratic equation, in order to determine the MPG/DNA ratio. The best fit yielded K_d values of 1.8×10^{-8} and 0.5×10^{-8} M for the pJ3O-antisense-Hucdc25C plasmid and pRL-SV40 plasmid respectively, with a maximal quenching of fluorescence of 28 and 32% respectively, obtained at saturating concentrations. (**B**) Agarose gel-shift assay. The pRL-SV40 plasmid encoding the reporter protein *R.reniformis* luciferase was incubated for 20 min at 25°C in PBS buffer with different concentrations of MPG corresponding to a charge ratio ranging between 1 and 15, as indicated above each lane. The preformed complexes were analyzed by electrophoresis on agarose gel (1% w/v) stained with ethidium bromide. Lane 1, plasmid DNA control in the absence of MPG; lanes 2–7, charge ratios of 2, 3, 5, 7, 10 and 15 respectively. (**C**) DNase I and nuclease protection assays. The pRL-SV40 plasmid was incubated at 25°C in PBS in the presence of increasing concentrations of MPG corresponding to charge ratios shown in (A), as indicated above each lane. The preformed MPG/DNA complexes were treated with DNase I (0.5 μg/ml) and the residual plasmids were extracted with phenol–chloroform and analysed by agarose gel electrophoresis (1% w/v). Lanes 1 and 2, purified DNA, untreated and treated with DNase I respectively. A total DNA protection was obtained for an MPG/DNA charge ratio of 3 (lane 5). (**D**) Stability against serum. The preformed MPG/DNA complexes were incubated for 5 h in the presence of cell culture medium supplemented with 10% serum. Lane 1, control plasmid; lane 2, plasmid incubated in the presence of serum. Full DNA protection was obtained for a charge ratio of 5.

might take place between peptides, which, as such, most likely formed a 'cage' around the molecule of DNA.

In an attempt to confirm this hypothesis, we investigated the formation of peptide/DNA complexes by monitoring their electrophoretic mobility as a function of the positive (MPG)/negative (DNA) charge ratios (between 0:1 and 15:1) on an agarose gel (1% w/v) stained with ethidium bromide. As shown in Figure 1B, the electrophoretic mobility of DNA was completely abolished for a charge ratio 3-fold higher than the neutralisation charge ratio (lane 3). The lack of migration suggested the formation of a large complex between MPG and the DNA, in agreement with the results obtained in the fluorescence titration experiments.

We further hypothesised that the formation of a such a peptide 'cage' around the DNA might prevent its degradation by nucleases. As such, we evaluated the nuclease-protective effect of MPG by incubating complexes of MPG/DNA varying in their charge ratio with DNase I, and analysing the subsequent integrity of DNA on an agarose gel. As shown in Figure 1C (lane 5), in MPG/DNA complexes with a charge ratio up to 2:1, DNA was subject to complete degradation by DNase I, whereas at higher ratios the concentration of MPG was sufficient to fully protect DNA, in which case the DNA migration band was perfectly detectable. Similar experiments performed with 10% FCS instead of DNase I revealed that DNA was fully degraded in the presence of serum (lane 2) up to a charge ratio of 5:1 (MPG/DNA), required for full protection of the DNA (Fig. 1D, lane 6), instead of 3:1 in the presence of DNase I. The difference observed with nuclease versus serum suggests that in the serum not only nucleases, but also proteases, take part in the degradation process; this difference may, however, also reflect the differential protection of the plasmid to purified DNase I versus to serum nucleases.

Finally, we sought to provide further evidence for the model of the peptide 'cage' of MPG surrounding nucleic acids, using quasielastic light scattering (QELS). The results of our preliminary experiments are in agreement with this model, as they reveal the existence of particles of MPG/DNA with an average diameter of 200–300 nm, detected for a charge ratio of 3:1 (data not shown).

