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Characterization of a novel pH-sensitive peptide that enhances drug release from folate-targeted liposomes at endosomal pHs

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Abstract

Although liposomes have proven useful for the delivery of drugs and gene therapy vectors, their potencies are often compromised by poor unloading following uptake into their target cells. We have consequently explored the properties of a novel 29-residue amphipathic peptide that was designed by arrangement of hydrophobic and hydrophilic residues to disrupt liposomes at lower peptide concentrations than previously tested peptides. The peptide was indeed found to promote pH-dependent liposome unloading with improved efficiency. A peptide of the same sequence, but half the length, however, promoted pH-dependent permeabilization only at much higher concentrations. Further characterization of the longer peptide revealed that release of liposome contents (i) occurred at a pH of $\sim$6, (ii) became less efficient as the size of the encapsulated cargo increased, and (iii) was moderately suppressed in cholesterol-containing liposomes. Use of this peptide to enhance the cytotoxicity of cytosine arabinoside encapsulated in folate-targeted liposomes demonstrated an increase in drug potency of $\sim$30-fold. Gene expression by a serum-stable folate-targeted liposomal vector was also measurably enhanced by inclusion of the peptide. We conclude that intracellular unloading of liposomal contents can be significantly improved by co-encapsulation of an optimally designed, pH-sensitive peptide.

Keywords: Liposome; pH sensitive peptide; Folate receptor; Drug delivery; Gene delivery

1. Introduction

Liposomes have shown considerable promise as vehicles for the delivery of small molecular mass drugs to tumors [1–3]. Indeed, the potency and normal tissue toxicity of doxorubicin has been significantly improved by encapsulating the drug into long circulating liposomes [4,5]. Unfortunately, the efficacy of liposomal drug delivery has not achieved its full potential, in part because of the slow escape of the encapsulated drugs following uptake of the liposomes by target cells [6–8]. However, target cell endocytosis generally traffics the liposomes into endosomes [9,10] and the low pHs characteristic of these compartments may be exploited for liposome unloading. Such efforts to accelerate drug release have largely focused on the development of pH-sensitive lipids that are rapidly hydrolyzed or protonated following endocytosis into an acidic intracellular compartment [11–13]. Alternatively, pH-dependent peptides that mimic viral fusion peptides have also

Abbreviations: ara-C, cytosine arabinoside; Chol, cholesterol; DOTAP, dioleyltriaminopropane; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEG, polyethyleneglycol; PI, propidium iodide

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been designed to enable release of liposome contents at acidic pHs [14–16].

The mechanism by which fusogenic peptides mediate liposome unloading probably involves a change in peptide conformation following exposure to acidic pHs [17]. Thus, at neutral pH, the peptides are largely seen in random coil conformations, but at pH values near 5, the peptides convert predominantly to amphipathic helices [18]. As a consequence of this conformational transition, these peptides insert into the liposome bilayer and self-associate, causing leakage of encapsulated contents [18]. Because the pH dependence of the conformational transition can be highly cooperative, amphipathic peptides have the potential of displaying no membrane activity during liposome circulation in the blood (pH 7.4), but inducing quantitative permeabilization following entry of the liposome into the cell (pH 4.5–6.5).

The GALA peptide was the first peptide to be specifically designed for pH-dependent liposome unloading [19], and various related peptides have since been synthesized and characterized for their pH-dependent pore-forming properties [20,21]. We have also synthesized a 30-amino-acid GALA-type peptide, called EALA, and demonstrated its ability to promote the specific unloading of liposomes in endosomes of the folate receptor endocytic pathway [22]. With this peptide, liposomes could be readily constructed that were stable during storage in neutral buffer for longer than 1 month, but still unloaded their contents upon endocytosis by target cells.

Although EALA allowed for good liposome stability and pH-dependent unloading properties, we still felt motivated to improve its potency in order to minimize the number of peptides required to facilitate liposome permeabilization. For this purpose, we explored the hypothesis that the number of peptides per liposome required to enable cargo unloading might depend on the fraction of the peptide's surface area occupied by hydrophilic groups (see Section 3). Based on this concept, we have designed a peptide with superior pH-dependent membrane-permeabilizing properties. In this paper, we characterize the novel peptide's liposome-destabilizing capacity under a variety of conditions and examine its ability to enhance the potency of encapsulated cytotoxic drugs and gene therapy vectors.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (98% purity), cholesterol, *n*-glutaryl-phosphatidylethanolamine (glutaryl-PE), and dioleyltrimethylammonium-propane (DOTAP) were all purchased from Avanti Polar Lipids (Alabaster, AL). Polyethyleneglycol-bis-amine (H_2N-PEG-NH_2) M_r 3400 was purchased from Shearwater Polymers (Huntsville, AL). Texas Red dextrans of $M_r \sim 10\,000$ and $\sim 40\,000$ were from Molecular Probes (Eugene, OR). [^{3}H]Thymidine was purchased from American Radiolabeled Chemicals (St. Louis, MO). KB cells were a generous donation from the Purdue University Cancer Center, and all cell culture media and reagents were obtained from Gibco BRL (Gaithersburg, MD). Cytosine β-D-arabinofuranoside (ara-C) and all other reagents were from Sigma Chemical Co. (St. Louis, MO).

2.2. Peptide synthesis

The 29-amino-acid peptide, H_2N-(AALEALAEA-LEALAEALEALAEAAAAGGC)-CO_2H, and the previously characterized 30-amino-acid EALA peptide, H_2N-(AALAEALAEALAEALAEALAEALA-AAAGGC)-CO_2H, were synthesized by the Purdue University Peptide Synthesis Facility using solid phase methodology based on an Fmoc strategy. The 15-amino-acid peptide H_2N-(ALEALAEALEA-LAEA)-$CONH_2$ was synthesized by Peptidogenic (Livermore, CA). All peptides were purified to greater than 95% homogeneity, as determined by analytical high-performance liquid chromatography, and characterized by fast atom bombardment mass spectrometry.

