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Influence of Membrane-Active Peptides on Lipospermine/DNA Complex Mediated Gene Transfer

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To explore whether endosomal release presents a major barrier to lipospermine-mediated gene delivery, acidic membrane-active peptides derived from influenza virus or artificial sequences were incorporated into DNA/dioctadecylamidoglycylspermine (=Transfectam) complexes. Depending on the cell line used, gene expression levels are approximately 3−30-fold higher than those obtained by applying DNA complexed to optimal amounts of Transfectam alone. In addition, gene transfer efficiency of DNA complexes with lower amounts of Transfectam (1.5−2 charge equiv) is increased by a factor of up to 1000 by peptides INF6 (influenza virus derived sequence) and INF10 (artificial sequence). The helper lipids 1,2-dioleoylphosphatidylethanolamine, egg phosphatidylethanolamine, and 1,2-dioleoyl-*rac*-glycerol also can enhance the gene transfer. Thus, endosomal escape seems to be only a moderate barrier for optimized, positively charged DNA/Transfectam complexes, but a substantial bottleneck for less positively charged complexes.

INTRODUCTION

Gene therapy relies on strategies that allow efficient and safe introduction of genetic information into human cells. Although most gene therapy protocols use recombinant viral vectors, the limitations of those biological vectors have prompted the design of a great variety of new synthetic systems, among which mono- and polycationic amphipathic molecules, able to complex DNA, have proven to be very attractive (Felgner et al., 1987, 1994; Behr et al., 1989; Leventis and Silvius, 1990; Gao and Huang, 1991; Rose et al., 1991; Hawley-Nelson et al., 1993; Solodin et al., 1995). This class of vectors has been shown to be applicable for *in vivo* administration in experimental animals (Brigham et al., 1989; Alton et al., 1993; Zhu et al., 1993; Canonico et al., 1994; Liu et al., 1995; Schwartz et al., 1995; Thierry et al., 1995) and in clinical trials (Nabel et al., 1993; Caplen et al., 1995). Despite the advances, the efficiency of these vectors, based on number of administered genes, remains orders of magnitude behind that of viral vectors. Efforts to further improve lipid-based gene transfer have been primarily focused on the direct modification of the cationic lipid (acyl chains, spacer arm, hydrophilic part; Felgner et al., 1994; Remy et al., 1994); the mechanism and the limiting steps of gene delivery remain unclear. Packaging of DNA into compact particles, uptake into the cell, release from internal vesicles, release of the DNA from the cationic lipid, and transfer into the nucleus are considered important steps of DNA delivery. Complexation of cationic lipids and DNA results in the formation of nucleolipidic particles (Behr, 1994; Sternberg et al., 1994; Gao and Huang, 1996), which bind to the cell membrane surface by charge interaction, and endocytosis seems to be an efficient and the major delivery pathway (Legendre and Szoka, 1992; Behr, 1993; Zhou and Huang, 1994; Wrobel and Collins, 1995; Zabner et al., 1995) in many cell types. However, subsequent steps, i.e. the transport of DNA from the endosome to the cytoplasm and thence to the nucleus (Zabner et al., 1995) and the release of DNA from the cationic lipid (Zabner et al., 1995; Xu and Szoka, 1996), are considered to represent bottlenecks in successful gene transfer. Release of DNA from endosomes is suggested to proceed by destabilization of the endosomal membrane triggered by mixing the cationic lipids with the cellular anionic lipids (Leventis and Silvius, 1990; Xu and Szoka, 1996); it is, however, unclear how efficiently this mechanism proceeds.

The goal of this work was to evaluate whether gene expression mediated by the lipospermine Transfectam (dioctadecylamidoglycylspermine, DOGS[1]) (Behr et al., 1989) can be further enhanced by adding compounds that should facilitate the transfer across the endosomal membrane. As membrane-destabilizing agents we used amphipathic peptides (Plank et al., 1994; Mechtler and Wagner, 1997) as well as helper lipids (Duzgunes et al., 1989; Leventis and Silvius, 1990; Felgner et al., 1994; Zhou and Huang, 1994; Remy et al., 1995) added to Transfectam/DNA. Amphipathic peptides were previously found to strongly (up to ≥1000-fold) enhance polycation-based gene transfer (Plank et al., 1994; Gottschalk, 1996; Mechtler and Wagner, 1997; Zauner, 1995). As reported in this paper, we found that incorporation of peptides into Transfectam/DNA complexes results in (only) 3−30-fold higher gene expression as

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[1] Abbreviations: DOG, 1,2-dioleoyl-*rac*-glycerol; DOGS, dioctadecylamidoglycylspermine = Transfectam; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; EPC, egg phosphatidylcholine; EPE, egg phosphatidylethanolamine; FCS, fetal calf serum; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; HBS, Hepes-buffered saline (150 mM NaCl, 20 mM Hepes, pH 7.3); HBTU, [*O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate); MOG, 1-monooleoyl-*rac*-glycerol; SM, sphingomyelin.

