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The Influence of Endosome-disruptive Peptides on Gene Transfer Using Synthetic Virus-like Gene Transfer Systems*

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Christian Plank, Berndt Oberhauser, Karl Mechtler, Christian Koch, and Ernst Wagner‡

From the Research Institute of Molecular Pathology, Dr. Bohr-Gasse 7, A-1030 Vienna, Austria

The process by which viruses destabilize endosomal membranes in an acidification-dependent manner has been mimicked with synthetic peptides that are able to disrupt liposomes, erythrocytes, or endosomes of cultured cells. Peptides containing the 20 amino-terminal amino acid sequence of influenza virus hemagglutinin as well as acidic derivatives showed erythrocyte lysis activity only when peptides were elongated by an amphipathic helix or by carboxyl-terminal dimerization. Interestingly, peptides consisting of the 23 amino-terminal amino acids of influenza virus hemagglutinin were also active in erythrocyte lysis. When peptides were incorporated into DNA complexes that utilize a receptor-mediated endocytosis pathway for uptake into cultured cells, either by ionic interaction with positively charged polylysine-DNA complexes or by a streptavidin-biotin bridge, a strong correlation between pH-specific erythrocyte disruption activity and gene transfer was observed. A high-level expression of luciferase or interleukin-2 was obtained with optimized gene transfer complexes in human melanoma cells and several cell lines.

One limiting step to receptor-mediated gene delivery (1–3) is the exit of DNA from endosomes (4–6). We have previously generated synthetic complexes that mimic the entry of viruses into cells (7, 8). These complexes contain a ligand for endocytosis into the target cell and a membrane-destabilizing function for entry into the cytoplasm. They consist of plasmid DNA, a polylysine-conjugated ligand (transferrin or a synthetic ligand binding to the asialoglycoprotein receptor), and polylysine-conjugated peptides derived from the amino-terminal sequence of influenza virus hemagglutinin HA-2. The influenza peptides were found to substantially augment (up to >500-fold) receptor-mediated gene transfer in a series of cell lines. Triggered by an acidic environment, the peptides can disrupt liposomes (7, 9). This is consistent with our working hypothesis that the endosome-disruptive properties of these peptides are responsible for the enhanced gene expression. In this communication, we address the question of whether influenza virus-derived peptides are able to disrupt endosomes of living cells. We present new peptide derivatives with pH-specific, increased membrane disruption activity and describe their use in a modified version of the gene transfer system. The influence of the peptide sequences and organization of the DNA complex on membrane disruption and gene transfer has been investigated.

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‡ To whom correspondence and reprint requests should be addressed. Tel.: 43-1-797-30-521; Fax: 43-1-798-71-53.

EXPERIMENTAL PROCEDURES

Materials

The plasmid pCMVL, coding for the *Photinus pyralis* luciferase gene, has been described in Ref. 8. Streptavidin-poly-L-lysine conjugates and human transferrin-poly-L-lysine conjugates TfpLys^{290} were prepared as reported (6, 10). Conjugates contained streptavidin and pLys^{290} at a molar ratio of 3.5:1. Electroneutral DNA complexes are obtained with 4 μg of TfpLys^{290} (*i.e.* 4 μg of transferrin conjugated to 2 μg of polylysine, calculated as hydrobromide salt) and 3 μg of DNA.

Peptide Synthesis

Peptides were assembled on an Applied Biosystems 431A synthesizer by using *p*-alkoxybenzylalcohol resin (Bachem, 0.97 mmol/g) as solid support and Fmoc[1]-protected amino acids (Bachem). Amino acids were coupled by the HBTU activation method (11) or by the standard Fmoc/HOBT chemistry (12). The following protecting groups were used: (Trt)Asn, (Trt)Cys or (*t*-Bu)Cys, (*t*-Bu)Glu, (Trt)His, (*t*-Bu)Ser. The peptides were cleaved from the resin, and the side chain protecting groups were removed (except (*t*-Bu)Cys) with trifluoroacetic acid/water/phenol/thioanisol/ethanedithiol (10:0.5:0.75:0.5:0.25). Crude peptides were precipitated by dropwise addition of 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.

Peptides INF1 to INF4, INF6, INF7—Crude peptides were dissolved in 1 ml of 20 mM ammonium bicarbonate, pH 8, containing 10 μl of β-mercaptoethanol. The solution was subjected to gel filtration (Sephadex G-10, HBS, pH 7.3). The pooled peptide fractions were diluted 2-fold with water and loaded onto a Mono Q column (Pharmacia LKB Biotechnology Inc. HR 5/5; buffer A: 20 mM HEPES, pH 7.3; buffer B: A plus 3 M NaCl; gradient elution 0.3 ml/min, 0–50% B in 30 min; peptides were eluted at 0.6–0.8 M NaCl). Biotinylated INF3 was obtained by addition of 0.2 μmol of biotin-maleimide (Sigma; 10 mM in dimethylformamide) to 0.2 μmol of INF3 (mercapto form) in 80 μl of HBS. The reaction was complete within a few seconds as judged by qualitative Ellman's assay (13). The reaction mixture was subjected to gel filtration on Sephadex G-10, with 20 mM HEPES, pH 7.3, as eluent.

Dimeric Peptides INF3DI and INF4DI—The mercapto form of the peptide (approximately 0.5 μmol/ml in 0.8 M NaCl, 20 mM HEPES, pH 7.3) was reacted with 10 eq of 2,2′-dithiobispyridine (10 mM in ethanol) at room temperature overnight. Excess reagent was removed by gel filtration (Sephadex G-10, HBS) to obtain the (2-pyridylthio)-Cys peptides. Dimeric peptides were prepared by mixing equimolar amounts of the pyridylthio-modified peptide with the mercapto form of peptide in 1 M NaCl, 20 mM HEPES, pH 7.3, concentrating to half of the original volume by evaporation in a Speed Vac (Savant) and reaction overnight. The resulting material was desalted (Sephadex G-10, HBS) and finally purified by ion exchange chromatography (Pharmacia Mono Q, HR-5/5; gradient elution 20 mM HEPES, pH 7.3, 0.1–1.5 M NaCl; INF3DI was eluted at 1.0 M NaCl).