2.3. Synthesis of folate-PEG-PE conjugate

Folate-PEG-PE was synthesized as described previously [23], with the following modifications. (1) After coupling 1 equivalent folic acid to H_2N-PEG-NH_2 with dicyclohexylcarbodiimide, the product was purified on a G-15 size exclusion column in ddH_2O. (2) The purified folate-PEG-NH_2 was conjugated directly to n-glutaryl-PE via carbodiimide coupling. (3) Following completion of the conjuga-

tion reaction, the chloroform was evaporated and the resulting solid was dissolved in ddH_2O for purification by passage through a CL4B column. The early micellar fraction was collected and lyophilized to yield the final product, folate-PEG-PE.

2.4. Preparation of small unilammelar vesicles

All liposomes were comprised of 100% egg phosphatidylcholine with the exception of propidium iodide encapsulating liposomes, which contained 0, 20 or 40 mol% cholesterol, and ara-C liposomes which contained 0.5 mol% folate-PEG-PE for tumor targeting. All lipids were first dissolved in chloroform and then dried to a thin film by rotary evaporation. For the unloading studies, thin films were rehydrated by addition of phosphate-buffered saline (PBS) (pH 7.4), containing propidium iodide (2 mg/ml) or Texas Red dextran of M_r 10 000 or 40 000 Da (5 mg/ml). Propidium iodide (PI) and dextran-containing liposomes did not contain peptide, rather peptides were added externally to pre-formed liposomes in solution. In order to test the ability of the 29-residue peptide to enhance delivery of a cytotoxic drug, liposomes containing ara-C were prepared with co-encapsulated peptide. Folate-targeted ara-C liposomes were rehydrated in PBS containing ara-C (10 mg/ml) with or without the 29-amino-acid peptide (2 mg/ml). Rehydration was accomplished by vigorous vortexing followed by five cycles of freezing and thawing. All liposome preparations were then sized by extrusion ten times through a 100-nm pore size polycarbonate membrane using a high-pressure extruder (Lipex Biomembranes, Vancouver, BC, Canada). Finally, liposomes were separated from unencapsulated material by passage through a size exclusion column equilibrated in PBS. CL4B size exclusion resin was used in all cases except for Texas Red dextran 40 000 M_r containing liposomes, which were purified using CL6B resin. Final encapsulation efficiency of the 29-residue peptide, as determined by amino acid analysis, was approximately 0.1%, yielding an intraliposomal peptide concentration of $\sim$10 μM.

Liposomal ara-C concentration was determined by measuring the drug's absorbance at 272 nm on a Shimadzu Biospec-1601 UV-Visible spectrophotometer. Empty liposomes were used as a background for this determination. All liposomes were stored at 4°C and used within 1 week of preparation.

2.5. PI release assay

Although a method for quantitating the unloading of liposomal contents from endosomal compartments of cultured cells has already been reported [22], a related, but simpler approach was developed for the rapid screening of various peptide formulations for their abilities to permeabilize liposomes. PI is a pH-insensitive membrane impermeable dye that fluoresces only upon binding polynucleic acids [22]. Its fluorescence is consequently negligible when entrapped in the aqueous lumen of liposomes, but intense when released into a solution containing calf thymus DNA. For analysis of peptide-induced PI release, PI-containing liposomes in PBS were added to one of the following buffers: 100 mM citrate, pH 4.0 or 4.5; 100 mM citrate/phosphate, pH 5.0 or 5.5; 100 mM phosphate, pH 6.0, 6.5, or 7.0; or PBS, pH 7.4. Small volumes of peptide stock solutions in PBS (pH 8.0) were then added to the liposome suspensions to achieve the desired lipid to peptide molar ratios, and suspensions were stirred at room temperature for various time periods. Calf thymus DNA dissolved in PBS was then added to the liposome suspensions (0.02 mg/ml final concentration), and the fluorescence was measured (released propidium iodide fluorescence) in an Aminco Bowman Series 2 luminescence spectrometer using 495-nm excitation and 515-nm emission wavelengths. A sample containing liposomes plus DNA in the appropriate buffer, but without the addition of peptide, was subtracted from each test sample to account for the minimal background fluorescence of PI that remained encapsulated (fluorescence of blank). Further, it was confirmed that the fluorescence of PI did not increase when combined in solution with relevant concentrations of the 29-residue peptide. To determine the maximum PI release possible for each sample, 0.1% of reduced Triton X-100 detergent was added to the liposome suspension at the end of each experiment and the fluorescence was again measured (maximum possible fluorescence). Percent PI released was calculated as (released fluorescence−fluorescence of blank)×100/(maximum possible fluorescence).

2.6. Dextran release assay

Dextrans were chosen as macromolecular markers for sizing peptide pores because they are membrane-impermeable, water-soluble, and available in a range of sizes. To quantitate the peptide-induced release of 10 000 and 40 000 Da dextrans from liposomes, dextran-loaded liposomes in PBS were added to buffers at specific pHs, peptide was then added, and samples were stirred as described for the PI release assay. Liposomes were then passed through a CL4B or CL6B size exclusion column (equilibrated in PBS) to separate released dextran from liposomal dextran. Fractions were collected until all dextran had eluted from the column, and reduced Triton X-100 detergent (0.01% final concentration) was added to each fraction to eliminate liposome-related quenching and light-scattering effects. The fluorescence of each fraction was then determined by exciting at 595 nm, while monitoring emission at 615 nm, and percent dextran released was calculated at each pH as follows: (sum of fluorescence of non-liposome fractions) × 100/(sum of fluorescence of all fractions).

2.7. Cell culture

Folate receptor positive KB cells were cultured in folate-deficient Minimal Essential Medium (FDMEM) to allow for sustained and elevated expression of the folate receptor. Medium contained 10% heat-inactivated fetal calf serum (FCS), penicillin (50 units/ml), streptomycin (50 μg/ml), 2 mM L-glutamine and non-essential amino acids at 37°C in a 5% CO_2 humidified atmosphere. For ara-C cytotoxicity studies, cells were seeded 24 h prior to each experiment in 24-well plates at approximately 25% confluence. In a typical liposomal vector transfection experiment, cells were seeded 48 h before transfection in 24-well plates at 15–20% confluence.