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Table 1. Sequences and Origins of the Different Membrane-Active Peptides

peptide	origin	specificity for low pH	sequence (N → C terminus)	ref
melittin	peptide from bee venom	no	GIGAV LKVLT TGLPA LISWI KRKRQQ	*a*
INF6	influenza HA-2 (see *b*)	no	GLF GAI AGFI ENGW EGMI DGWYG	*b*
INF5	dimeric HA-2 acidic mutant	yes	GLF EAI EGFI ENGW EGnI DG]K GLF EAI EGFI ENGW EGnI DG	*b*
INFA	influenza HA-2 alanine mutant	no	GLF EAI EAFI ENAW EAMI DAWYG	*c*
EGLA-I	artificial sequence	yes	GLFL GLA [EGLA]$_4$ EGL EGLA GGSC	*c*
INF10	influenza HA-2 EGLA hybride	yes	GLF ELA EGLA ELGW EGLA EGWYGC	*c*

[a] Benachir and Lafleur (1995). [b] N-terminal sequences of influenza virus hemagglutinin subunit HA-2 [see Plank et al. (1994)]; n, norleucine; K, carboxyl-terminal lysine modified at N^{α} and N^{ϵ}. [c] Mechtler and Wagner (1997).

compared to optimized, positively charged Transfectam/DNA complexes. This suggests that for Transfectam/DNA complexes the escape of DNA from endocytic vesicles is an existing, but minor, bottleneck. However, transfection of more electroneutral formulations is strongly increased by peptides and helper lipids by up to 1000-fold, indicating that for these particles endosomal escape is a limiting step.

MATERIALS AND METHODS

Materials. The plasmid pCMVL, coding for the *Photinus pyralis* luciferase gene under control of the cytomegalovirus enhancer/promoter, has been described (Plank et al., 1992). Endotoxin content was measured by the *Limulus amebocyte* lysate assay (BioWhittaker, Walkersville, MD). Lipopolysaccharide content of pCMVL used in transfection experiments was 0.2 endotoxin unit/μg of DNA. Transfectam was synthesized as described (Behr et al., 1989) and is available from Promega (Madison, WI). Chloroquine, bafilomycin A_1, DOPE, MOG, DOG, EPE, EPC, cholesterol, and melittin (from bee venom) were obtained from Sigma (St. Louis, MO).

Peptide Synthesis. Peptides described in Table 1 were assembled on an Applied Biosystems 433 synthesizer with feedback monitoring by using fluorenylmethoxycarbonyl (Fmoc)-protected amino acids. Amino acids were coupled by the HBTU activation method (Fastmoc^{U}; 0.25 mmol scale; Knorr et al., 1989). The following side chain protecting groups were used: (Boc)Lys, (t-Bu)Glu, (t-Bu)Asp, and (Trt)Asn.

The synthesis and purification of the INF5 and INF6 peptides are described in Plank et al. (1994). The peptides INFA, INF10, and EGLA-I were synthesized according to the method of Mechtler and Wagner (1997). In brief, for EGLA-I, an HMP-resin (TentaGel R PHB; 0.22 mmol/g, Rapp Polymere) was chosen, using a mixture of 70% *N*-methylpyrrolidone/30% dimethylformamide (DMF) as solvent. Peptides INFA and INF10 were synthesized on a Cys(Trt) preloaded aminomethylated polystyrene resin with a *p*-carboxytrityl chloride linker (0.52 mmol/g; PepChem, Tübingen, Germany) using DMF as solvent.

The peptides were cleaved from the resin, and side chain protecting groups were removed with a mixture of trifluoracetic acid/water/phenol/thioanisole/ethanedithiol (10:0.5:0.75:0.5:0.25) for 1.5 h at room temperature. Crude peptides were precipitated by dropwise addition of diethyl ether and were collected by centrifugation. Peptides were washed three times with ether and subsequently dried under a stream of argon followed by high vacuum. Crude peptides, dissolved in 1 M triethylammonium bicarbonate (pH 9), were subjected to gel filtration (Sephadex G-10; 20 mM triethylammonium acetate, pH 7.3). The purified peptide fractions were freeze-dried in a Speedvac (Savant) and stored as a powder at −80 °C.

The purity of the peptides was determined by analytical reversed phase HPLC, and peptide identities were confirmed by time-of-flight mass spectroscopy performed with a Finnigan MAT Lasermat instrument. Purified peptides were stored at −80 °C as lyophilized powder or in a 75:25 (v/v) HBS/glycerol mixture. Peptide INF6 lost its biological activity when stored in solution, most likely by structural changes involving aggregation (changes in HPLC profile). The membrane disruption activity of the peptides was tested in liposome and erythrocyte lysis assays (Plank et al., 1994; Mechtler and Wagner, 1997).

Liposome Leakage Assay. The ability of peptides INF6 and INF10, in free form or associated with Transfectam, to disrupt liposomes was assayed by the release of calcein from liposomes loaded with a self-quenching concentration of calcein. Liposomes (lipid compositions in molar ratio: EPC/EPE/cholesterol/SM, 10:3:5:2) were prepared as described (Mechtler and Wagner, 1997) by reversed phase evaporation with an aqueous phase of 100 mM calcein (dissolved by addition of 3.75 equiv of sodium hydroxide) and 50 mM NaCl and extruded through a 100 nm polycarbonate filter to obtain a uniform size distribution. The liposomes were separated from unincorporated material by gel filtration on Sepharose 4B with an isoosmotic buffer (200 mM NaCl, 25 mM HEPES, pH 7.3). For the leakage assay in 96 well microtiter plates, the liposome stock solution was diluted (10 μL/mL) in assay buffer (200 mM sodium chloride containing 20 mM citrate, pH 5.5, or 20 mM HEPES, pH 7.3). Fivefold serial dilutions of the test samples (stock solutions in 100 μL of buffer: 5 μg of peptide INF6 or 7.5 μg of INF10, either in free form or associated with 7.5 μg of Transfectam) were prepared in a 96 well microtiter plate (rows B−G). Row H was left as blank. Eighty microliters of the serial dilution of the test samples was added to 100 μL of the liposome solution in another 96 well microtiter plate (final lipid concentration: 15 μM) and, after 20 min of incubation at room temperature, assayed for calcein fluorescence at 515 nm (excitation 495 nm) on a microtiter plate fluorescence photometer (Perkin-Elmer). The value for 100% leakage was obtained by addition of 1 μL of a 10% Triton X-100 solution to row A; row H (liposomes without peptide) was defined as 0% leakage.