Peptide INF5—The synthesis of dimeric influenza peptide INF5 was performed starting with 230 mg of TentaGel™ S-PHB resin (Rapp Polymere; 0.27 mmol/g) using the HBTU activation method (1 mmol scale).

[1] The abbreviations used are: Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; FCS, fetal calf serum; HBS, HEPES-buffered saline (150 mM NaCl, 20 mM HEPES, pH 7.3); pLys^{290}, poly(L-lysine) with an average chain length of 290 lysine monomers; *t*-Bu, *tert*-butyl; TEAB, triethylammonium bicarbonate; TfpLys, transferrin-poly(L-lysine) conjugate; Trt, trityl; HPLC, high performance liquid chromatography; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; HA, hemagglutinin.

The first amino acid to be coupled was N^{α},N^{ϵ}-di-Fmoc-lysine. This results in the synthesis of a peptide-head-to-head-dimer with a carboxyl-terminal lysine as linking amino acid.

Peptides GALA, GALA-GLF, GALA-INF1, and GALA-INF3—The Cys-*t*-Bu protected peptide GALA was dissolved in 100 mM TEAB and further purified on a Nucleosil 500–5C4 column (0.1% aqueous trifluoroacetic acid, gradient of 0% to 80% acetonitrile). The peptide eluted at about 50% acetonitrile. The crude mercapto form of peptide GALA (5 mg, obtained by deprotecting the corresponding Trt-Cys peptide) was dissolved in 100 µl of 100 mM TEAB, pH 8, containing 1 µl of β-mercaptoethanol and purified by gel filtration (Sephadex G-10, 100 mM TEAB, 0.5 mM EDTA) and ion exchange chromatography (Mono Q Pharmacia, buffer A: 20 mM HEPES, pH 7.3; buffer B: A plus 3 M NaCl; gradient elution 0–100% B; the peptide was eluted at 1.5 M NaCl). Peptides GLF GALA-INF1 and GALA-INF3 were synthesized in an analogous manner.

The purity of the peptides was determined by analytical reverse phase HPLC, and peptide identities were confirmed by time-of-flight mass spectrometry (14) performed with a a Finnigan MAT Lasermat instrument. Purified peptides were stored at −80 °C as a lyophilized powder or in 40% glycerol. Peptides INF1, INF6, and INF7 lost their biological activity when stored in solution, most likely by structural changes involving aggregation (changes in HPLC profile). The amount of mercapto groups in peptides was determined using 5,5′-dithiobis(2-nitrobenzoic acid) and measurement at 412 nm (13). When monomeric forms of peptides were used in subsequent experiments, mercapto groups were blocked by reaction with 1.3 to 5 eq of *N*-(hydroxyethyl)-maleimide (1 h, room temperature). Excess maleimide was removed by gel filtration (Sephadex G-25, 100 mM TEAB, pH 8).

Liposome Leakage Assay

Liposomes were prepared from egg phosphatidylcholine (Avanti Polar Lipids) by reverse phase evaporation (15) with an aqueous phase of 100 mM calcein (dissolved by addition of 3.75 eq of sodium hydroxide) and 50 mM NaCl, extruded through a 100-nm polycarbonate filter (16) and purified by gel filtration on Sephadex G-25 with an iso-osmotic buffer (200 mM NaCl, 25 mM HEPES, pH 7.3). The liposome stock solution was diluted to a lipid concentration of 45 µM in 1.8 × assay buffer (360 mM NaCl, 36 mM sodium citrate of appropriate pH). A serial dilution of the test peptide in water (highest concentration: 5–20 µg/100 µl; dilution steps of 1:5) was prepared in a 96-well microtiter plate by transferring 20 µl of the peptide solution from one well to the next well and diluting with 80 µl of H_2O. An aliquot of 100 µl of liposome solution was added to 80 µl of the dilutions of peptides (final lipid concentration: 25 µM) and, after 30 min at room temperature, was assayed for fluorescence at 515 nm (excitation 495 nm) on a microtiter plate fluorescence spectrometer (Perkin-Elmer Cetus). The value for 100% leakage was obtained by addition of 1 µl of a 10% Triton X-100 solution. Sigmoid curves of leakage activity as a function of the peptide concentration were obtained. The leakage units were calculated as reciprocal values of the peptide concentration, where 50% leakage was observed (*i.e.* the volume (µl) of liposome solution, which is 50% lysed, per µg of peptide).

Erythrocyte Lysis Assay

Freshly prepared human erythrocytes were washed with HBS and resuspended in a 2 × assay buffer of the appropriate pH (300 mM NaCl, 30 mM sodium citrate) at a concentration of approximately 7×10^7/ml. An aliquot of 75 µl was added to 75 µl of a serial dilution of the peptide in water in a 96-well microtiter plate and incubated for 1 h at 37 °C with constant shaking. After removal of the unlysed erythrocytes by centrifugation (1000 × *g*, 5 min), 100 µl of the supernatant were transferred to a new microtiter plate, and hemoglobin absorption was determined at 450 nm (background correction at 750 nm). 100% lysis was determined by adding 1 µl of a 10% Triton X-100 solution prior to centrifugation. The hemolytic units were calculated as the reciprocal value of the peptide concentration, where 50% leakage was observed (*i.e.* the volume (µl) of erythrocyte solution which is 50% lysed, per µg of peptide).