2.8. [^{3}H]Thymidine incorporation assay

Triplicate wells containing KB cells in FDMEM were incubated for 4 h at 37°C with folate-targeted liposomes containing ara-C with or without co-encapsulated peptide. 1 mM folic acid was pre-added to selected wells as a competitive ligand to demonstrate that uptake of the liposomes was mediated by the folate receptor. Following incubation, cells were washed three times with PBS and incubated an additional 24 h in FDMEM containing 1 μCi [^{3}H]thymidine per well [24]. After this second incubation, cells were again washed three times in PBS and dissolved in 0.2 M NaOH containing 1% Triton X-100. Liquid scintillation counting was used to determine the amount of radioactive thymidine incorporated by the cells in each well, and percent growth inhibition was calculated relative to the maximum thymidine incorporation by cells in untreated wells.

2.9. Plasmid preparation

Escherichia coli (XL1 blue) cells were transformed with pGL3 vector containing the luciferase reporter gene (Promega, Madison, WI) and selected in an ampicillin rich growth medium at 37°C overnight. A single clone of transfected *E. coli* was further proliferated in LB broth (containing 100 μg ampicillin/ml) at 37°C with vigorous agitation and aeration for 18 h. *E. coli* cultures with $OD_{600} > 3.0$ were harvested and pGL3 plasmid DNA was purified by alkaline lysis and use of Biggest prep purification kit (5 prime → 3 prime Inc., Boulder, CO).

2.10. Preparation of cationic liposome/DNA complexes

Liposomes for transfection studies were prepared by drying a chloroform solution of DOTAP (1 μmol), cholesterol (0.67 μmol), and 0.5 mol% folate-PEG-PE (targeted) or PEG-PE (non-targeted) under vacuum to a thin film. The film was hydrated by addition of 1.0 ml sterile 5% dextrose in 5 mM HEPES (pH 8.0). Liposomes were prepared by first vortexing the hydrated lipid for 1 min and then rotating the sample in a water bath at 50°C for 45 min and then 35°C for 10 min. The suspension was allowed to stand at room temperature overnight and then subjected to sonication in a bath sonicator to obtain a clear emulsion [25]. Peptide and DNA were dissolved in sterile 5% dextrose in 5 mM HEPES (pH 7.4) at peptide/DNA weight ratios ranging from 0.1–1.6. Liposome/peptide/DNA complexes were induced to self-assemble by vortexing equal volumes of the DOTAP/cholesterol suspension and the peptide/DNA mixture dissolved in sterile 5% dextrose in

5 mM HEPES (pH 7.4) for 1 min, followed by incubation at room temperature for 15 min.

2.11. Transfection of cells with DOTAP/cholesterol liposomal vectors

Immediately prior to transfection, KB cells were washed with 0.5 ml of FDMEM ± 10% FCS, and then incubated for 4 h at 37°C with DNA/liposome complexes with or without peptide in 400 μl FDMEM ± 10% fetal calf serum (FCS). After 4 h incubation, the medium containing any free DNA/liposome complexes was removed and the cells were returned to culture with 0.5 ml FDMEM+10% FCS. Thirty-six hours after transfection, cells were analyzed for reporter gene expression using the Luciferase Assay system (Promega, Madison, WI). Measurement of relative light units (RLU) of the luciferase enzymatic product was performed on a Microtiter Plate Luminometer (Dynex Technologies, Chantilly, VA) and reported relative to the concentration of cell lysate protein in each sample. Protein concentration was determined using the BCA assay (Pierce Chemical Co., Rockford, IL).

3. Results

3.1. Design of an optimal peptide sequence for membrane permeabilization

Because the peptide variants examined in this study were nearly 90% α-helical (determined by circular dichroism, data not shown), the peptide sequences were fit to an idealized helical wheel in an effort to predict their geometries of self assembly within a phospholipid bilayer. When negatively charged glutamic acid residues are distributed over a large angle of an amphipathic peptide helix, multiple copies of the peptide may have to associate within a lipid bilayer to avoid energetically unfavorable contacts between glutamic acid side chains and bilayer lipids (see Fig. 1A). Consequently, the resulting peptide-lined pore is expected to be large. In contrast, when the hydrophilic glutamic acid residues constitute a smaller angle of the peptide helix, membrane pores could conceivably be assembled with fewer peptide monomers, enabling permeabilization at lower peptide concentrations (Fig. 1A). Assuming an average radial rotation of ∼100°/residue and a helical diameter of ∼10 Å, we determined that the amino acid sequence requiring the fewest monomers per aqueous pore was AALEALAEALEALAEALEALAEAAAAGGC. As seen in the helical wheel of Fig. 1B, this peptide localizes all hydrophilic residues within a 90° angle on one side of the helix. Consequently, a tetramer should hypothetically constitute the preferred oligomer in the membrane (Fig. 1A). This 29-amino-acid peptide plus a truncated 15-residue version of the same sequence were, therefore, constructed and examined for their liposome permeabilization properties (Fig. 1B).

3.2. The novel 29-amino-acid peptide catalyzes rapid pH dependent liposome permeabilization to both propidium iodide and a 10 000 Da dextran

In order to determine whether the newly designed 29-residue peptide was membrane active, it was added to preformed liposomes containing either a low molecular mass solute (propidium iodide, PI) or a 10 000 Da Texas Red-labeled dextran. After acidification of the suspension to pH 4, release of liposome contents was evaluated as a function of time. As seen in Fig. 2, release of both PI and dextran from the liposomes occurred rapidly, reaching a maximum within ∼10 min of peptide addition. However, whereas PI was able to fully escape the liposome interior, the high molecular mass dextran was not. Importantly, release kinetics were similar for the 15-amino-acid peptide and the previously described EALA peptide (data not shown). Because of these similar kinetics, percent release was subsequently determined 10 min following peptide addition in all other studies. Importantly, no leakage of liposome contents could be detected during similar incubations at neutral pH, suggesting that the liposomes might remain stable during circulation in the blood.

3.3. The novel 29-amino-acid peptide is significantly more potent than EALA

Our objective in designing a new peptide was to identify a sequence that could promote liposome unloading at lower peptide concentrations than the pre-

viously tested EALA peptide. To evaluate whether this objective was realized, the 29-amino-acid peptide was mixed with PI-loaded liposomes at a lipid to peptide ratio of 10 000:1 and compared with EALA at the same peptide ratio. As expected, neither peptide promoted measurable content release at neutral pH; however, as pH was lowered into the endosomal range (pH 4–6), the newly designed peptide was significantly more effective than EALA (Fig. 3). Since these two peptides demonstrated indistinguishable behavior at higher peptide concentration (data not shown), it is conceivable that glutamic acid placement is only critical when very small amounts of peptide are present. However, since a liposome's carrying capacity can often limit its usefulness, minimization of the volume consumed by the lytic peptide constitutes a desirable objective.