Preparation of the Cationic Lipid/DNA Complexes with/without Membrane-Active Peptides or Helper Lipids. Complexes of DNA and Transfectam were prepared as described (Barthel et al., 1993). Briefly, plasmid DNA (3 μg) and the desired amount of Transfectam were each diluted into 75 μL of 150 mM NaCl and gently mixed. After 10−20 min, the two solutions were mixed. After an additional 10 min, the mixture was diluted with serum-free medium to a final volume of 2 mL; 1 mL of the transfection mixture was put on each well of the duplicate.

We use the term "charge equivalent" to indicate the amount of lipid used for a transfection; 1 charge equiv corresponds to the amount required to neutralize all of

the negative charges carried by the phosphate groups of the plasmid. For example, 3 μg of DNA corresponds to 9 nmol of negative charges; the charge ratio is calculated by taking into account the fact that 1 mol of Transfectam carries three positive charges, as three ammonium groups are protonated at physiological pH; according to this calculation, 3 μg of nucleic acid is neutralized by 3 nmol (3.8 μg) of Transfectam.

Membrane-active peptides (0.5 or 1 mg/mL solutions in HBS/glycerol, 3:1) were added to the preformed Transfectam/DNA complexes; after a 10−20 min period, the transfection volume was adjusted to 2 mL with culture medium, and 1 mL of this transfection mixture per well was pipetted onto the cells.

Helper lipids (DOPE, DOG, EPC, EPE, MOG) and cholesterol were diluted in ethanol containing a trace of methylene chloride (10 μL in 1 mL ethanol). The Transfectam/DNA/helper lipid complexes were formed by mixing the desired amounts of Transfectam/helper lipid (the amounts of helper lipids used are given in equivalents (mole/mole) to Transfectam) prior to dilution with the DNA solution.

Chromatography of Peptide/Transfectam/DNA Complexes. To demonstrate that the negatively charged peptides (at physiological pH) are bound via electrostatic interactions to the Transfectam 2 equiv/DNA complex, we subjected purified INF5 (90 μg) or Transfectam 2 equiv/60 μg of DNA with/without 90 μg of INF5 to gel filtration (Superose 12 HR10-30, Pharmacia, 10 × 300 mm, HBS, flow rate 0.5 mL/min). Free peptide was detected by spectrophotometry measuring at 280 and 230 nm.

Cell Culture. Media, horse serum, and fetal calf serum (FCS) were from Gibco-BRL (Gaithersburg, MD). Culture media were supplemented with 2 mM L-glutamine and antibiotics. Human melanoma cells (H225) were kindly provided by S. Schreiber and G. Stingl (University of Vienna, Austria). BNL CL.2 (mouse embryonic liver cells), A549 (human lung carcinoma cells), and M-3 (Cloudman S91 melanoma cells; clone M-3) were obtained from ATCC (Rockville, MD). H225 cells were cultured in Roswell Park Memorial Institute (RPMI) medium 1640/10% FCS/1 mM sodium pyruvate, A549 cells in Dulbecco's Modified Eagle Medium (DMEM)/10% FCS, BNL CL.2 cells in high-glucose DMEM/10% FCS, and M-3 cells in Ham's-F10 medium/15% horse serum/5% FCS.

Transfection of Cells. Fifty thousand to seventy-five thousand cells per well for 24 well plates (Nunc, Roskilde, Denmark) and 150 000 cells per well for 6 well plates were plated the day before transfection. For all experiments, the final transfection volume was 1 mL per well. After 3−4 h, transfection medium was replaced with fresh medium containing 10% FCS. Luciferase activity was assayed 24 h after transfection. Each experiment was carried out several times; within a series, experiments were done in duplicate.

Luciferase Activity. Cells were harvested after 24 h in 150−200 μL of 250 mM Tris (pH 7.3)/0.5% Triton X-100. The cell lysate was then transferred to 1.5 mL Eppendorf tubes and centrifuged for 5 min at 10000*g* to pellet debris. Luciferase light units were recorded (using a Clinilumat LB9502 instrument from Berthold, Bad Wildbad, Germany) from an aliquot of the supernatant (20 μL) with 10 s integration after automatic injection of freshly prepared luciferin solution (Cotten et al., 1991). Luciferase background (150−250 light units) was subtracted from each value, and the transfection efficiencies were expressed as total light units per well and are the means of duplicates. The Bradford dye-binding assay (Bio-Rad, Hercules, CA) was used to quantify the protein content.