MPG promotes efficient delivery and expression of nucleic acids in different cell lines

We next evaluated the ability of MPG to deliver DNA into different cell lines including HS-68 and NIH 3T3 fibroblasts, C2C12 myoblasts and cos-7 cells using the pRL-SV40 reporter system encoding *R.reniformis* luciferase, and compared the efficiency of transfection with that of the commonly used Lipofectamine®-based technique (5,6). pRL-SV40 (0.5 μg) was first incubated with different concentrations of MPG, corresponding to positive (MPG)/negative (DNA) charge ratios from 1:1 to 20:1. Cultured cells ($0.5–1 \times 10^6$) were then overlaid with the preformed MPG/DNA complexes in 500 μl DMEM for 1 h in the presence or absence of FCS. Following this transfection step, fresh DMEM supplemented with serum was added and the expression of luciferase was monitored 24 h later in a luminometric assay. The efficiency of transfection in the absence of serum for different concentrations of MPG, as judged by the expression of luciferase, is reported in Figure 2A. Maximal transfection and expression of luciferase were obtained for a peptide/DNA charge ratio >5:1, corresponding

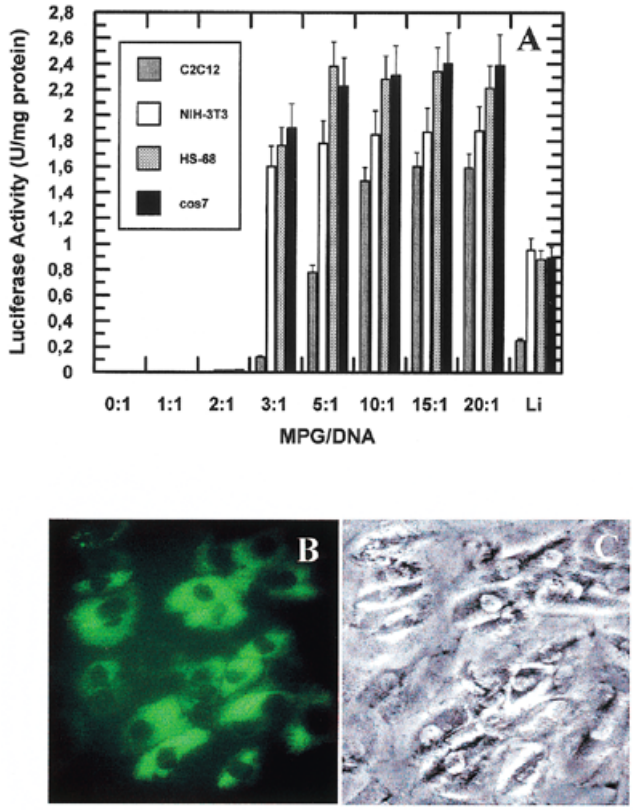

Figure 2. MPG-mediated pRL-SV40 and pcDNA3.1NT-GFP plasmid delivery. (**A**) Different cell lines were incubated in the presence of preformed MPG/DNA complexes (varying in the MPG/DNA charge ratio up to 20:1, corresponding to an MPG concentration of 1.6 µM; DNA being the reporter pRL-SV40 plasmid encoding *R.reniformis* luciferase) for 1 h at 38°C, after which they were replaced in DMEM supplemented with 10% FCS. Twenty-four hours later, cell extracts were prepared and their luciferase activity was determined and reported as a function of total protein. Similar transfection experiments were performed as controls, using Lipofectamine® and for 4 h transfection, as described in the commercial transfection protocol. Maximal transfection efficiency using MPG was obtained for a charge ratio of 10:1, yielding luciferase activities of 2.4, 2.45 and 1.9 U/mg of protein for HS-68, COS-7 and NIH 3T3 cells, respectively. For C2C12 cell lines, maximal transfection was obtained for a charge ratio of 15:1, yielding a luciferase activity of 1.6 U/mg of protein. Using Lipofectamine® this value was at least twice and seven times lower, corresponding to a luciferase activity of ~0.9 and 0.3 U/mg of protein. Each result corresponds to the average of four separate experiments. HS68 fibroblasts cultured on glass coverslips in DMEM supplemented with 10% FCS were transfected with MPG/pcDNA3.1NT-GFP complexes (ratio 5:1). After 24 h transfection, the cells were analysed by fluorescence and phase-contrast microscopies. (**B**) GFP expression; (**C**) corresponding phase-contrast image.

to a concentration of MPG of 0.4 µM, with a specific luciferase activity between 1.9 and 2.42 U/mg of protein for COS-7, NIH 3T3 and HS-68 cell lines. Once again, these results are consistent with the model of a peptide 'cage' surrounding the DNA molecule being required for its efficient transfection. In the case of C2C12 cells, maximal transfection was obtained for a charge ratio of 15:1, which corresponds to a peptide concentration of 1.2 µM, with a specific luciferase activity of 1.6 U/mg of protein.