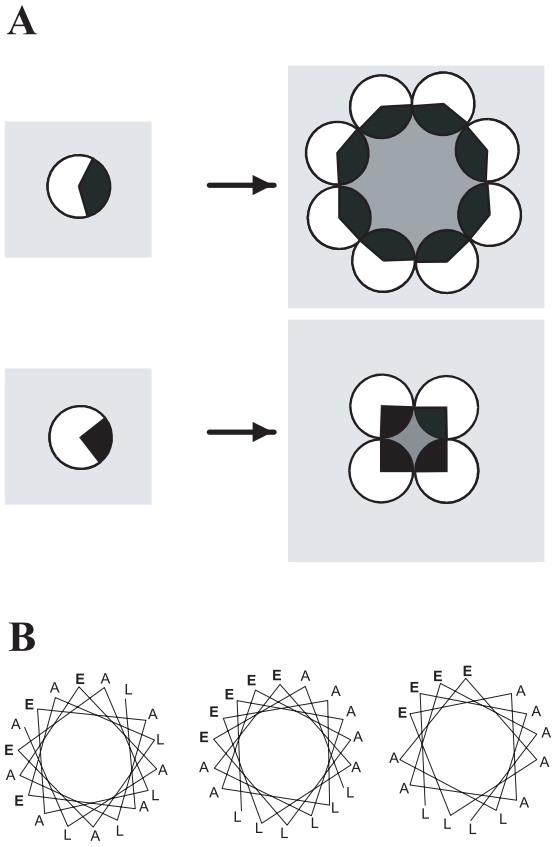

Fig. 1. (A) Diagram showing the effect of hydrophilic residue arrangement on potential pore formation. Amphipathic peptides in helical conformation are represented by circles. The liposomal bilayer and aqueous pore volume are shown in light gray and dark gray, respectively. For peptides with hydrophilic charged residues distributed along a wide angle on the helix (top left), a large number of molecules will be needed to form an aqueous pore in a lipid bilayer (top right). When charged residues are confined to a smaller angle (bottom left), hydrophilic interactions should be satisfied by the formation of a smaller pore at lower peptide concentration (bottom right). (B) Comparison of partial sequence helical wheel diagrams of EALA, and the novel 29- and 15-amino-acid peptides. EALA (left) possesses glutamic acid residues distributed over a large angle and interspersed with hydrophobic residues (similar to GALA), whereas the 29- and 15-amino-acid peptides (middle and right helical wheels, respectively) possess glutamic acid residues confined within a smaller angle of the helix.

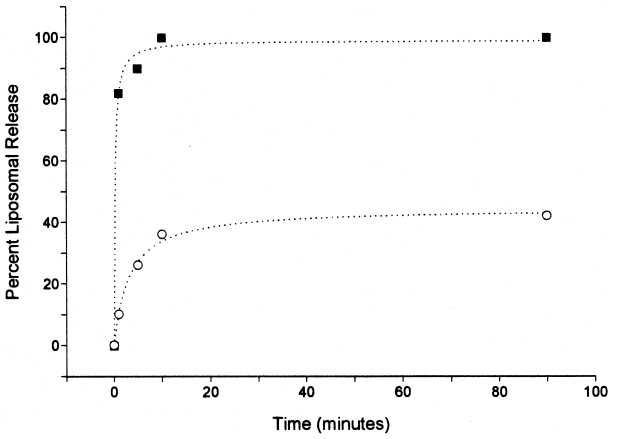

Fig. 2. Liposomal release of both propidium iodide (PI) and a 10 000 M_r dextran is complete after 10 min incubation with the 29-amino-acid peptide. Egg phosphatidylcholine liposomes encapsulating either PI (■) or 10 000 M_r Texas Red-labeled dextran (○) were incubated with the 29-amino-acid peptide at a 100:1 lipid to peptide ratio. Leakage of either marker from liposomes was assessed at varying time points following acidification to pH 4. PI leakage was measured following the addition of calf thymus DNA to the incubated solution and dextran leakage was determined using size-exclusion chromatography and fluorescence detection, as described in Section 2.

3.4. The 29-amino-acid peptide is significantly more active than the 15-amino-acid truncated version at low pH, and less active at physiological pH

We also wished to evaluate whether a shorter version of the same sequence might retain the desirable properties of the 29-amino-acid peptide, thereby consuming even less of the liposome's internal volume. For this purpose, a 15-residue peptide was obtained and compared with the above peptide of the same sequence. Both the 29- and 15-amino-acid peptides were found to destabilize liposomes with the desired pH dependence at relatively high peptide concentra-

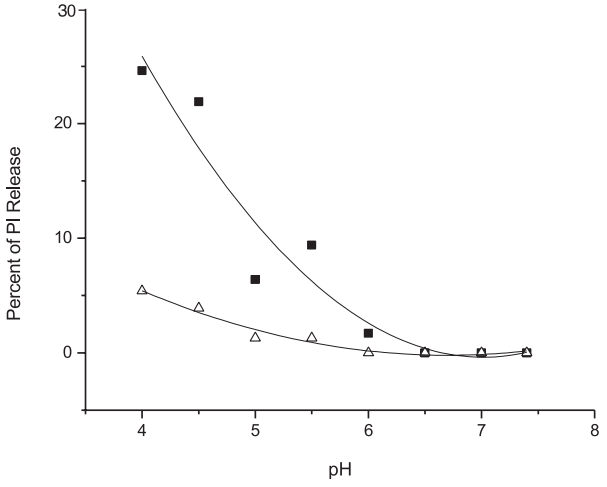

Fig. 3. The 29-amino acid peptide promotes more efficient PI release from liposomes compared to EALA. Either the 29-amino acid peptide (■) or EALA (△) was incubated with egg phosphatidylcholine liposomes encapsulating propidium iodide (PI) for 10 min at a lipid to peptide ratio of 10 000:1 and a lipid concentration of 2.9 mM. Leakage of PI from liposomes was determined 10 min later, as described in Section 2.

tions (Fig. 4a). However, as the lipid to peptide molar ratio increased from 50:1 to 100:1 and 500:1, the 15-residue peptide declined in potency whereas the 29-residue version did not (Fig. 4b,c). Although peptides of intermediate length were not examined, these data demonstrate that continual truncation of the 29-residue peptide will eventually lead to loss of activity.