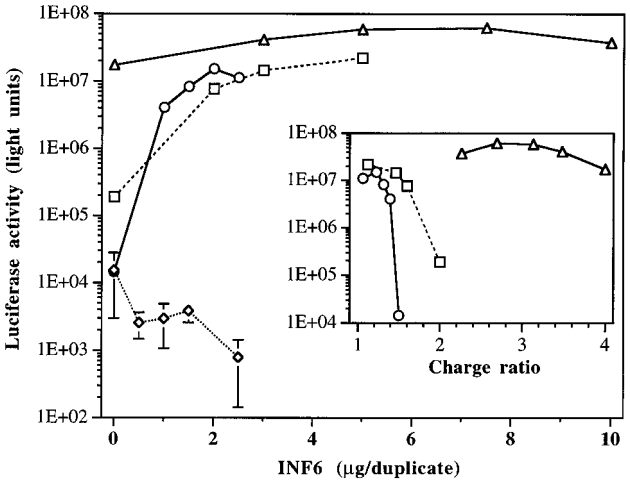

Figure 1. Transfection efficiency of Transfectam/DNA/INF6 complexes. Complexes of 1 (◇), 1.5 (○), 2 (□), and 4 (△) charge equivalents of Transfectam/3 μg of pCMVL in the presence of increasing amounts of the membrane-active peptide INF6 were used. DNA complexes were mixed with RPMI 1640 culture medium and added to the human melanoma cells H225 (75 000 cells per well in 24 well plates). After 4 h, the transfection medium was replaced by fresh RPMI containing 10% FCS. The cells were harvested 24 h after transfection and assayed for luciferase activity. Total luciferase activity of the cells is shown and is the mean of duplicates (±SD). (Insert) Representation of the luciferase activity versus the calculated charge ratio (Transfectam/DNA + INF6; +/−) of the different complexes. As the negatively charged peptide INF6 (four negative charges per molecule at neutral pH) associates with the positively charged lipospermine/DNA complex, the charge ratio +/− is modified.

Flow Cytometry. Plasmid DNA was incubated with the fluorescent intercalator dye YOYO-1 (Molecular Probes, Eugene, OR; *ca.* 1 dye molecule/300 bp), after which complexes were mixed as described above. The complexes were added to 300 000 H225 cells per well (6 well plates) at either 4 °C (cell surface association) or 37 °C (cell surface association and cellular uptake) for 4 h. The cells were washed twice with cold PBS and harvested with 1 mM EDTA in PBS. Cells were then analyzed on a FACScan (Becton Dickinson, San Jose, CA).

RESULTS

Increased Efficiency of Lipospermine/DNA Complexes through Membrane-Active Peptides. Complexes of plasmid pCMVL (encoding a luciferase reporter gene) and 1, 1.5, 2, or 4 charge equiv of Transfectam in combination with various amounts of the membrane-active peptide INF6 (see Table 1) were prepared. As the influenza-derived peptide INF6 has four negative charges at pH 7, it will associate, via electrostatic interactions, with the cationic lipid/DNA particles. Almost all of the peptide was found to bind to the transfecting particles (as determined by chromatography, see Materials and Methods). Transfection efficiencies were determined by using human H225 melanoma cells (Figure 1) and several other cell lines (Figure 2). As shown in Figure 1, a 100−1000-fold increase could be observed upon addition of INF6 when 2 and 1.5 equiv, respectively, of lipid were used. In optimal conditions (4 equiv) only a slight enhancement (up to 5-fold) could be obtained in H225 cells. No enhancement was observed when neutral particles (1 charge equiv) were used, presumably because only few peptides can bind to these complexes. In fact, interactions with the cell membrane might be reduced

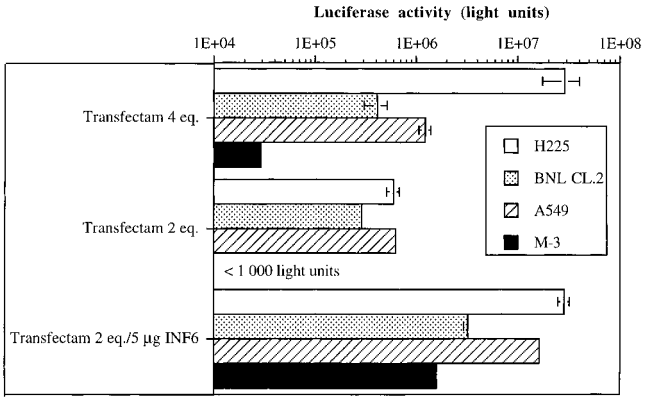

Figure 2. Peptide effect can be observed on several cell lines. The efficiency of Transfectam 2 equiv/pCMVL/5 μg of INF6 complexes was tested on H225, BNL CL.2, and A549 cells (100 000−150 000 cells plated per well in 6 well plates) and on M-3 cells (60 000 cells per well plated in a 24 well plate coated with 0.1% gelatin). Total luciferase activity of the cells is shown and is the mean of duplicates (±SD).

because residual positive charges on the complex required for cell interaction have been masked by the peptide association. The insert of Figure 1 presents the transfection efficiency of Transfectam/DNA/INF6 complexes versus the theoretical charge ratio (±) of the complexes. High luciferase expression can be achieved with almost electroneutral lipid-based vectors.

We tested several other cell lines to determine whether the effects of the membrane-active peptide on lipospermine-mediated gene transfer is specific for H225 melanoma cells or not (see Figure 2). The results obtained on mouse embryonic liver cells (BNL CL.2), human lung carcinoma cells (A549), and murine melanoma cells (M-3) demonstrate that the enhancement of transfection efficiency through addition of INF6 to DNA/2 equiv of Transfectam is a more general phenomenon. The extent of increase in gene expression mediated by the peptide is cell type dependent; while in H225 cells with DNA/2equiv of Transfectam/INF6 similar results were obtained as with DNA/4equiv of Transfectam (see also Figure 1), in the other tested cell lines approximately 10−50-fold higher expression levels were obtained.

To determine whether the peptide might influence the association of the DNA complexes with the cell, flow cytometry studies of various transfection complexes were performed on H225 cells. For this purpose the plasmid DNA was labeled with the intercalator YOYO-1 (Rye et al., 1992; Hirons et al., 1994) before formation of the lipid/DNA complex. As shown in Figure 3, the association of the Transfectam/DNA complexes with the cell surface varies with the charge ratio: with 2 equiv, a heterogeneous cell population is found, while at 4 equiv a classical Gauss curve is obtained. Addition of membrane-active peptides to 2 equiv (or 4 equiv, not shown) of Transfectam does not significantly modify the association with the cell (compare parts B and C of Figure 3), at either 37 or 4 °C (not shown). These experiments suggest that the peptide acts as expected; that is, it improves the release of the complexes from the endosomes.