The degree of transfection using MPG was at least twice and seven times higher than that obtained with Lipofectamine® for fibroblasts or COS-7 and C2C12 myoblasts, respectively. These transfection data with MPG reveal that the preformed MPG/DNA complex is efficiently driven into the cytoplasm of cells and that the presence of MPG molecules around the DNA does not modify the potential of its gene product to be expressed. As a control, we verified that in the absence of MPG no transfer of DNA occurred, confirming that free DNA cannot cross the cell membrane alone and that it is most likely rapidly degraded in the presence of serum, as already described in Figure 1D.

Finally, we evaluated the impact of serum in the cell culture medium on the efficiency of transfection of MPG. Experiments with an MPG/DNA charge ratio of 10:1 were performed in the presence of 10% FCS, a concentration commonly used in cell culture. As shown in Table 1, the efficiency of MPG remained insensitive to this percentage of serum in HS-68 and COS-7 cell lines and was reduced only by 10 and 16% in C2C12 and NIH 3T3 cells. In contrast, when transfections were preformed with Lipofectamine™ in the presence of 10% FCS, the efficiency of gene delivery was dramatically reduced, by up to 60%.

Table 1. MPG-mediated pRL-SV40 plasmid delivery

Cell lines	Luciferase activity (U/mg protein)	
	– serum	+ serum
cos-7	2.38 ± 0.4	2.4 ± 0.3
HS-68	2.3 ± 0.25	2.25 ± 0.12
NIH 3T3	1.9 ± 0.3	1.6 ± 0.15
C2C12	1.52 ± 0.24	1.37 ± 0.18

Different cell lines were incubated in the presence of preformed MPG/DNA complexes (charge ratio of 10:1) in the presence or absence of 10% FCS, DNA being the reporter pRL-SV40 plasmid encoding *R.reniformis* luciferase for 1 h at 37°C, after which they were replaced in DMEM supplemented with 10% FCS. Twenty-four hours later, cell extracts were prepared and their luciferase activity was determined and reported as a function of total protein. The results correspond to the averages of four separate experiments

The efficiency of MPG to deliver DNA into HS68 fibroblasts was determined with the pcDNA3.1NT-GFP expression plasmid encoding GFP. HS68 cells were transfected as described for luciferase, in the presence of 10% FCS. GFP expression was monitored by fluorescence microscopy 24 h after transfection. Figure 2B presents a typical field of transfected cells, which reveals that >90% of the cells expressed GFP, based on the phase-contrast image reported Figure 2C.

Cytotoxicity of MPG

In order to generalise the use of MPG as an efficient delivery agent, the degree of cytotoxicity of MPG and of the MPG/DNA complex (charge ratio 5:1) were evaluated in several cell lines, including HS-68 and NIH 3T3 fibroblasts, C2C12 myoblasts,