Importantly, a tendency to destabilize liposomes at neutral pH constituted another disadvantage of the shorter peptide. Thus, at pH 7.4, where very low levels of leakage were observed with the longer peptide, we consistently observed elevated (as high as 20%) PI release with the shorter peptide. Liposome instability at physiological pH is obviously an undesirable characteristic of any strategy designed to promote liposome unloading only following endocytosis.

3.5. *Both novel peptides exhibit decreased activity in cholesterol-containing membranes*

Cholesterol can be incorporated into liposomes to enhance stability and is often used when increased membrane rigidity is needed to prolong circulation time in vivo [4]. To characterize the above peptides' behavior in cholesterol-containing membranes, both long and short peptides were added to liposomes containing 0, 20, or 40 mol% cholesterol and examined for their pH-induced membrane lytic properties. As seen in Fig. 5, cholesterol was found to decrease PI release induced by both the 29 and 15-amino-acid peptides. However, whereas the activity of the shorter peptide was severely compromised by increasing cholesterol content, the activity of the longer peptide was only mildly affected. Thus, 40% cholesterol has been commonly used in liposomes for in vivo applications, and at this cholesterol concentration and pH 4.5, the 29-residue peptide retained 75% of its initial activity, whereas the shorter homolog displayed only 20% of its activity.

3.6. *The 29-amino-acid peptide can form pores large enough to release macromolecular dextrans from liposomes*

Because the 29-amino-acid peptide consistently demonstrated superior properties to both EALA and the 15-residue peptide, we decided to investigate only the 29-amino-acid peptide further. In order to explore the size of the membrane holes generated by the 29-residue peptide, the peptide was incubated with liposomes encapsulating 10 000 and 40 000 M_r dextrans and dextran efflux was monitored. As seen in Fig. 6, both dextrans were released from the liposomes in a pH-dependent manner, however, the smaller dextran escaped more efficiently than the larger one. Further, neither dextran was completely discharged from liposomes during the 10-min incubation period, even though PI could be entirely released under identical conditions. It should also be noted that dextran release was only observed at very high concentrations of peptide (lipid to peptide molar ratio of 50:1). The increased retention of larger molecules by the perforated liposomes suggests that peptide pore sizes are heterogeneous, with larger pores forming only at higher peptide concentrations. As a result of this, some liposomes may contain no openings large enough to pass the macromolecular dextrans.

3.7. *Encapsulation of the 29-amino-acid peptide into folate-targeted liposomes containing ara-C increases their toxicity toward cultured cancer cells ~30-fold*

In each of the above unloading experiments, pH

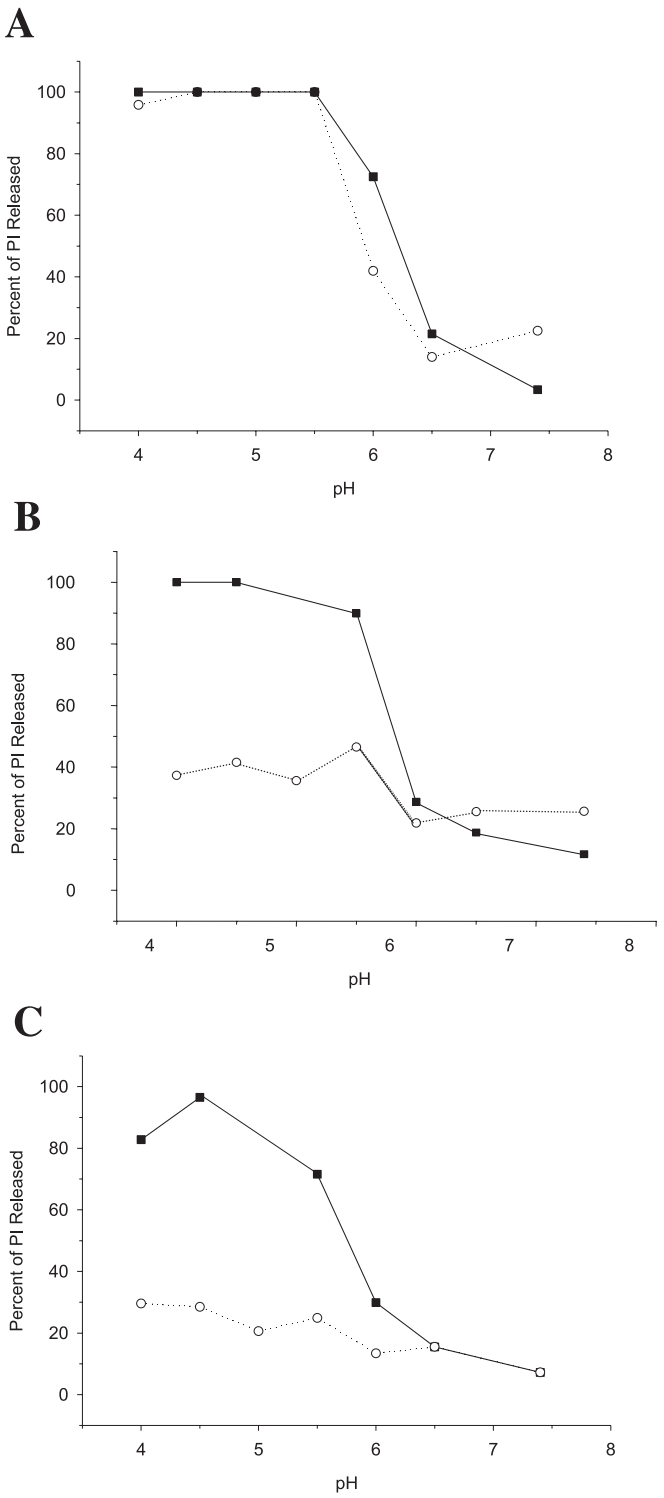

Fig. 4. Both long and short peptides induce pH-dependent unloading of propidium iodide from liposomes. Either the 29-amino-acid peptide (■) or the truncated 15-amino-acid peptide (○) was incubated with egg phosphatidylcholine liposomes encapsulating propidium iodide (PI) for 10 min at various pH. Leakage of PI from the liposomes was assessed at various lipid to peptide ratios: 50:1 (A), 100:1 (B), and 500:1 (C) as described in Section 2. Lipid concentration was maintained at 2.9 mM.

