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 carboxy-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 obtained. 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 polyamine-conjugated ligand (transferin 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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EXPERIMENTAL PROCEDURES

Materials
The plasmid pCMVl, coding for the PhoInius pyralis luciferase gene, has been described in Ref. 8. Streptavidin-poly-L-lysine conjugates and human transferrin-poly-L-lysine conjugates TfpLys and LysA were prepared as reported (6, 10). Conjugates contained streptavidin and LysA at a molar ratio of 3.5:1. Electroneutral 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 p-alkoxycarbonyl chloride (Bachem, 0.97 mmol/g) as solid support and 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: (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/thioanisole/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, INF5, 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 + 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-peptide. Dimeric peptides were prepared by purified 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 (11 mmol scale).

1 The abbreviations used are: Fmoc-(9-fluorenyl)methoxycarbonyl; FCS, fetal calf serum; HBS, HEPES-buffered saline (150 mM NaCl, 20 mM HEPES, pH 7.3); pLysA, 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, tri-tyl; HPLC, high performance liquid chromatography; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; HA, hemagglutinin.

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The first amino acid to be coupled was N\textsuperscript{\textcircled{N}}-di-Fmoc-lysine. This results in the synthesis of a peptide-head-to-head-dimer with a carboxyterminal 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 5OC4 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 deprotecing 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, 5 µM 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 µl NaCl). 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 Laserf 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 measured 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-hydroxysuccinimide esters. Finally, nonreacted N-hydroxysuccinimide and 1 M NaCl were 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 HEPES, pH 7.3; 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 µl NaCl). Peptides GLP GALA-INF1 and GALA-INF3 were synthesized in an analogous manner.

**Transfection of Cells**

BNL CL2 hepatocytes were grown in DMEM with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 µg/ml gentamicin, 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 µl (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-5258) corresponds to 10\textsuperscript{4} light units.

**Biospecific Interaction Analysis**

The interaction between peptide GALA-INF3 and polypeptide was examined by surface plasmon resonance detection system (BIACORE\textsuperset{TM}, 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 mM N-hydroxysuccinimide and 1 mM 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 mM 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% aqueous ethanalamine 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/µl in HBS, flow rate 1 µl/min) to detect the levels of unspecific binding (report point i). Then, ionically bound polypeptide was removed by washing with 1.5 mM NaCl (report point ii, in HBS). The surface was treated with 100 µl 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 mM NaCl and PBS (report point v). A 1 µl aliquot of HBS was injected to observe the formation of the ionic complex with the surface-bound peptide or the control surface. Loosely bound polypeptide was washed away with HBS, pH 7.3 (report point 2). To examine the behavior of the peptide-polypeptide complex at acid 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 polypeptide was observed after washes with 1.5 mM 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 amphiphatic 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 3) and 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. 1A). Surprisingly, in the erythrocyte lysis assay,
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 (INFBDI, INFSDI, INF3DI2) or by direct synthesis of the dimer starting from a carboxyl-terminal lysine modified 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. 2a) in fluorescence microscopy. When an endosomolytic peptide is included in the cell culture slide, and the fluorescence was spread over the cytoplasm (Fig. 2, a and b). 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-
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 and complex formation 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 μg 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. Next, we analyzed the association of polylysine and peptide GALA-INF3 by using a surface plasmon resonance detector (24) and asked whether the complexes 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 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. 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 INF6 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 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 hydrophilic domains (about 1–3 amino acids) interrupted by hydrophobic 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 pre-

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

ferin-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.

To the positively charged DNA complex, the negatively charged peptide was added to provide the endosome disrupting activity (Fig. 3a). These complexes were added to cell culture medium and incubated with BNL CL2 cells. A strong correlation of 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 CL2 cells (Fig. 3c). Inhibition of the endosomal acidification by blocking the endosomal proton pump with bafilomycin also considerably reduced peptide-mediated gene expression (Fig. 3d). The gene transfer efficiency of DNA/polylysine/polylysine 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.

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 TlpLysZm in 250 μl of HBS, followed by mixing with 20 μg of pLysZm 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 C1.2 cells; complexes consisted of 6 μg of pCMVL, 3 μg of TlpLysZm, 10 μg of pLysZm, and 30 μg of peptide INF3).

Fig. 4. Use of the metabolically stable peptide all-(α)-INF3DI consisting of (α)-amino acids. Transfection efficiencies of complexes containing 6 μg of pCMVL-DNA, 4 μg of TlpLysZm, 10 μg of (α)-pLysZm or (α)-pLysZm, and 30 μg of (α)-INF3DI or α-INF3DI, using 100,000 BNL C1.2 cells cultured in 6-well plates. Left bar, no peptide; center bar, (L)-INF3DI; right bar, (D)-INF3DI.

Fig. 5. Erythrocyte leakage activity depends on ratio of peptide:polylysine. Solutions of indicated amounts of pLysZm (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).

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. 4). 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 amphipathic sequence (19), have erythrocyte lysis activity nearly as high as the dimeric influenza peptides.

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abilities of the synthetic GALA peptide (19-211, elongated by Phe) in membrane destabilization as reported in Refs. 17 and cytole lysis activity of all investigated peptides. Shows the highest and pH-specific liposome leakage activity. Changes in the monomeric peptides (substitution of Gly-Gly in GALA sequence was found; the designed peptide GALA-GLF, acidic mutant thereof, INF7, were synthesized. The small changes in the residues. A marked synergism of Gly-Leu-Phe with the acidification reason, a new, more efficient version of gene transfer complexes 10-fold larger amounts of genetically controlled conditions and slowly form fine precipitates. 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 bafilomycin, which specifically blocks the endosomal proton pump and, consequently, the acidification of endosomes. The efficiency of endosomal disruption is strongly dependent on a high concentration of membrane-active peptide. At a concentration of approximately 100 μg 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 low 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-disturbing 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 peptide 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 (Fig. 3d). 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 (Fig. 3d).

The physical nature of the DNA complexes considerably influences their biological activity. Positively charged DNA complexes containing excessive polylysine are more readily released than a 70-kDa dextran.

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*a EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; **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 TpLys300, 5 μg of pLys200, 10 μg of INF3DI or 40 μg of INF3DI, or INF5) or peptides bound to a biotin-streptavidin interaction (3 μg of pCMVL, 1.5 μg of TpLys300, 3 μg of streptavidin-pLys200, 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-2tet, 1.5 μg of TpLys300, 5 μg of pLys200, 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 (Biotinex 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 others (17, 18) used, might play an important role in the interactions 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 monomereric 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,L,N-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. The ability of the peptides of fusion proteins 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-disturbing 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 peptide 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 (Fig. 3d). 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 (Fig. 3d). 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).
et al. discussions. We further appreciate the critical reading the charged polylysine chains. Transfections of NIH 3T3 fibro- pGShIL-2tetr. We thank Prof. Max L. Birnstiel for support and helpful membra... 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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REFERENCES

42. Eiser, A. F. (1980) Immunology Today 12, 316-318