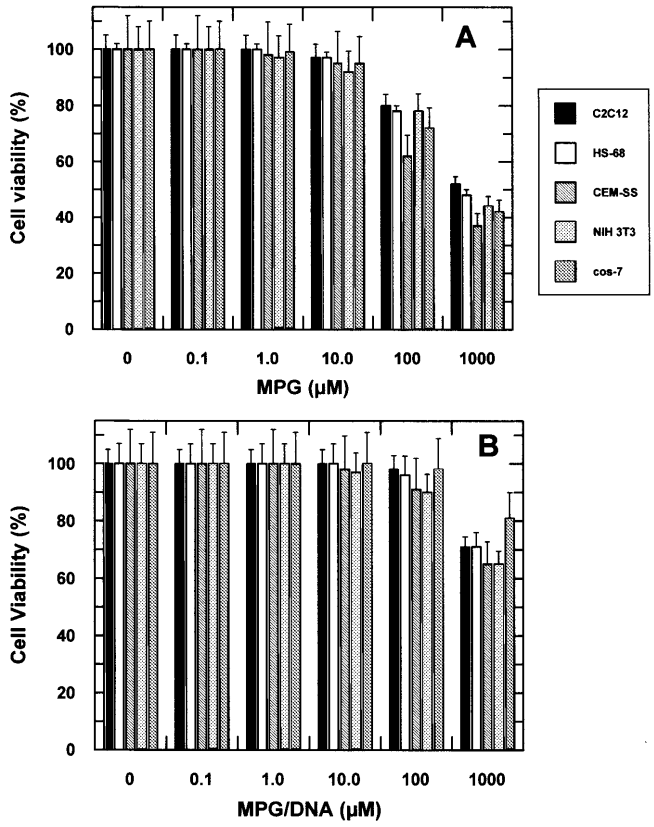

Figure 3. Cytotoxicity of MPG. HS-68, NIH 3T3, C2C12, cos-7 and CEM-SS cell lines were incubated with 0.1 μM to 1 mM MPG alone (**A**) and with MPG complexed with DNA (pRL-SV40 plasmid encoding *R.reniformis* luciferase) in a peptide/DNA ratio of 5:1 (**B**), at 37°C in DMEM supplemented with 10% FCS. The cytotoxicity of MPG alone and of MPG/DNA complexes was evaluated in MTT colorimetric assays and the results were expressed as percentages of dye reduction in cell lines incubated without MPG. The results correspond to averages of four separate experiments.

COS-7 kidney cells and human CEM-SS lymphoblasts. As shown in Figure 3A, addition of MPG to the culture medium up to a concentration of 10 μM, a concentration well above the K_d of the interaction between MPG and nucleic acids, did not induce any cytotoxic effects over a period of 48 h at 37°C, as already observed for mollicutes (wall-less bacteria) (32). At much higher concentrations of 0.1 and 1 mM MPG, however, cell viability decreased by ~25 and 60%, respectively. In contrast, when complexed with plasmid DNA (in this case pRL-SV40 at a 5:1 ratio), no cytotoxicity could be observed at a concentration of 0.1 mM MPG; cell viability decreased by only ~40% at a concentration of 1 mM (Fig. 3B). Hence, at the concentration of MPG required for large-scale efficient gene delivery (1–2 μM), no cytotoxicity occurs when MPG is complexed with a plasmid at a 5:1 ratio. These data therefore indicate not only that MPG alone is not cytotoxic at the concentrations required for efficient gene delivery, but moreover that binding of MPG to DNA actually reduces its cytotoxicity at higher concentrations.

MPG-mediated delivery of a plasmid carrying full-length antisense cdc25C into mammalian fibroblasts promotes cell cycle arrest at the G_2/M transition.