Fluorescence Microscopy

Fluorescein isothiocyanate dextran of average molecular weight 71,200 (Sigma FD-70S) was dissolved in DMEM (Life Technologies Inc.) and subjected to gel filtration (Sephadex G-25 PD-10; DMEM) to remove low molecular weight components. BNL Cl.2 cells were split into Lab-Tek chamber slides (Nunc 177402) at a density of 10,000 cells per well and grown overnight in DMEM plus 10% FCS. The medium was removed, and 150 µl of DMEM containing 5 mg/ml FITC dextran and 0.5 mg/ml peptide INF3DI or INF4DI was added. Cells were incubated at 37 °C for 15 min. The incubation medium was removed and the cells were incubated in fresh DMEM plus 10% FCS for an additional 15 min at 37 °C. The cells were fixed in phosphate-buffered saline containing 3% *p*-formaldehyde for 3 min, air-dried, and mounted with coverslips in Mowiol™. Fluorescence microscopy was performed with a Zeiss Axiophot microscope using a CCD camera (Photometrics, Tucson, AZ). Where indicated, cells were preincubated with 200 nM bafilomycin A_1 (Sigma B-8281, 200 × stock solution in dimethyl sulfoxide) in DMEM plus 10% FCS for 30 min. Subsequent steps were as described above, with the exception that the medium always contained 200 nM bafilomycin.

Transfection of Cells

BNL Cl.2 hepatocytes were grown in DMEM with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine, plated at a density of 300,000 cells per 6-cm dish or 100,000 cells per 3-cm dish or per well of a 6-well plate. The medium was replaced by 500 µl of DNA transfection complexes mixed with 1.5 ml or 0.5 ml (when using 6-cm or 3-cm dishes, respectively) of medium plus FCS. After 4 h at 37 °C, the transfection medium was replaced by 4 ml (or 2 ml) of DMEM with 10% FCS. Harvesting of cells and luciferase assays were performed 24 h after transfection as described (3). The light unit values shown in the figures represent the total luciferase activity of the transfected cells. A standard of 2 ng of luciferase (Sigma L-5256) corresponds to 10^6 light units.

Biospecific Interaction Analysis

The interaction between peptide GALA-INF3 and polylysine was examined using a surface plasmon resonance detection system (BIAcore™, Pharmacia). Changes of mass on the sensor surface (a gold film coated with a layer of carboxymethyl-dextran) correlate with changes of an optical signal presented as resonance units. The carboxyl groups at the sensor surface were activated by injecting 50 µl of an aqueous solution containing 0.25 M *N*-hydroxysuccinimide and 1 M *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide at a flow rate of 5 µl/min. The *N*-hydroxysuccinimide-ester intermediates were reacted with cysteamine (50 µl of a 4 M solution in HBS, flow rate 5 µl/min) to introduce mercapto groups on the surface. The activation procedure was repeated five times in order to minimize unmodified carboxyl groups. Finally, nonreacted *N*-hydroxysuccinimide esters were blocked with 1 M aqueous ethanolamine hydrochloride, pH 8.5.

For interaction analysis, both the mercapto surface and, as control, an unmodified surface were used. Response units are listed in Table II. The sensor surface (report point i) was rinsed with $pLys^{290}$ (10 ng/µl in HBS, flow rate 1 µl/min) to detect the levels of unspecific binding (report point ii). Then, ionically bound polylysine was removed by washing with 1.5 M NaCl (report point iii, in HBS). The surface was treated with a 1 M solution of dithiothreitol followed by HBS. For coupling the peptide GALA-INF3, 35 µl of 0.5 mg/ml peptide (thiopyridine form) were injected (report point iv), followed by three washes with 1.5 M NaCl and HBS (report point 1). Again, $pLys^{290}$ in HBS was injected to observe the formation of the ionic complex with the surface-bound peptide or the control surface. Loosely bound polylysine was washed away with HBS, pH 7.3 (report point 2). To examine the behavior of the peptide-polylysine complex at acid pH, the sensor was washed with CBS (citrate-buffered saline, 150 mM NaCl, 15 mM sodium citrate buffer, pH 5; report point 3). Full dissociation of complexed polylysine was observed after washes with 1.5 M NaCl followed by HBS (report point 4).

RESULTS

Starting from the amino-terminal fusogenic peptide sequence of influenza hemagglutinin subunit HA-2 (Fig. 1*A*), we have synthesized a series of amphipathic peptides (see Table I) and tested their membrane disruption activity in liposome and erythrocyte leakage assays (Fig. 1, *B* and *C*). Peptide INF1 containing the 20 amino-terminal amino acid sequence of HA-2 shows a 10-fold increased liposome leakage activity at pH 5 compared to pH 7. Peptides INF2 (glycine to glutamic acid substitution at position 4) or INF3, INF4 (glycine, alanine to glutamic acid substitutions at positions 4 and 7) have even higher pH specificity with leakage activity detectable only at the lower pH. This is consistent with data of analogous peptides (7, 17, 18) and with the model that additional glutamic acids at positions 4 and 7 (jointly with the acidic residues at positions 11, 15, and 19) should further destabilize an α-helix at neutral pH (see Fig. 1*A*). Surprisingly, in the erythrocyte lysis assay,

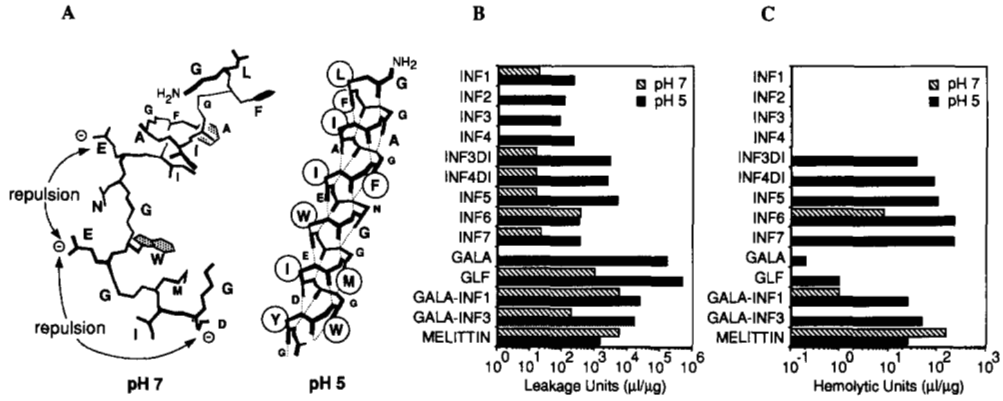


FIG. 1. **Amino-terminal fusogenic peptide of influenza hemagglutinin subunit HA-2 and synthetic derivatives.** *A*, conformation at neutral pH, according to data from x-ray analysis (50, 51) and α-helical conformation at low pH, consistent with Refs. 18 and 52. *B*, leakage assays using phosphatidylcholine liposomes. *C*, erythrocyte lysis assays. Assays at pH 7 and pH 5 were performed as described under "Experimental Procedures."