Enhancement Is Peptide Sequence Dependent. Previous work with ligand/polylysine DNA complexes in association with membrane-destabilizing peptides showed that peptides with specificity for low pH were the most useful (Plank et al., 1994; Mechtler and Wagner, 1997). Our preliminary results (described above) led us to test a series of membrane-active peptides (Table 1) in association with 2 charge equiv of Transfectam.

The peptides INF6, INFA, and melittin, which possess a good membrane disruption activity but no specificity for low pH (Plank et al., 1994; Mechtler and Wagner, 1997), displayed different behaviors: INFA and INF6 (negatively charged) increased the transfection efficiency of 2 charge equiv of Transfectam 10- and ~200-fold respectively, while melittin (positively charged) only slightly increased the luciferase expression (Figure 4). Moreover, melittin was highly toxic at 2.5 μg/mL. The peptides INF5 and INF10, which can efficiently disrupt membranes at pH 5.0 (Plank et al., 1994; Mechtler and Wagner, 1997), gave better results than the INFA peptide but were less efficient than the INF6 derivative. The artificial pH-specific peptide EGLA-I, in which one of the alanine residues in the repeat of GALA (Parente et al., 1988a,b) has been replaced by glycine (Mechtler and Wagner, 1997), gave results comparable to those of the INF5 peptide but had less activity than INF6.

Thus, the most efficient peptide in combination with Transfectam appeared to be peptide INF6 whose membrane-destabilizing activity is pH independent. To confirm that the peptides are still active when ionically bound to the cationic lipid/DNA complex, we measured leakage activity of the two most efficient peptides, INF10 (Figure 5A) and INF6 (Figure 5B), either in free form or associated with Transfectam, using calcein-loaded liposomes of natural lipid composition (EPC/EPE/cholesterol/SM) at neutral or acidic pH. As can be seen, the peptides efficiently release calcein also in combination with Transfectam, although the activity is diminished in quantity. In quality, the characteristics of the peptides are retained: INF10 shows specificity for acidic pH, whereas INF6 is effective at both pH levels.

To check whether the peptides may act at the level of acidic endocytic vesicles, we measured the level of reporter gene expression obtained with Transfectam 2 equiv/DNA + peptide in the presence or absence of the specific inhibitor of the vacuolar proton pump bafilomycin A_1 (Bowman et al., 1988; Yoshimori et al., 1991). As expected, the INF10 formulation (with specificity for low pH) was more sensitive to bafilomycin (5-fold decreased gene expression) than the INF6 formulation (see Figure 6). Note that transferrin-polylysine/acidic-peptide mediated gene transfer was shown to be also inhibited approximately 5-fold by bafilomycin (Plank et al., 1994; Zauner et al., 1995). Bafilomycin did not decrease the efficiency of 4 charge equiv of Transfectam. This latter observation is consistent with the buffering properties of the spermine headgroup (Behr, 1994).

Combination with Neutral Lipids (Helper Lipids) Affects the Transfection Efficiency. The efficiency of Transfectam/DNA complexes near electroneutrality is strongly increased when 1.5−2 equiv of the helper lipid DOPE (moles per mole to Transfectam) is added (Remy et al., 1995). We tried to find other lipids able to increase the efficiency of Transfectam when such suboptimal amounts were used. The combination of 2 charge equiv of Transfectam with 1−3 equiv (mol/mol) of DOPE, EPE, or DOG resulted in 10−20-fold higher gene expression levels (Figure 7). MOG enhanced expression approximately 4-fold, whereas EPC and cholesterol could not improve the transfection efficiency on H225 cells. An additional increase—up to 7-fold—of the Transfectam/DOPE formulation could be obtained by mixing DOG (1 mol % to DOPE) with these two lipids (data not shown). The effect of DOPE and DOG relies presumably on their ability to induce fusion and to stabilize H_{II} phases (Duzgunes et al., 1989; Siegel et al., 1989). The addition of DOPE to 2 charge equiv of Transfectam completely changes the FACScan profile (compare parts B and D of Figure 3): the cell population is more homogeneous and