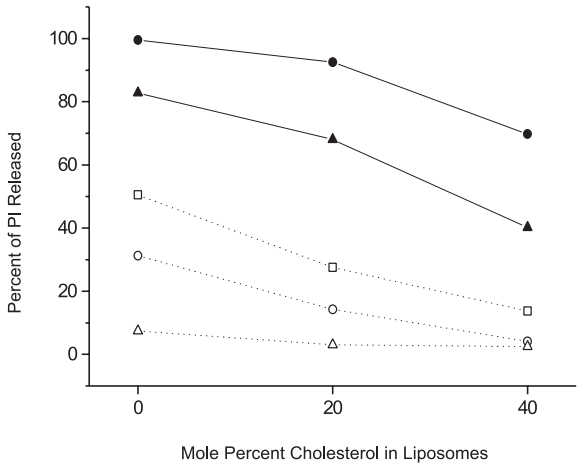

Fig. 5. Cholesterol reduces peptide-induced pH-dependent unloading of PI. Egg phosphatidylcholine liposomes containing 0, 20 or 40 mol% cholesterol were incubated with the 29 (solid lines and symbols) or 15 (dotted lines and open symbols) -residue peptide at pH 4.0 (squares), 4.5 (circles), or 5.0 (triangles). Leakage of PI from the liposomes was assessed after 10 min incubation, as described in Section 2. Lipid concentration was maintained at 0.1 mM, and lipid to peptide ratio was 100:1.

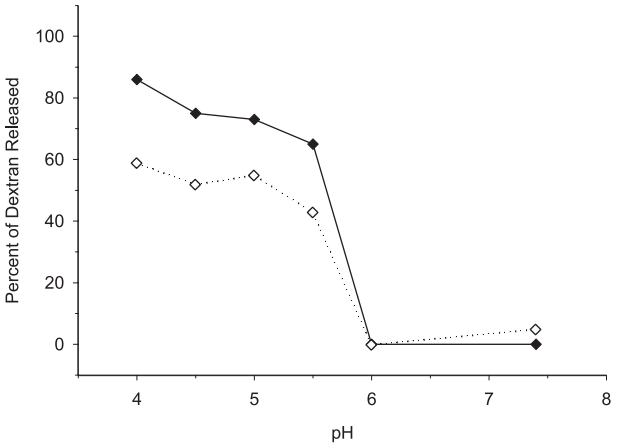

Fig. 6. The 29-amino-acid peptide induces pH-dependent unloading of encapsulated dextrans from liposomes. Egg phosphatidylcholine liposomes encapsulating 10 000 M_r (♦) or 40 000 M_r (◇) Texas Red-labeled dextrans were incubated for 10 min with the 29-residue peptide at a 50:1 lipid to peptide ratio. Dextran leakage was assessed following incubation at various pH. Lipid concentration was maintained at 2.9 mM.

was artificially changed to mimic the acidification which occurs during receptor-mediated liposome endocytosis by a folate receptor-expressing cancer cell. To test whether the 29-residue peptide might actually function in this manner in a living cell, the peptide was co-encapsulated with the anti-cancer drug, cytosine arabinoside, in folate-targeted liposomes (containing 0.5 mol% folate-PEG-PE), and the liposomes were incubated with folate receptor-expressing human KB cells [26]. As seen in Fig. 7, the 29-amino-acid peptide measurably enhances the toxicity of ara-C to cancer cells. Thus, under conditions where folate-targeted liposomes containing ara-C exhibit little toxicity towards KB cells, co-encapsulation of the peptide results in 30% growth inhibition. Furthermore, liposomes containing the 29-residue peptide, but no ara-C, demonstrated negligible toxicity (data not shown), indicating that cytotoxicity was not caused by the peptide. The delivery of folate-targeted liposomes containing peptide and ara-C was also examined using 2 mM free folic acid in the cell culture medium as a competitive inhibitor of the folate receptor. As expected, the toxicity of these liposomes was $<5\%$ of the non-competed sample (data not shown). This result confirms that liposome uptake and the subsequent cytotoxicity is mediated by the folate receptor [27].

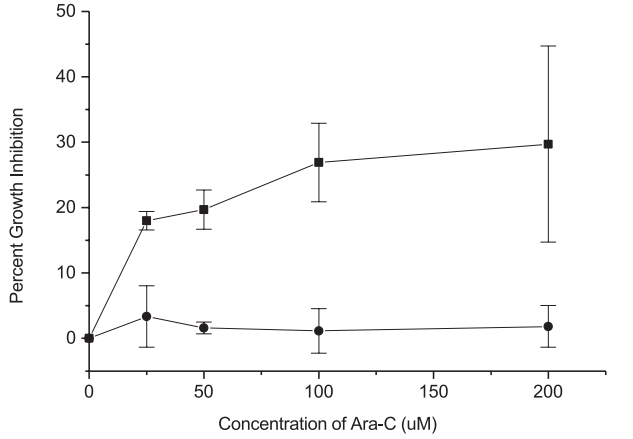

Fig. 7. The toxicity of liposomal ara-C to cultured KB cells is enhanced by the 29-amino-acid peptide. Folate-targeted egg phosphatidylcholine liposomes (containing 0.5 mol% folate-PEG-PE) encapsulating ara-C only (●) or ara-C and 29-amino-acid peptide (■) were incubated with KB cells for 4 h. Ara-C concentration, reported on the *x*-axis, was determined by UV absorbance of detergent-lysed liposomes in solution. Cytotoxicity was determined 24 h later using a [^{3}H]thymidine incorporation assay, as described in Section 2.