TABLE I
Sequences of peptides

Influ HA2, amino-terminal sequence of influenza virus X-31 (H3N2) hemagglutinin subunit HA-2; INF1 and INF2 are described in Ref. 7; GALA, analogous to peptide sequence reported in Refs. 19–21; mp, 3-mercaptopropionic acid; n, norleucine; C-C or CC], cysteine disulfide bond;]K, carboxyl-terminal lysine modified at N^{α} and N^{ϵ}.

Influ HA2	GLF GAI AGFI ENGW EGMI DGWYG- - - -
INF1	GLF GAI AGFI ENGW EGMI DGGGC
INF2	GLF EAI AGFI ENGW EGMI DGGGC
INF3	GLF EAI EGFI ENGW EGMI DGGGC
INF3DI	GLF EAI EGFI ENGW EGMI DGGGC]
	GLF EAI EGFI ENGW EGMI DGGGC
INF3DI2	GLF EAI EGFI ENGW EGMI DGGGC-mpGG]K
	GLF EAI EGFI ENGW EGMI DGGGC-mpGG
INF3DI3	GLF EAI EGFI ENGW EGMI DGGGC-mpGGG]K
	GLF EAI EGFI ENGW EGMI DGGGC-mpGGG
INF4	GLF EAI EGFI ENGW EGnI DGCA
INF5	GLF EAI EGFI ENGW EGnI DG]K
	GLF EAI EGFI ENGW EGnI DG
INF6	GLF GAI AGFI ENGW EGMI DGWYG
INF7	GLF EAI EGFI ENGW EGMI DGWYG
GALA	WEA ALA EALA EALA EHLA EALA EALEALAAGGSC
GALA-GLF	GLFG ALA EALA EALA EHLA EALA EALEALAAGGSC
GALA-INF1	GLF GAI AGFI ENGW EGLA EALA EALEALAAGGSC
GALA-INF3	GLF EAI EGFI ENGW EGLA EALA EALEALAAGGSC

none of the peptides INF1–INF4 showed significant activity. The liposome preparations and erythrocytes differ in lipid composition, membrane surface charge, and modification; erythrocyte membranes contain negatively charged, sialic acid-modified glycoproteins and may be less accessible to the peptides. Dimerization of the influenza peptides either by disulfide bond formation at the carboxyl-terminal cysteines (INF2DI, INF3DI, INF4DI) or by direct synthesis of the dimer starting from a carboxyl-terminal branching lysine (INF5) resulted in an approximately 20-fold enhanced liposome leakage activity and high, pH-specific lysis of erythrocytes. Another acidic amphipathic peptide, GALA, designed by the group of Frank Szoka (19–21), showed an even higher, pH-specific liposome leakage activity. Peptide GALA-GLF, which contains the three amino-terminal amino acids of influenza HA-2, has the highest liposome leakage activity. These two peptides, however, have a 500-fold lower erythrocyte lysis activity than the influenza peptide dimers. Peptides GALA-INF1 and GALA-INF3 were designed by replacing the first 16 amino acids of GALA with the amino-terminal sequence either of INF1 (influenza wild type) or of INF3. This elongation of the influenza sequences yields peptides with pH specificities similar to the short influenza sequences INF1 or INF3, but also with erythrocyte lysis activities nearly as high as the dimeric influenza peptides. Peptides INF6 and INF7 containing the sequence of the 23 amino-terminal amino acids of HA-2 show the highest erythrocyte lysis activity. When compared to peptide INF1 or INF3, the small elongation including the hydrophobic residues Trp and Tyr at the carboxyl terminus of INF6 or INF7 results in a surprising (>1000-fold) increase in activity. Melittin (22), a peptide of known high, but not pH-specific, activity, was included in the assays as standard.

The capacity of peptides to disrupt endosomes was tested by incubating cultured cells (BNL Cl.2 hepatocytes or NIH 3T3 cells) with medium containing high molecular weight (70,000) FITC-dextran with or without peptide (INF3DI, INF4DI, or GALA-INF3) for 15 min at 37 °C, followed by a 15-min incubation with normal medium. FITC-dextran, when taken up into cells by fluid phase endocytosis, accumulates in internal vesicles that appear as bright spots (Fig. 2*a*) in fluorescence microscopy. When an endosomolytic peptide is included in the culture medium, no bright vesicles were found in most areas of the cell culture slide, and the fluorescence was spread over the cell indicating that the FITC-dextran had been released into the cytoplasm (Fig. 2, *b* and *d*). The presence of bafilomycin, an inhibitor of vacuolar ATPases (23) which specifically blocks the endosomal proton pump, also blocked the peptide-mediated release of FITC-dextran from internal vesicles (Fig. 2*c*).