Efforts in gene therapy focus essentially on the development of strategies to target and knockout specific cellular components responsible for genetic malformations, aberrant development, disorderly growth or malignant proliferation of cells. Both the fast penetration of MPG/nucleic acids into cells and the lack of its cytotoxicity favour its use as an efficient, non-toxic and non-hazardous delivery agent in cellular applications. In an attempt to validate the potential use of MPG in gene therapy, we assessed its ability to deliver pJ3O-antisense-Hucdc25C, a plasmid carrying the full-length antisense cDNA encoding human cdc25C, which has been shown to knock out the mitotic function of human cdc25C and consequently to arrest cells at the G_2/M transition when microinjected into mammalian fibroblasts (20). The dual-specificity phosphatase cdc25C plays a key role in the control of cell cycle progression, as a renowned mitotic inducer required to promote entry into mitosis (20,33,34). Moreover, cdc25 proteins have been shown to possess an oncogenic potential and as such constitute excellent targets for gene therapy (35,36). Synchronised human HS-68 fibroblasts were grown into late G_1 phase (12–14/24–25 h cycle in this cell line) and then either mock-transfected with MPG alone, or transfected with MPG pre-incubated with the pJ3O plasmid vector, with pJ3O-sense Hucdc25C, or with pJ3O-antisense Hucdc25C, as described above for the pRL-SV40 plasmid in the presence of serum. Following transfection, cells were incubated for another 14 h before fixation, i.e. at 23–24 h post-refeeding, at which time non-transfected cells normally entered mitosis, and the levels of cdc25C and of cdk2 were evaluated by western blotting. Loss of cdc25C function, and consequent cell cycle arrest was monitored by counting the number of cells exhibiting mitotic phenotypes, with respect to the number observed in non-transfected or mock-transfected cells. Figure 4A represents the average percentage of mitotic phenotypes observed in each type of transfection experiment. Transfection of MPG or of the pJ3O vector clearly had no effect on the ability of cells to enter mitosis, as compared to non-tranfected cells. Similarly, transfection of the pJ3O-sense Hucdc25C construct did not alter the number of mitotic counts 24 h post-refeeding. In contrast, transfection of pJ3O-antisense Hucdc25C with MPG for only 1 h resulted in the efficient inhibition (70%) of entry into mitosis. The levels of cdc25C and cdk2 expression are reported in Figure 4B. Western blot analysis reveals that in control cells (lane 1) or in cells overlaid with 1 μg of pJ3O vector (lane 2), or pJ3O-sense Hu-cdc25C (lane 3) both cdc25C and cdk2 were normally expressed, confirming that MPG and control vectors do not affect cell progression. In contrast, when cells were transfected with pJ3O-antisense Hcdc25C [0.5 μg (lane 3) or 1 μg (lane 4)] levels of cdc25C protein were strongly reduced with 0.5 μg of DNA and completely abolished with 1 μg of antisense. We finally investigated the effect of MPG-mediated transfection of pJ3O-antisense Hcdc25C on the levels of Hu-cdc25C mRNA by comparison with those of cells mock-transfected with pJ3O vector, by northern blotting. As reported in Figure 4C, the normal levels of Hu-cdc25C mRNA observed in mock-transfected cells are dramatically reduced in cells transfected with MPG/pJ3O-antisense Hcdc25C complex, whereas control mRNA of