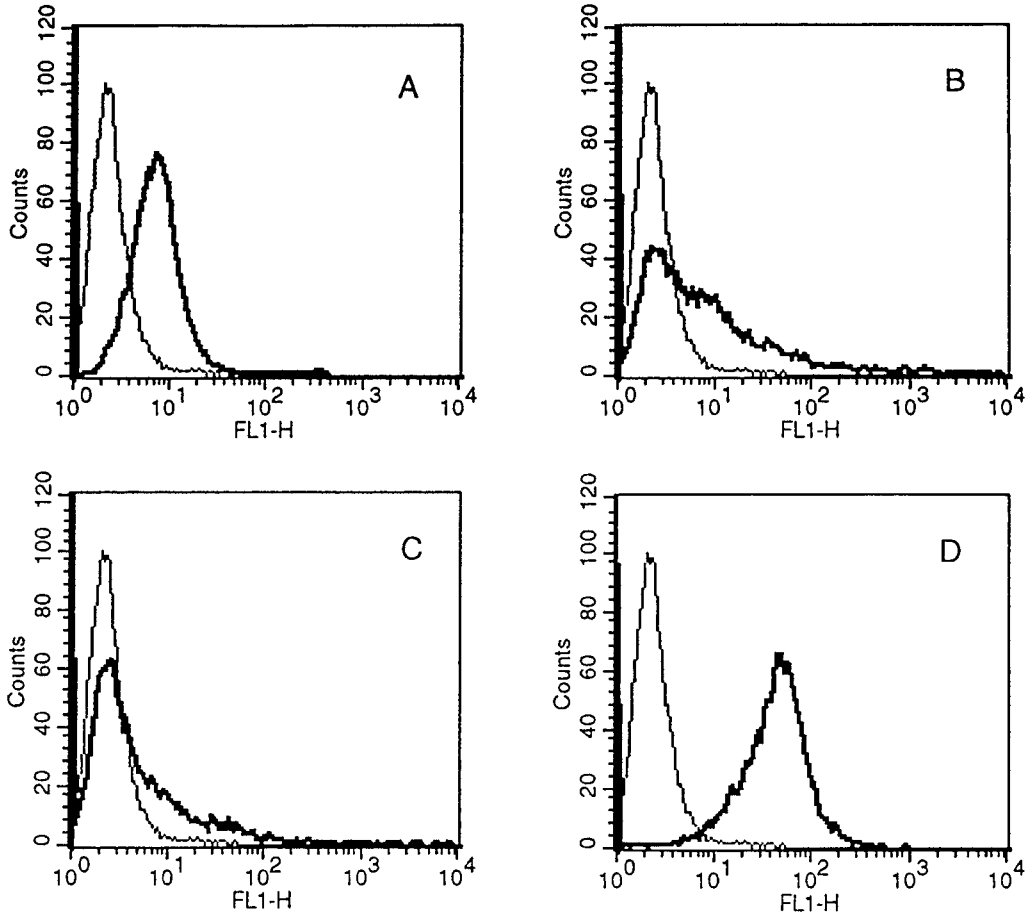

Figure 3. FACS analysis. Three hundred thousand H225 cells were incubated in the presence of transfection complexes containing 1.5 μg of YOYO-1-labeled pCMVL and either Transfectam 4 (A) or 2 equiv (B), Transfectam 2 equiv/1.5 μg of INF5 (C), or Transfectam 2 equiv/1.5 equiv of DOPE (D). After 4 h, the cells were washed twice with PBS, harvested with 1 mM EDTA/PBS, and analyzed by FACS (thick lines). Cells incubated with YOYO-1-labeled DNA alone were indistinguishable from unstained control cells and are shown as negative control (thin lines).

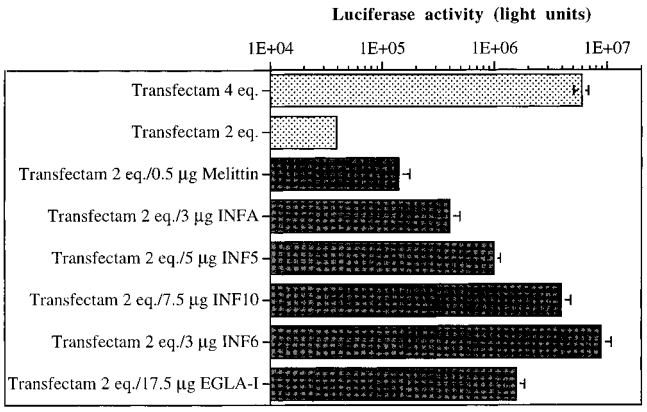

Figure 4. Several peptides are able to increase the efficiency of Transfectam 2 equiv. Increasing amounts of peptide were added to Transfectam 2 equiv/3 μg of DNA complexes (as described under Materials and Methods and Figure 1). The best formulation for each peptide is compared to Transfectam 4 and 2 charge equivalents. Total luciferase activity of the cells is shown and is the mean of duplicates (±SD).

the cell association and uptake are higher with than without helper lipid.

DISCUSSION

The efficiency of DNA transfer mediated by cationic lipids or polycations is dependent on many factors, including size and structure of complexes, immunogenicity, stability in serum and against intracellular enzymes, binding and uptake into the cell, release into the cytoplasm and transfer into the nucleus, or the release of the DNA from the cationic carrier.

Transfection based on receptor-mediated endocytosis of polylysine conjugate/DNA particles is thought to be particularly limited in efficiency by the accumulation of DNA complexes in intracellular vesicles. Methods to destabilize the endosomal membrane, such as addition of replication-defective adenoviruses (Curiel et al., 1991), membrane-active peptides derived from influenza virus (Wagner et al., 1992; Plank et al., 1994) or from rhinovirus (Zauner et al., 1995), and—in some cell types—the use of lysosomotropic agents (Wagner et al., 1994) or glycerol (Zauner et al., 1996), have been shown to strongly enhance the level of gene expression.

There is mounting evidence that DNA delivery seems to take place by endocytosis also in cationic lipid-based gene transfer (Felgner et al., 1987, 1994; Behr et al., 1989; Leventis and Silvius, 1990; Gao and Huang, 1991; Rose et al., 1991; Hawley-Nelson et al., 1993; Solodin et al., 1995; Legendre and Szoka, 1992; Behr, 1993; Zhou and Huang, 1994; Wrobel and Collins, 1995; Zabner et al., 1995). Cationic lipid-based gene transfer has been found to be efficient in a great variety of cells; in contrast to polylysine-based gene transfer, it has been less clear whether the release of the DNA from endosomal compartments is limiting this method of gene transfer (Leventis and Silvius, 1990; Zabner et al., 1995; Xu and Szoka, 1996). This also may depend on the nature of the particular applied cationic lipid or lipid mixture.