A modified version of gene transfer complexes was generated that contains the membrane-disruptive peptides ionically bound (Fig. 3*a*). Half of the negative charge of the plasmid DNA encoding a luciferase reporter gene was saturated with trans-

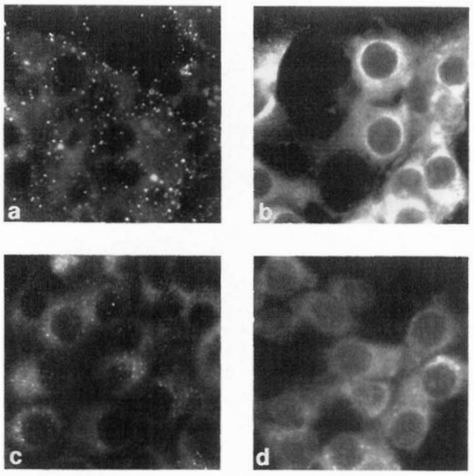


FIG. 2. **Peptide-mediated release of 70-kDa FITC-dextran from endosomes.** Internalized FITC-dextran without (*a*) or with peptide INF3DI (*b*) or INF4DI (*d*); with peptide INF4DI and bafilomycin (*c*).

ferrin-polylysine to provide a ligand for receptor-mediated endocytosis. The remaining nucleic acid charge was utilized for binding an excess of polylysine. The binding of excess polylysine is kinetically controlled; these complexes are not formed when electroneutral, polylysine-saturated DNA complexes have been provided with an excess of polylysine.[2] To the positively charged DNA complex, the negatively charged peptide was added to provide the endosome disrupting activity (Fig. 3*a*). These complexes were added to cell culture medium and incubated with BNL Cl.2 cells. A strong correlation of gene transfer efficiency (see Fig. 3*b*) with the capacity of peptides to lyse erythrocytes in an acidic environment was found. Using the dimeric influenza peptides INF3DI, INF4DI (Fig. 3*d*), INF5 (see Fig. 6), or monomeric peptide INF7 (data not shown), highest gene expression levels (more than 5000-fold higher than in the absence of peptide) were obtained. Peptides GALA-INF3 and GALA also mediate gene transfer, with an approximately 2-fold or 20-fold lower efficiency. Monomeric influenza peptides INF3 (or INF2, INF4, not shown) do not significantly enhance gene transfer. Peptides that possess a high hemolytic potential but do not display the strong specificity for low pH (GALA-INF1, melittin; or INF6, not shown) or lack the negative charges necessary for binding to the DNA-complex (melittin) show only moderate to insignificant augmentation of gene expression. Introduction of spacer sequences between the two coupled influenza peptides INF3 gradually decreases the activity of the dimeric peptides (INF3DI2, INF3DI3) both in erythrocyte membrane disruption and gene transfer to BNL Cl.2 cells (Fig. 3*c*). Inhibition of the endosomal acidification by blocking the endosomal proton pump with bafilomycin also considerably reduced peptide-mediated gene expression (Fig. 3*d*). The gene transfer efficiency of DNA/polylysine/peptide complexes lacking transferrin was, on average, 8-fold lower (data not shown).

Synthetic protease-resistant peptides (D)-INF2DI and (D)-INF3DI consisting of (D)-amino acids showed leakage activities indistinguishable from their counterparts synthesized from (L)-amino acids (data not shown). In order to test whether degradation in lysosomal compartments influences the gene transfer process, DNA complexes containing (L)-pLys or (D)-pLys and (L)-INF3DI or (D)-INF3DI were assembled and applied to cultured cells (Fig. 4). The transfection efficiencies did not significantly change when the metabolically more stable (D)-peptide and (D)-polylysine were used.

[2] C. Plank, B. Oberhauser, K. Mechtler, C. Koch, and E. Wagner, unpublished observation.

Ionic binding of peptides to polylysine modifies their behavior in membrane disruption (see Fig. 5*A*). Electroneutral mixtures of GALA-INF3 and polylysine showed a lower erythrocyte lysis activity (approximately 3-fold lower than peptide alone). Furthermore, at this ratio, precipitates were formed (see Fig. 5*B*). During the first hour of most gene transfer experiments described in Fig. 3 also a slow formation of up to 1 μM large precipitates, consisting of DNA and polylysine, was observed. To determine whether the precipitates or soluble complexes are the active components in gene transfer, transfection complexes were fractionated by centrifugation into a metrizamide cushion containing the precipitates and a supernatant fraction containing soluble complexes; the transfection efficiency with the soluble fraction was 20-fold higher than with the precipitate fraction.[2] Next, we analyzed the association of polylysine and peptide GALA-INF3 by using a surface plasmon resonance detector (24) and asked whether the ionic complexes dissociate at acidic pH, when the peptide is protonated. Peptide GALA-INF3 was bound covalently to a sensor modified with carboxymethyldextran. Care was taken that carboxymethyl groups, which also may ionically interact with polylysine, were blocked by modification with ethanolamine. Polylysine injected through a microflow cell was bound to the peptide-coated sensor surface, resulting in a change of an optical signal (see Table II). Polylysine remained bound when washed with neutral, physiological 150 mM salt buffer, whereas about half of the polylysine was eluted by an acidic 150 mM salt buffer, pH 5. Residual polylysine was released by washing with high salt buffer.

Polylysine-rich, positively charged DNA complexes can be used in transfection of cell lines but show toxic effects in primary cells.[2] For gene transfer to primary human melanoma cells (Fig. 6), we generated less charged transfection complexes by using two alternative strategies. Firstly, biotinylated, monomeric peptide INF3-biotin was incorporated into a transfection complex via streptavidin-biotin interaction using a complex of DNA with TfpLys and streptavidin-pLys. Alternatively, transfection complexes with saturating amounts of ionically bound peptides were used. Using peptide INF5 at a peptide: polylysine charge ratio of approximately 4:1, no precipitates were formed during transfection, and high expression levels of the luciferase reporter gene (Fig. 6*A*) or a human interleukin-2 gene (Fig. 6*B*) were observed. Using peptide INF6 (low pH specificity) or INF7 (high specificity for low pH) in an analogous experiment, 2% or 70% of the luciferase expression with INF5 was obtained.

