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

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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.

EXPERIMENTAL PROCEDURES

Materials

The plasmid pCMV1, 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™ and Lys™ were prepared as reported (6, 10). Conjugates contained streptavidin and Lys™ at a molar ratio of 3.5:1. Electrophoretic DNA complexes are obtained with 4 µg of TfpLys™ (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 Fmoc-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: (t-Bu)Cys, (t-Bu)Glu, (t-Bu)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/pheno//thioanisol/ethanediol (10:0.5:0:5:0.25). 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 µm of biotin-maleimide (Sigma; 10 mM in dimethylformamide) to 0.2 µm of INF3 (mercapto form) in 80 µl of HBS. The reaction was complete within a few seconds as judged by qualitative Eilman’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 in 0.8 M NaCl, 20 mM HEPES, pH 7.3) was reacted with 10 eq of 2,2'-dithiobispropionitrile (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 µ 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 Polymer, 227 mmol/g) using the HBTU activation method (1 mmol scale).

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Gene Transfer Enhanced by Endosomolytic Peptides

The first amino acid to be coupled was \( N^\text{Ac}-\text{Fmoc-lysine.} \) 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-4-Bu protected peptide GALA was dissolved in 100 mM TEAB and further purified on a Nucleosil 500–504 column (0.1% aqueous trifluoroacetic acid, gradient of 0% to 80% acetonitrile). The peptide eluted at about 50% acetonitrile. The crude mercuric form of peptide GALA (5 mg, obtained by deprotecting the corresponding Trt-Cys peptide) was further purified on a Nucleosil 500C4 column (0.1% aqueous trifluoroacetic 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)-2-nitrobenzoic acid and measured by gel filtration (Sephadex G-25, 100 mM TEAB, pH 8). Peptides GLP 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 Finnigan MAT Lasermet 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)-2-nitrobenzoic acid and measured 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 calcium (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 a 1.8 x assay buffer (500 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 H₂O. 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. Sigmod curve of leakage activity as a function of the peptide concentration was obtained. The assay was repeated three times as a reciprocal 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 x assay buffer of the appropriate pH (300 mM NaCl, 30 mM sodium citrate) at a concentration of approximately 7 x 10⁸/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 x 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, 71,500 (Sigma FD-75) 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 C1.2 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 0.1% 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% formaldehyde for 3 min, air-dried, and mounted with coverslips in Mowiol™. Fluorescence microscopy was performed with a Zeiss Axios photomicroscope using a CCD camera (Photometrics, Tucson, AZ). Where indicated, cells were preincubated with 200 μM bafilomycin A (Sigma B-8281, 200 μg 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 μM bafilomycin.

**Transfection of Cells**

BNL C1.2 hepatocytes were grown in DMEM with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 μg/ml 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 DMEM and incubated for 24 h before 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 μg of luciferase (Sigma L-5286) corresponds to 10¹⁵ light units.

**Biospecific Interaction Analysis**

The interaction between peptide GALA-INF3 and polylin was examined for the transfected cells using the plasma 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 1-(hydroxyphenyl)alanine and 1 M 1-ethyl-N-((dimethylaminopropyl) carbodiimide at a flow rate of 5 μl/min. The N-(hydroxyphenylalanine-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-(hydroxyphenylalanine-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 PBS (10 ng/ml, HBS, flow rate 1 μl/min) to detect the levels of unspecific binding (report point ii). Then, ionically bound polylin 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 (thiopryidine form) were injected (report point iv), followed by three washes with 1.5 M NaCl and HBS (report point v). Again, pl was injected to observe the formation of the ionic complex with the surface-bound peptide or the control surface. Loosely bound polylin was washed away with HBS, pH 7.3 (report point 2). To examine the behavior of the peptide-polylin complex at acidic pH, the sensor was washed with PBS (citrate-buffered saline, 150 mM NaCl, 15 mM sodium citrate buffer, pH 5; report point 3). Full dissociation of complexed polylin 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. 1A), 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 of INF3), INF4 (glycine to 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. 1A). Surprisingly, in the erythrocyte lysis assay,
The liposome preparations and erythrocytes differ in lipid composition, carboxyl-terminal branching lysine (INF5) resulted in an amphipathic peptide, GALA, designed by the group of Frank Szoka (19-21), showed an even higher, pH-specific liposome leakage activity. These two peptides, however, have a different activity. Peptide GALA-GLF, which contains the three amino-terminal amino acids of influenza HA-2, has the highest membrane surface charge, and modification; erythrocyte membranes contain negatively charged, sialic acid-modified glycoproteins and may be less accessible to the peptides.