Using the lipospermine Transfectam, it was found in previous studies that transfection efficiency was optimal

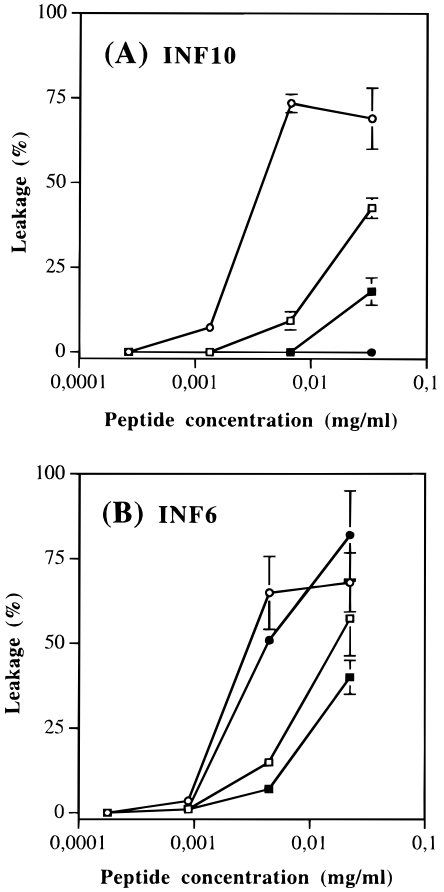

Figure 5. Liposome leakage assay. The leakage activity of peptides INF10 (A) and INF6 (B), in free form (circles) or associated with Transfectam (squares), was determined at pH 7.3 (solid symbols) and 5.5 (open symbols) on EPC/EPE/cholesterol/SM (10:3:5:2) liposomes loaded with 100 mM calcein.

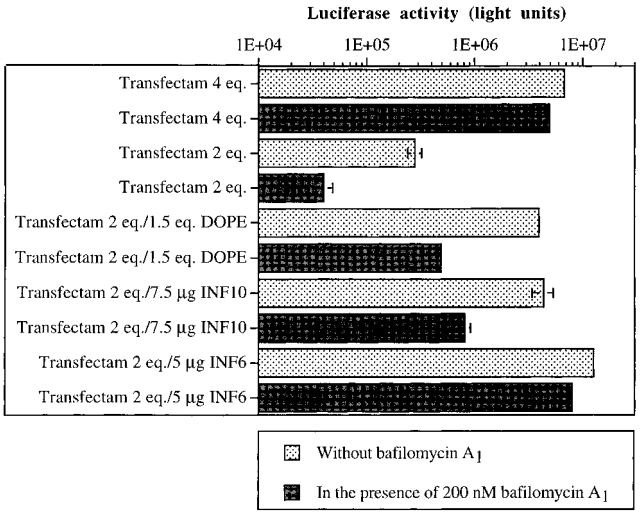

Figure 6. Effects of bafilomycin A_1 on the transfection efficiencies of Transfectam/DNA/peptide or DOPE complexes. Transfection of H225 cells was performed in the absence (light bars) or presence (dark bars) of bafilomycin A_1 (final concentration 200 nM). After 4 h of incubation, the medium was replaced by a fresh one containing 10% FCS. Total luciferase activity of the cells is shown and is the mean of duplicates (±SD).

when the charge ratio of cationic lipid to DNA was highly positive (i.e. about 3−6; Barthel et al., 1993). The positive charges promote binding and uptake of the complex into cells. In addition, Transfectam has endosome buffering capacity (pK_a of the least basic secondary amine ca. 5.4), thus protecting DNA from enzymatic

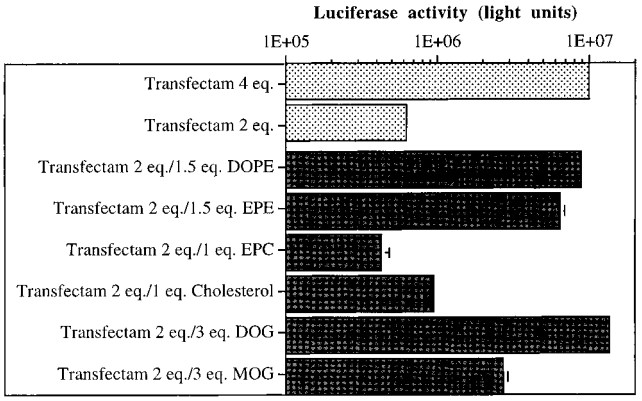

Figure 7. Helper lipids are able to increase the efficiency of 2 charge equiv of Transfectam. Transfection complexes were prepared as described under Materials and Methods. Total luciferase activity of the cells is shown and is the mean of duplicates (±SD).

degradation and potentially causing also some osmotic destabilization of buffered endosomes (Behr, 1994).

We were interested whether the efficiency of Transfectam-mediated plasmid delivery can be enhanced by incorporating influenza-derived peptides. This series of peptides has been previously shown to be able to disrupt liposomes, erythrocytes, or endosomes of cultured cells, enhancing receptor-mediated gene transfer up to approximately 1000-fold (Plank et al., 1994; Mechtler and Wagner, 1997). When optimal amounts of Transfectam are used in association with membrane-destabilizing peptides, a significant, but only moderate (3−30-fold), enhancement of the gene expression was obtained (Figures 1 and 2). Thus, in this context, escape from endocytotic vesicles seems not to be a major bottleneck of gene transfer. Related findings were reported by Kamata et al. (1994), who showed that two influenza-derived peptides can increase the level of gene expression of the commercially available formulation Lipofectin by up to a factor of 5.