DISCUSSION

Membrane fusion, membrane disruption, or the formation of transmembrane channels are essential steps of many biological processes (25, 26) such as the fusion of cells (27), of vesicles (28), or the sperm-egg fusion (29). These processes are also involved in the entry of viruses (30–34) and microorganisms (35, 36) into cells, in the cytolytic action of toxins of microorganisms (37, 38), defense toxins of insects (22, 39), or fish (40), in the immune system (41, 42), and the action of antibiotic peptides of insects (43), frogs (44), or vertebrates (45). The membrane-reorganizing activity often can be assigned to a short fusion domain of about 30 amino acids containing an alternating pattern of hydrophobic domains (about 1–3 amino acids) interrupted by hydrophilic domains. Under appropriate conditions, most of these fusion peptides are expected to form amphipathic α-helices that can interact with lipid membranes. In the case of several viruses that enter cells via endocytosis, this conformational change of the fusion domain is triggered by the low pH within the endosomes. Most acid-triggered fusion peptides contain acidic amino acids within their hydrophilic domains, whereas pH-insensitive peptides have no acidic residues and/or a pref-

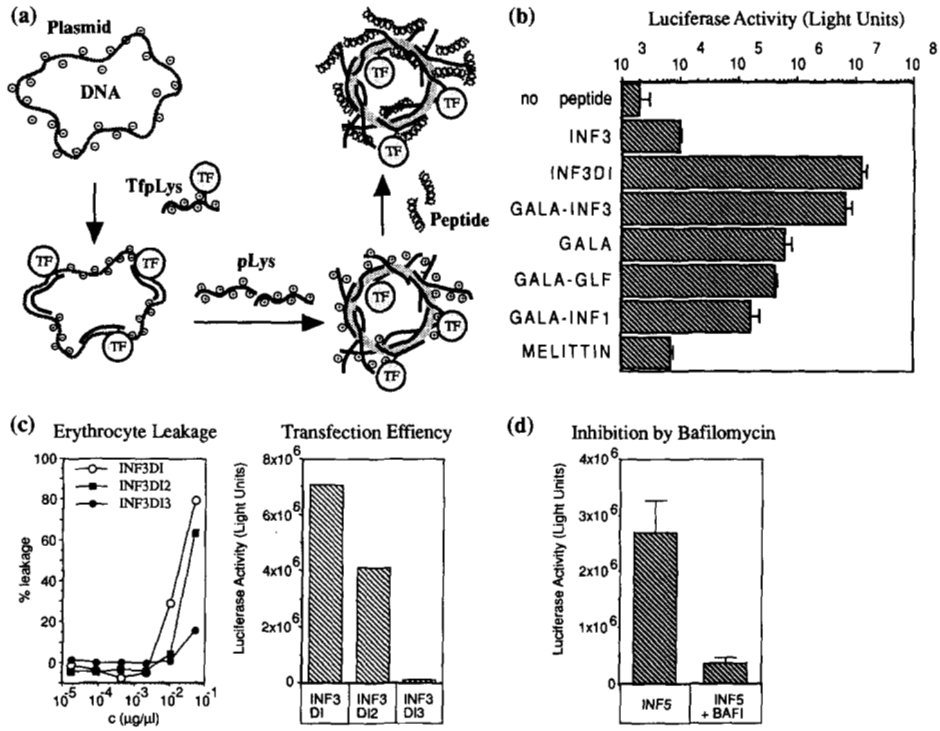


FIG. 3. **Transfection of BNL Cl.2 hepatocytes.** *a*, assembly of quaternary gene transfer complexes containing peptides ionically bound to polylysine. *b*, DNA complexes were prepared by mixing 6 µg of pCMVL-DNA in 250 µl of HBS with 4 µg of $TfpLys^{290}$ in 250 µl of HBS, followed by mixing with 20 µg of $pLys^{290}$ in 750 µl of HBS and optimized amounts of peptides (30 µg of INF3, 30 µg of INF3DI, 20 µg of GALA-INF3, 20 µg of GALA, 4 µg of GALA-GLF, 10 µg of GALA-INF1, or 3 µg of melittin) in 250 µl of HBS. All mixing steps were performed at 30-min intervals. After an incubation for another 30 min, complexes were mixed with 0.5 ml of DMEM plus 6% FCS and added to 300,000 cells. Harvesting of cells and luciferase assays were performed as described under "Experimental Procedures." *c*, erythrocyte leakage activity of dimeric peptides INF3DI, INF3DI2, or INF3DI3 and gene transfer efficiencies of corresponding complexes in 300,000 cells. *d*, inhibition of gene transfer by 200 nM bafilomycin (100,000 BNL Cl.2 cells; complexes consisted of 6 µg of pCMVL, 3 µg of $TfpLys^{290}$, 10 µg of $pLys^{290}$, and 30 µg of peptide INF5).

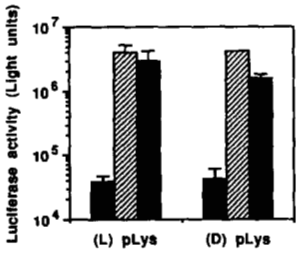
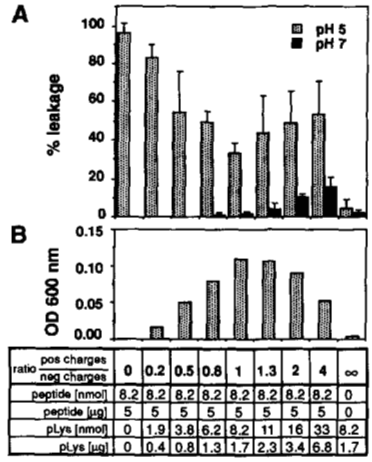


FIG. 4. **Use of the metabolically stable peptide *all*-(D)-INF3DI consisting of (D)-amino acids.** Transfection efficiencies of complexes containing 6 µg of pCMVL-DNA, 4 µg of $TfpLys^{290}$, 10 µg of (L)-$pLys^{290}$ or (D)-$pLys^{250}$, and 30 µg of (L)-INF3DI or (D)-INF3DI, using 100,000 BNL Cl.2 cells cultured in 6-well plates. *Left bar*, no peptide; *center bar*, (L)-INF3DI; *right bar*, (D)-INF3DI.



ratio pos charges / neg charges	0	0.2	0.5	0.8	1	1.3	2	4	∞
peptide [nmol]	8.2	8.2	8.2	8.2	8.2	8.2	8.2	8.2	0
peptide [µg]	5	5	5	5	5	5	5	5	0
pLys [nmol]	0	1.9	3.8	6.2	8.2	11	16	33	8.2
pLys [µg]	0	0.4	0.8	1.3	1.7	2.3	3.4	6.8	1.7