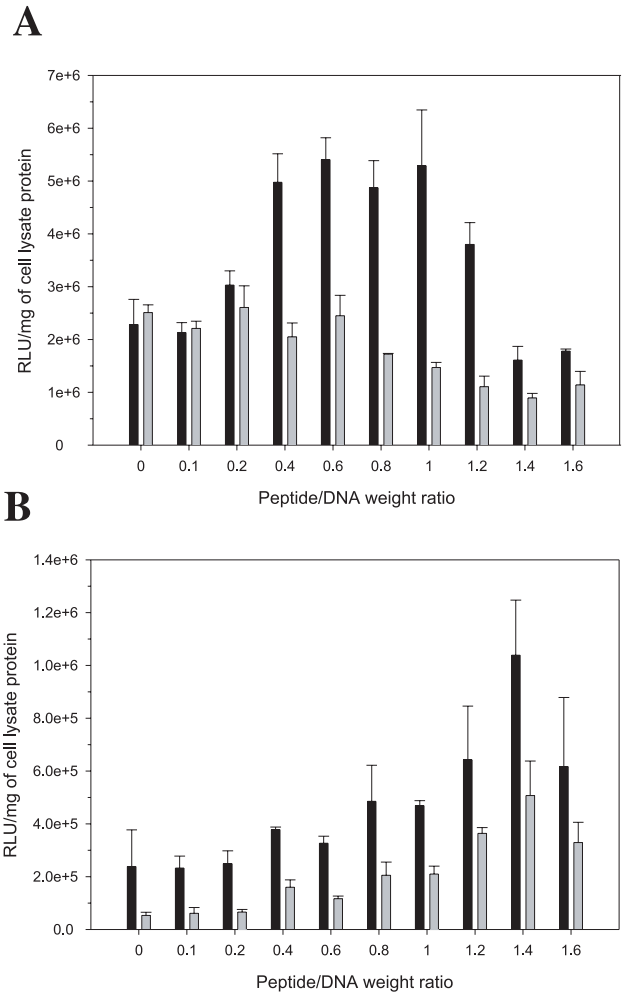

Fig. 8. The 29-amino-acid peptide enhances the folate-targeted transfection efficiency of DOTAP/cholesterol liposomes. Transfections were performed without serum (A) or in the presence of 10% fetal bovine serum (B). Transfection particles were prepared by complexing 0.1 μg pGL3 DNA mixed with peptide (at the indicated peptide/DNA weight ratios), with cationic liposomes consisting of 60% DOTAP and 40% cholesterol. Folate-targeted cationic liposomes contained 0.5% folate-PEG-PE (black bars) and non-targeted liposomes contained 0.5% PEG-PE (gray bars). KB cells in 96-well plates were transfected as described in Section 3, and assay results are expressed as RLUs per mg of cell lysate protein.

3.8. *The 29-amino-acid peptide enhances folate-targeted transfection of KB cells by serum-stable DNA-DOTAP/cholesterol complexes*

The encouraging results with peptide-assisted drug delivery led us to explore the utility of the 29-amino-acid peptide in enhancing the potency of various gene therapy formulations. For this purpose, DOTAP/cholesterol liposomes were complexed with different ratios of the peptide and a luciferase expression vector, and the complex was then examined for transfection efficiency following incubation with KB cells in the absence (Fig. 8a) or presence (Fig. 8b) of serum. Without serum or peptide present (Fig. 8a, first set of bars), no folate-targeted enhancement of transfection was observed (please note that with other liposomal vector formulations folate targeting significantly enhances transfection even in the absence of serum and peptide [11,28]). Thus, the cationic charge on the cationic liposomal vector complexes was apparently sufficient to dominate the binding and release of plasmid into the KB cells. However, in the case of folate-targeted liposomes, increasing the amount of peptide enhanced transfection up to an optimum peptide/DNA weight ratio of 1:1. With non-targeted liposomes, addition of the peptide did little to enhance the potency of the vectors. This result may have arisen because the non-targeted liposomal vectors could have entered the cytoplasm at an earlier stage of the endocytic pathway, perhaps by a pH-independent mechanism [29]. The result is also interesting, because it implies that endosome escape could constitute a critical variable governing the efficiency of targeted liposomal vectors. Thus, while the peptide cannot influence folate receptor binding or receptor-mediated endocytosis, without peptide catalysis of DNA escape from the endosomes, the folate-targeted vector was largely ineffective in promoting gene expression. It was also observed that at higher peptide/DNA ratios (above 1:1), the transfections decreased with both the both the targeted and non-targeted complexes, suggesting that high amount of peptide may interfere with proper cationic lipid/DNA complex formation.

In contrast to transfections in the absence of serum, analogous studies in the presence of serum indicated that without peptide present, a 100-fold reduction in non-targeted transfections but only a 10-fold reduction in targeted transfections was observed (Fig. 8b, first set of bars). Thus, while serum proteins likely coated the cationic vector complexes and reduced the binding ability of the non-targeted complexes, shielding of charge interactions in the targeted complexes was probably compensated in part by association of the folate targeting ligand with fo-

late receptors on the cancer cell surfaces. The above data suggest that the ~250 Å polyethylene glycol spacer between folic acid and the lipid is sufficient to enable the ligand to extend through the serum protein coat. However, serum protein shielding of the cationic charges on these vectors may still hinder their lipid mixing/fusion properties, resulting in the observed decrease in activity with both targeted and non-targeted complexes. Hence, the peptide was found to enhance both folate-targeted transfection and non-targeted transfection at an elevated serum content. This would suggest that some serum-coated complexes still enter cells non-specifically, but that they find themselves in low-pH endosomes where the peptide can enhance their unloading. In addition, increasing the amount of peptide did increase transfection, and a slightly higher peptide/DNA weight ratio of 1.4:1 permitted optimal transfection of these serum coated complexes.

4. Discussion

Many pH-sensitive peptides have been utilized as possible catalysts of liposome unloading at low pH [20,21,30–34]. Because none of these peptides exhibit the pH sensitivity and potency that would warrant their evaluation in clinical trials, we undertook the design of a peptide that would retain the pH sensitivity of previously studied peptides, yet exhibit a potency that would encourage its evaluation for clinical use. Based on the characteristics presented in Figs. 2–8, we suggest that the novel 29-residue peptide advances our progress toward this objective. Thus, the peptide (i) causes negligible liposome leakage at pHs >6.5, (ii) undergoes its lytic transition in the endosomal pH range, (iii) remains functional in cholesterol-containing liposomes, (iv) induces rapid leakage ($t_{1/2}<10$ min) upon acidification, (v) promotes release of both low and high M_r cargos, and (vi) functions at lower concentrations than the previously characterized EALA peptide.