The capacity of peptides to disrupt endosomes was tested by incubating cultured cells (BNL C1.2 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. 2a) 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. 2c).

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

FIG. 1. Amino-terminal fusogenic peptide of influenza hemagglutinin subunit HA-2 and synthetic derivatives. A, conformation at neutral pH. Assays at pH 7 and pH 5 were performed as described under “Experimental Procedures.”

TABLE I

Sequences of peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>INF1</td>
<td>GLF GAI AGFI ENGW EGM1 DGWYG-</td>
</tr>
<tr>
<td>INF2</td>
<td>GLF GAI AGFI ENGW EGM1 DGQC</td>
</tr>
<tr>
<td>INF3DI</td>
<td>GLF GAI AGFI ENGW EGM1 DGQC</td>
</tr>
<tr>
<td>INF3DI2</td>
<td>GLF GAI AGFI ENGW EGM1 DGQC</td>
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<tr>
<td>INF3DI3</td>
<td>GLF GAI AGFI ENGW EGM1 DGQC</td>
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<tr>
<td>INF4</td>
<td>GLF GAI AGFI ENGW EGM1 DGQC</td>
</tr>
<tr>
<td>INF5</td>
<td>GLF GAI AGFI ENGW EGM1 DGQC</td>
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<tr>
<td>INF6</td>
<td>GLF GAI AGFI ENGW EGM1 DGQC</td>
</tr>
<tr>
<td>INF7</td>
<td>GLF GAI AGFI ENGW EGM1 DGQC</td>
</tr>
<tr>
<td>GALA</td>
<td>GLF GAI AGFI ENGW EGM1 DGQC</td>
</tr>
<tr>
<td>GALA-GLF</td>
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</tr>
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<td>GALA-INF1</td>
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</tr>
<tr>
<td>GALA-INF3</td>
<td>GLF GAI AGFI ENGW EGM1 DGQC</td>
</tr>
</tbody>
</table>

activity. When compared to peptide INF1 or INF3, the small elongation including the hydrophobic residues Trp and Tyr at the carboxy 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.

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Gene Transfer Enhanced by Endosomolytic Peptides

Ionic binding of peptides to polylysine modifies their behavior in membrane disruption (see Fig. 5A). Electroneutral mixtures of GALA-INF3 and polylysine showed a lower cytotoxicity (activity approximately 3-fold lower than peptide alone). Furthermore, at this ratio, precipitates were formed (see Fig. 5B). 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 complex dissociate at acidic pH, when the peptide is protonated. Peptide GALA-INF3 was bound covalently to a sensor modified with carboxymethyl-dextran. Care was taken that carboxymethyl groups, which also may interactively 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. 6A) or a human interleukin-2 gene (Fig. 6B) 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 cytolitic 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-

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Gene Transfer Enhanced by Endosomolytic Peptides

FIG. 3. Transfection of BNL C1.2 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 TpLys® in 250 µl of HBS, followed by mixing with 20 µg of pLys® 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 INF4, 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 C1.2 cells; complexes consisted of 6 µg of pCMVL, 3 µg of TpLys® , 10 µg of pLys® , and 30 µg of peptide INF3).

One carefully studied fusogenic protein is the trimeric influenza virus hemagglutinin (HA). Endosomal acidification triggers a structural change which exposes the fusion peptide domain 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. 1A, left). In the endosome, upon protonation of the carboxyl groups, the fusion peptide is thought to adopt an α-helical conformation (Fig. 1A, 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.

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. 1C). 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 amphiphatic sequence (19), have erythrocyte lysis activity nearly as high as the dimeric influenza peptides.

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current for positively charged residues.

One carefully studied fusogenic protein is the trimeric influenza virus hemagglutinin (HA). Endosomal acidification triggers a structural change which exposes the fusion peptide domain 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. 1A, left). In the endosome, upon protonation of the carboxyl groups, the fusion peptide is thought to adopt an α-helical conformation (Fig. 1A, 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.

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. 1C). 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 amphiphatic sequence (19), have erythrocyte lysis activity nearly as high as the dimeric influenza peptides.
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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 calcine) 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 calcine was similarly released (data not shown). The release is inhibited by baflomycin, 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 nM 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. These findings of peptide-mediated 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 C1.2 hepatocytes strongly correlates with the capacity of peptides to lyse erythrocytes specifically under acidic conditions. INF3DI-mediated gene transfer to BNL C1.2 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 baflomycin, peptide-mediated gene expression was considerably reduced (Fig. 3d).

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.
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(\geq 1 \mu m 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 enhancing DNA delivery. Midoux et al. recently extending the concept of synthetic viral gene transfer for technical assistance. We are grateful to Peter Steinlein for the help in the Biacore analysis.

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