However, when we used less positively charged complexes, the addition of membrane-active peptides resulted in up to a 1000-fold increase in expression (1.5 charge equiv of Transfectam in the presence of 2 μg of INF6), reaching or surpassing the level obtained with the optimized amounts of cationic lipid (Figures 1 and 2). The peptide did not change binding of DNA to the cell (Figure 3). Consistent with the working hypothesis that the peptides act on an endosomal level, the effect mediated by the pH-specific peptide INF10 was sensitive to endosomal neutralization by bafilomycin A_1 (Figure 6). Among the peptides tested, only the acidic (negatively charged) ones gave good results in combination with Transfectam. This is in agreement with the hypothesis that for improved endosomal escape of DNA the peptides have to be in the same endocytotic vesicles as the lipid/DNA complexes, which is the case when (negatively charged) peptides are ionically bound to the positively charged Transfectam/DNA particles. The order of efficiency of peptides, when associated with 2 charge equiv of Transfectam, was found to be as follows: INF6 > INF10 > EGLA-I, INF5 > INFA > melittin (Figure 4). These results are different from those obtained with the ligand/polylysine-based system. While INF5 was found to be the best peptide in association with transferrin/polylysine, and approximately 50-fold more effective than INF6 (Plank et al., 1994; Mechtler and Wagner, 1997), in Transfectam-based gene transfer INF5 is 10 times less efficient than INF6. Peptide INF5 has membrane disruption activity only at acidic pH, whereas INF6 is also

active at neutral pH. In lipid-free polylysine-based gene transfer such an activity at neutral pH results in toxic side effects, which were not observed in cells transfected with peptide INF6 in the liposomal system. An additional possible explanation for the higher efficiency of peptide INF6 (compared to INF5 or INF10) is a reduced acidification of endosomes resulting from the buffer capacity of Transfectam (Behr, 1994).

It has recently been shown that highly efficient DNA complexes with low charge (2 equiv of Transfectam) can also be generated by the inclusion of the helper lipid DOPE (Remy et al., 1995). We compared this type of complex with the peptide-containing complexes. The enhancement by DOPE is thought to rely on its capacity for transition from the bilayer ($L\alpha$) phase to the inverted hexagonal (H_{II}) phase. The endosomal protonation promotes this transition, resulting in membrane-active activity (Allen et al., 1990; Litzinger and Huang, 1992; Farhood et al., 1995). Our FACS data suggest an additional, potentially important effect of the helper lipid: the cell association of the transfection complexes is far more pronounced when 1.5 equiv of DOPE is added to 2 charge equiv of Transfectam (Figure 3D) than when the lipospermine is used alone. This suggests that DOPE not only improves the escape of DNA from the endosomes but also increases cell association (and uptake) of the complexes. The existence of this second effect of DOPE is also supported by the fact that the Transfectam/DOPE efficiency is only partially abolished by the presence of the vacuolar proton pump inhibitor bafilomycin A_1 (Figure 6).

Besides DOPE, we used several other lipids in combination with Transfectam to compare their helper capacity with the activity of the membrane-active peptides. EPE and DOG, but not EPC or cholesterol, could enhance the gene expression in H225 cells (Figure 7) to an extent similar to that shown by peptides INF6 and INF10. The lipids that enhance are diacylglycerols, i.e. lipids which do not form stable lamellar structure due to a small headgroup surface. This reinforces the conclusion that the helper lipid has to possess a membrane destabilization activity.

These results show that the modes of action of Transfectam and polylysine after endocytosis of DNA complexes are different. In contrast to polylysine-mediated gene transfer, gene transfer mediated by optimized positively charged Transfectam/DNA complexes cannot be greatly enhanced by the addition of membrane-active peptides, DOPE, glycerol (Zauner et al., 1996), or replication defective adenoviruses[when added to Transfectam/DNA at up to 30 000 particles per cell, data not shown; see also Zabner et al. (1995)]. This finding is not unexpected and is in agreement with the hypothesis that a surplus of positively charged lipids in the DNA complex can directly destabilize the endosomal membrane by lipid mixing (Leventis and Silvius, 1990; Xu and Szoka, 1996) and/or osmotic stress resulting from endosomal protonation of the surplus of Transfectam. In keeping with these hypotheses, the transfection efficiency of DNA complexed with lower, suboptimum amounts of lipids (i.e. 1.5−2 charge equiv) can be increased up to 1000-fold by a membrane-active peptide (without influencing cell association). This confirms that for these particles endosomal escape is a limiting step, similar to what has been observed for polylysine-based gene delivery.

Efficient lipid/DNA transfection complexes that possess a charge ratio near neutrality may be useful for *in vivo* gene transfer. They might be prerequisite for selective, targeted liposomal gene transfer by specific receptor ligands (Remy et al., 1995; Compagnon et al., 1996). This is also supported by recently published findings of Lee and Huang (1996), who demonstrated that folate-targeted liposome/DNA complexes carrying a net positive charge lose their specificity. In addition, as has also been shown for the polylysine-based system (Plank et al., 1996), positively charged lipid/DNA complexes interact with serum complement; only electroneutral particles do not activate the complement system. This correlates well with the *in vivo* results obtained by Schwartz et al. (1995): they observed highest reporter gene expression when low positively charged Transfectam/DNA complexes (i.e. 0.8 or 1.8 equiv) were used in combination with the lipid DOPE. Thus, for *in vivo* applications, the charge ratio between the carrier and nucleic acids seems to be a key parameter, meaning that Transfectam/DNA/membrane-active peptides might be an alternative to other cationic lipid formulations.

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