A fundamental difference between the GALA-like peptides and our 29-amino-acid peptide lies in the distribution of glutamic acid residues around each peptide's helical wheel. In GALA and EALA, the glutamic acids are interspersed with leucine and alanine residues on the same face of the helix. In the new 29-amino-acid peptide, however, all glutamic acids are situated on the same surface of the α-helix and are not interrupted by intervening hydrophobic residues. Interestingly, although our data demonstrate that the 29-residue peptide is only membrane active at low pH, these sequence alterations afford 88% helical conformation to the peptide even at neutral pH, as determined by circular dichroism [35].

Because no EALA surface exposes solely hydrophilic residues, a well-defined geometry of self-assembly in the membrane may not occur. In contrast, the geometry of self assembly of the 29-amino-acid peptide in a bilayer should be more limited, since it has well defined hydrophilic and hydrophobic surfaces. It has been previously shown that 4–6 GALA peptide monomers are necessary for its assembly within the bilayer [36], whereas 8–12 monomers are needed for the formation of a transbilayer pore [31]. This is in accordance with our simple model for bilayer assembly (Fig. 1A) which predicts that roughly 8 monomers are necessary for pore formation with either GALA or EALA peptides. Using the same model, we hypothesized that a peptide with glutamic acid residues arranged around a smaller arc of the helical wheel (i.e., as in our novel peptide) would require fewer monomers for pore formation. This difference, however, would be expected to emerge only at very limiting peptide concentration, when sufficient peptide is available to form a tetrameric but not an octameric pore. Indeed, measurable distinctions between EALA and the 29-mer became evident at very low peptide concentrations, where the new peptide clearly outperformed EALA. While the difference in potency may be considered relatively minor, because very low peptide concentrations can maximize the volume available for co-encapsulated therapeutic molecules, this difference could impact the usefulness of the peptide in therapeutic applications.

We suggest the differences in liposome permeability promoted by EALA and the new peptide also support the hypothesis that a peptide with glutamic acid residues arranged on a smaller angle of the helical wheel will form smaller sized membrane pores, perhaps consisting of only a few peptide monomers per channel (Fig. 1A). Thus, the essentially quantitative release of PI from the liposomes demonstrated that an aqueous channel of at least 1.5 nm in diameter must have formed in virtually all peptide-con-

taining liposomes. In contrast, the limited efflux of the ~10 000 and ~40 000 M_r dextrans suggests that considerably fewer pores of large diameter were formed in the same liposomes. Still, the fact that the macromolecular solutes were able to escape the liposomes at all would suggest that the 29-residue peptide can form very large pores when sufficient peptide is present. However, the observation that neither dextran was quantitatively released from the liposomes further suggests that a measurable population of liposomes must exist with openings too small to accommodate any macromolecules.

Results from the studies with dextran-loaded liposomes also allowed us to better define the mechanism of action of our peptide. We have assumed that the activity of the 29-residue peptide was due to formation of pores in a bilayer. Indeed, using this peptide, molecules of increasing size were released with decreasing efficiency, suggesting a size filter exists in the efflux pathway. The presence of a permeability barrier with a strong size dependence argues more for a mechanism of pore formation than detergent-like membrane lysis or destabilization [31].

The 15-residue truncated peptide was also found to facilitate the pH-dependent unloading of PI from liposomes, but only at significantly higher concentrations than required by its longer counterpart. Whether the 29-residue version is, in fact, already at its optimal length is not certain, since no other homologous peptides were tested. The 29-residue peptide was originally designed to just barely span the bilayer. Thus, it was felt that a significantly shorter peptide would not extend to both aqueous compartments and would consequently be energetically unable to reside within the bilayer, thereby rendering it membrane inactive. This assumption was obviously wrong, as indicated in Fig. 4. In fact, Puyal and coworkers have shown that the weak membrane destabilizing activity of their 14-amino-acid fusogenic peptide could be enhanced 1000-fold if the peptide was directly linked to the liposomes [37]. We also noticed that higher local concentrations of the peptide allowed for greater membrane lytic activity. Conceivably, direct conjugation of the 15-amino-acid peptide to a lipid head group could also resurrect this peptide's usefulness.

As noted above, membrane lytic peptides may either be covalently attached to a lipid head group or co-encapsulated with the drug inside the liposome. For our applications, we have chosen the co-encapsulation strategy, since in addition to promoting liposome unloading, the peptide may subsequently become available to insert into the endosomal bilayer and further enhance cytoplasmic delivery of the drug [22]. This latter component of the peptide's activity is likely to further enhance the cytotoxicity of the folate targeted liposomal ara-C preparation. Obviously, further work is also needed to determine the optimum lipid composition for use with the 29-residue peptide for in vivo drug delivery applications. Clinical or 'stealth' liposomes have a very rigid bilayer containing solid-phase lipid and a high percentage of cholesterol. While these liposomes may be more difficult to permeabilize than the egg phosphatidylcholine liposomes tested in this study, inclusion of cholesterol in the latter formulation caused only minor reduction in peptide potency.

A major limitation of non-viral gene delivery has historically been the poor release of DNA from endosomal compartments. Previous workers have documented the ability of pH-sensitive peptides to enhance the transfection efficiency of non-targeted fusogenic liposomal vectors [38]. Only recently have serum-stable complexes been used in conjunction with amphipathic peptides to enhance transfection [39–41], but none of these preparations were tumor targeted. We have demonstrated the feasibility of employing a co-entrapped lytic peptide to enhance the specific receptor-targeted transfection of cancer cells. The fact that our formulation was also serum stable offers hope that the peptide might be applied for in vivo use.

In summary, we have characterized a novel peptide that was designed to self-associate into small pore-forming oligomers within membrane bilayers upon acidification to endosomal pHs. Our data reveal that this peptide retains the optimal pH sensitivity of GALA-like peptides, but maintains its potency at lower concentrations. Since it has also demonstrated improved utility in combination with serum stable, folate-targeted, liposomal gene therapy vectors, we suggest that further characterization and optimization of the system with in vivo applications is warranted.

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