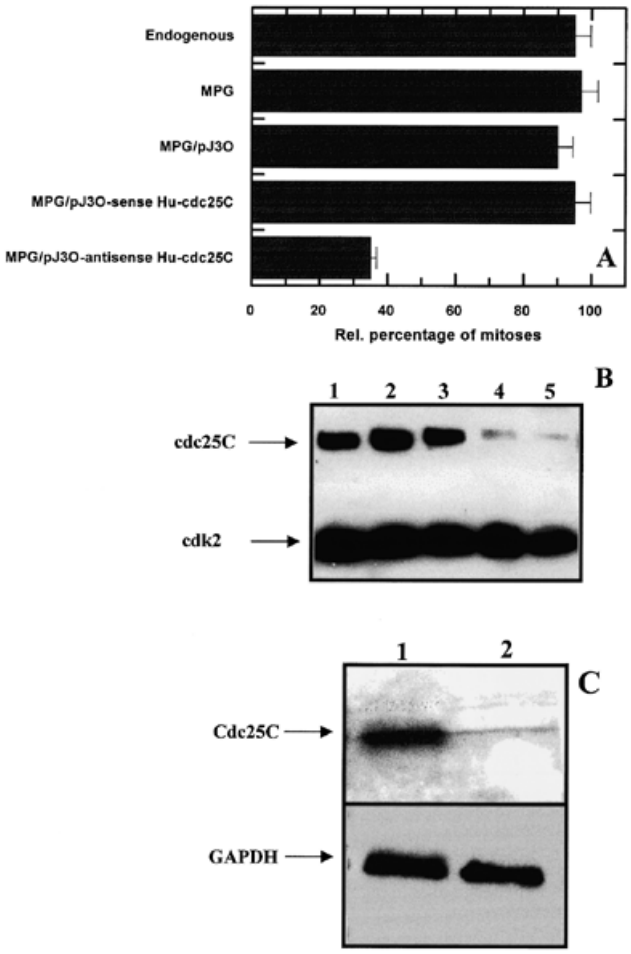

Figure 4. MPG-mediated delivery of antisense human cdc25C into mammalian fibroblasts. HS68 fibroblasts cultured in DMEM supplemented with 10% FCS were synchronised through serum starvation for 48 h, and grown into late G_1 (12–14 h post-refeeding), prior to transfection. Cells were then either mock transfected (with MPG peptide alone), or transfected with MPG/pJ3O plasmid, MPG/pJ3O-sense Hucdc25C or MPG/pJ3O-antisense Hu-cdc25C, constructs encoding full-length sense or antisense human cdc25C, respectively, under the control of an SV40 promoter. After 1 h transfection in the presence of serum, fresh DMEM supplemented with 10% serum was added and cells were incubated for another 12–14 h, then fixed with methanol and stained with an antibody against tubulin and with Hoechst. The ability of transfected cells to progress into mitosis was assessed as a function of the number of mitotic phenotypes observed (centrosome duplication, spindle formation, metaphase plate, chromosome segregation, midbody). (**A**) The average number of mitotic counts for each transfection experiment (repeated at least five times) was normalised with respect to the number of mitotic phenotypes counted in a population of non-transfected cells, 24 h post-refeeding. The results for each type of transfection experiment are consequently expressed as the relative percentage of mitotic phenotypes. (**B**) Forteen hours after transfection, cells were scraped from their dishes and the protein levels of cdc25C and cdk2 were evaluated by western blotting. Protein samples were separated by 12.5% SDS–PAGE, transferred onto a polyvinylidene difluoride membrane and probed with either polyclonal rabbit anti-human cdc25C or polyclonal rabbit anti-human cdk2, followed by horseradish peroxydase-conjugated goat anti-rabbit secondary antibody. Lane 1, control cells; lanes 2 and 3, cells mock-transfected with 1 µg of pJ3O vector and 1 µg of pJ3O-sens-Hu-cdc25C; lanes 4 and 5, cells transfected with 0.5 and 1 µg of pJ3O-antisense Hcdc25C respectively. (**C**) Northern blot analysis of Hu-cdc25C gene expression. Total RNA was prepared from cells transfected with 1 µg of pJ3O (lane 1) or 1 µg of pJ3O antisense Hu-cdc25C (lane 2) and analysed by northern blot as indicated in the Materials and Methods. The northern blot was subsequently hybridised with a GAPDH probe as control to normalise loading sample.

GAPDH is present at the same levels in both cases. These data indicate that antisense Hu-cdc25C RNA efficiently forms a hybrid with sense Cdc25C mRNA which is then rapidly degraded. The mechanism by which double-stranded hybrid antisense RNA/sense mRNA is degraded may involve a double-stranded RNA specific RNase. Taken together, these results confirm that the block to mitotic progression observed in the presence of pJ3O-antisense Hucdc25C is effectively due to an antisense effect, which leads to a decrease in the expression of cdc25C. Moreover, in none of the conditions described are the levels of cdk2 affected, which confirms the specificity of the antisense construct. Finally, as for the luciferase reporter system, the efficiency of antisense-Hucdc25C was dependent on the MPG/DNA charge ratio. When a population of cells was transfected with an MPG/antisense DNA charge ratio of 5:1, for instance, cell cycle progression was arrested in >80% of the cells.

CONCLUSIONS

In this study, we describe a new, potent method for the efficient delivery of nucleic acids into a series of different cell lines, based on the use of a single short peptide termed MPG. MPG not only presents high affinity for nucleic acids, but also protects them against nucleases. From the results presented above, we suggest that the rapid self-assembly between MPG and DNA first involves electrostatic interactions, as already proposed for cationic peptides (10–13,16,17), and then promotes further peptide–peptide interactions, leading to the formation of a protective 'cage' around the DNA molecule. Based on these results, we propose that MPG is an excellent tool for the generalised delivery of nucleic acids, as well as of oligonucleotides, into cultured cells. Finally, given that MPG provides an efficient means to target essential cell cycle proteins with oncogenic potential, such as cdc25C, it will be useful for targeting cellular components involved in genetic malformations, and other diseases. We therefore believe that MPG technology is of prime interest for the development of new gene therapy strategies.

ACKNOWLEDGEMENTS

We thank Prof. M. Dorée and Prof. R. S. Goody for fruitful discussions and continuous support to this work. We also thank Dr P. Jay and S. Chaloin for precious technical advice and the gift of reagents. This work was supported in part by the CNRS, by the GDR 1153 'Peptides et Protéines amphipathiques' and by grants from the Association pour la Recherche sur le Cancer (ARC-contract 1244), from the Ligue Nationale Contre le Cancer and the Agence Nationale de Recherche sur le Sida (ANRS).

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