FIG. 5. **Erythrocyte leakage activity depends on ratio of peptide: polylysine.** Solutions of indicated amounts of $pLys^{290}$ (0–6.8 µg) in 50 µl of 150 mM NaCl were mixed with 5 µg of GALA-INF3 in 50 µl of 150 mM NaCl and incubated for 30 min at room temperature in a 96-well microtiter plate. Two wells were filled with 5 µg of polylysine in 100 µl of 150 mM NaCl without peptide. *A*, erythrocyte lysis assays were performed by the addition of 75 µl of erythrocytes in 150 mM NaCl and sodium citrate buffer, pH 7.0 or pH 5.0, to each well. *B*, mixtures of GALA-INF3 and polylysine were prepared as described above, diluted with 200 µl of 150 mM NaCl, and used for turbidity measurement (absorbance at 600 nm).

erence for positively charged residues.[3]

One carefully studied fusogenic protein is the trimeric influenza virus hemagglutinin (HA). Endosomal acidification triggers a structural change which exposes the fusion peptide domain located at the amino terminus of the subunit HA-2 (31, 46). Within the fusion peptide, the repulsions of negatively charged acidic side chains (at positions 11, 15, and 19) prevent the formation of an α-helix at neutral pH (Fig. 1*A*, *left*). In the endosome, upon protonation of the carboxyl groups, the fusion peptide is thought to adopt an α-helical conformation (Fig. 1*A*, *right*). The amphipathic helices of the virus penetrate the endosomal membrane of the host cell, which finally leads to fusion of the viral membrane with the endosomal membrane. Synthetic peptides containing 16 to 20 amino acids of the influenza HA-2 amino terminus have been reported (17, 18) to fuse phosphatidylcholine liposomes and also cause leakage of aqueous liposomal contents at pH values lower than 6.

Our use of similar synthetic peptides as endosome-disrupting agents in gene transfer has already been described (7, 8). The surprising inability of HA-2-related peptides INF1–INF4 to release hemoglobin from erythrocytes prompted us to construct dimers of these peptides (INF3DI, INF4DI and INF5), to mirror the manner in which these peptides are presented by the virus (46). These dimers showed enhanced liposome leakage activity and, most important, a high and pH-specific activity in erythrocyte lysis (Fig. 1*C*). It appears that the enhanced erythrocyte leakage activity observed with dimeric peptides is due to the elongated α-helix of the dimer, rather than to the cooperative interaction of the monomers. Introduction of a nonhelical spacer sequence between the two fusion sequences (INF3DI3) strongly reduced erythrocyte lysis activity; peptides GALA-INF1 and GALA-INF3, containing the 16 amino-terminal HA-2 residues elongated with 14 amino acids of an artificial amphipathic sequence (19), have erythrocyte lysis activity nearly as high as the dimeric influenza peptides.

[3] B. Oberhauser, C. Plank, and E. Wagner, manuscript in preparation.

TABLE II
Interaction analysis response units

Report points		Mercapto surface (EDC/NHS/cysteamine)[a]		Control surface (carboxymethyl dextran)	
		Relative RU	ΔRU	Relative RU	ΔRU
i	HBS	0		0	
ii	pLys	646	646	2446	2446
iii	NaCl, HBS	22	−624	−2	−2448
iv	GALA-INF3	6669	6647	6	8
1	NaCl, HBS	6430	−239	4	−2
2	pLys, HBS	8393	1963	2466	2462
3	CBS,[b] pH 5	7653	−740	2759	293
4	NaCl, HBS	6375	−1278	32	−2727

[a] EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodimide; RU; resonance units.
[b] CBS, citrate-buffered saline.

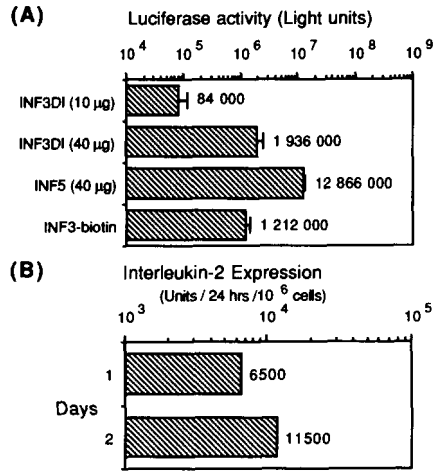


FIG. 6. **Gene transfer to human melanoma cells.** *A*, luciferase expression. Transfection complexes with peptides ionically bound to polylysine (3 μg of pCMVL, 1.5 μg of TfpLys^{290}, 5 μg of pLys^{290}, 10 μg of INF3DI or 40 μg of INF3DI, or INF5) or peptides bound through a biotin-streptavidin interaction (3 μg of pCMVL, 1.5 μg of TfpLys^{290}, 3 μg of streptavidin-pLys^{290}, 10 μg of INF3-biotin) were used. DNA complexes were mixed with 0.5 ml of RPMI 1640 (Life Technologies, Inc.) containing 10% FCS, and added to human melanoma cells (100,000 cells in 6-well plates). After 4 h, the medium was replaced by fresh medium. The cells were harvested 24 h after transfection and assayed for luciferase activity. *B*, expression of human interleukin-2 (IL-2). Transfection complexes of 3 μg of pGShIL-2tetr, 1.5 μg of TfpLys^{290}, 5 μg of pLys^{290}, and 40 μg of INF5 were applied as described in *A*. At day 1 and day 2 after transfection, the amounts of interleukin-2 secreted into the culture medium within 24 h were determined by an enzyme-linked immunosorbent assay (Biokine™ IL-2 test kit, T Cell Diagnostics, Cambridge, MA). Values are given in BRMP units per million cells; 1 BRMP unit is equal to 40 pg of IL-2.

In the light of these results, we re-examined the HA-2 sequence and suspected that the aromatic residues Trp-21 and Tyr-22, which are not included in the synthetic peptides we and others (17, 18) used, might play an important role in the interaction with the lipid membrane. To test this notion, peptides INF6, consisting of the 23 residues of the fusion peptide, and an acidic mutant thereof, INF7, were synthesized. The small changes in the monomeric peptides (substitution of Gly-Gly in INF1 and INF3, for Trp-Tyr) resulted in a dramatic change in membrane disruption behavior, yielding the highest erythrocyte lysis activity of all investigated peptides.

The importance of the free amino terminus (H_2N-Gly-Leu-Phe) in membrane destabilization as reported in Refs. 17 and 47 prompted our investigation of the membrane-disruptive abilities of the synthetic GALA peptide (19–21), elongated by these residues. A marked synergism of Gly-Leu-Phe with the GALA sequence was found; the designed peptide GALA-GLF shows the highest and pH-specific liposome leakage activity. However, peptides GALA-GLF and GALA have far lower erythrocyte lysis activity than the most effective influenza peptides. The contrasting behavior of GALA (and GALA-GLF) in liposome and erythrocyte leakage may be explained by the findings of Parente *et al.* (21) that GALA forms small channels and mediates the release of small molecules (such as calcein) but not larger molecules (such as hemoglobin).

The ability of the peptides to disrupt endosomes of cultured cells and release co-internalized compounds to the cytoplasm has been examined by the release of fluorescent compounds. Upon fluid phase pinocytotic uptake of peptides INF3DI or INF4DI with FITC-dextran, the fluorescent matter was shown to be released (Fig. 2). The low molecular weight fluorophore calcein was similarly released (data not shown). The release is inhibited by bafilomycin, which specifically blocks the endosomal proton pump and, consequently, the acidification of endosomes. The efficiency of endosome disruption is strongly dependent on a high concentration of membrane-active peptide. At a concentration of approximately 100 μM peptide, the majority of endocytic vesicles were disrupted within 15–30 min; at 3-fold lower concentration, no significant leakage was detectable by fluorescence microscopy. The findings of endosomal release were further confirmed (53) in experiments *in vitro* by the peptide- and pH-dependent release of biotin-dextran from endosomes that had been isolated from cells incubated with biotin-dextran, peptide GALA-INF3, and ammonium chloride. These experiments also revealed that a 10-kDa dextran is more readily released than a 70-kDa dextran.

For us, a major incentive to study the endosome-disruptive properties of fusion peptides has been their potential applicability in synthetic, virus-like gene transfer systems. Supplying unconjugated monomeric influenza peptides INF1 or INF2 (7), but also the more potent peptides INF3DI, INF5, or GALA-INF3 (data not shown) to the transfection medium containing transferrin-polylysine/DNA complexes does not considerably enhance gene transfer. We believe that the peptide-mediated release of (≥100 nm) large DNA complexes from endosomes is a less frequent event than the release of small compounds and, as the peptides disrupt membranes in a highly concentration-dependent manner, requires strategies that concentrate membrane-destabilizing peptides in sufficient quantities and active form into the vesicles containing the DNA to be delivered. Our first synthetic, virus-like gene transfer complexes with enhanced activity contained polylysine-conjugated influenza peptides INF1 or INF2 (7). It was fortuitous that the modification of these monomeric influenza peptides with polylysine not only provided DNA-binding property, but also substantially enhanced the membrane disruption activity. The numbers of peptides per DNA complex were low because of technical reasons; the transfection efficiency, however, was found to correlate with an increasing number of peptides linked to polylysine. For this reason, a new, more efficient version of gene transfer complexes was generated that contains up to 10-fold larger amounts of negatively charged, membrane-disruptive peptides ionically bound to polylysine (see Fig. 3). Luciferase gene transfer efficiency in BNL Cl.2 hepatocytes strongly correlates with the capacity of peptides to lyse erythrocytes specifically under acidic conditions. INF3DI-mediated gene transfer to BNL Cl.2 cells resulted in approximately 10-fold higher expression levels (approximately 35 light units per cell) than were obtained with polylysine-conjugated influenza peptides (7, 8). When the endosomal acidification was inhibited with bafilomycin, peptide-mediated gene expression was considerably reduced (Fig. 3*d*).

The physical nature of the DNA complexes considerably influences their biological activity. Positively charged DNA complexes containing excessive polylysine are prepared under kinetically controlled conditions and slowly form fine precipitates

(≥1 μm in size) with reduced gene transfer activity. The ionic interaction of peptide with polylysine also may have a negative effect on membrane disruption activity (see Fig. 5). However, precipitates during DNA complex formation can be minimized by applying saturating amounts of fusion peptide which block the charged polylysine chains. Transfections of NIH 3T3 fibroblasts or B16 melanoma cells with these optimized DNA complexes containing INF5 resulted in luciferase gene expression levels (approximately 350 light units per 3T3 cell; approximately 150 light units per B16 cell, data not shown) considerably higher than those obtained with the same complexes in BNL Cl.2 cells. The importance of the transferrin receptor in the endocytotic uptake of DNA complexes varies with cell type, so that the efficiency of transfection into BNL Cl.2 cells was reduced 8-fold in the absence of transferrin, compared to a 1.5- or 2-fold reduction in 3T3 or B16 cells (data not shown). The absence of toxicity has also allowed the transfection of primary human melanoma cells with high efficiency (up to 130 light units per cell; or 11,500 units of IL-2 per million cells, see Fig. 7).

Recently, other groups have also demonstrated the use of membrane-active peptides in enhancing DNA delivery. Midoux *et al.* (48) reported that synthetic influenza peptides are able to enhance receptor-mediated gene delivery. Haensler and Szoka (49) describe the use of the GALA peptide coupled to positively charged polymer particles for DNA delivery. We and others are currently extending the concept of synthetic viral gene transfer systems (i) to the use of other membrane-destabilizing peptides, *e.g.* derived from picorna viruses, and (ii) to other ways of displaying these peptides in an ordered fashion thought to be required for optimal entry.

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