Octaarginine- and Octalysine-modified Nanoparticles Have Different Modes of Endosomal Escape

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The present study examines the role of surface modification with an octaarginine peptide (R8) in liposomal escape from endocytic vesicles, using octalysine (K8) as a control cationic peptide; the mechanism of endosomal escape of liposomes was also investigated. Gene expression of condensed plasmid DNA encapsulated in R8-modified nanoparticles was more than 1 order of magnitude higher than that of K8-modified nanoparticles, and 2 orders of magnitude higher than gene expression using unmodified nanoparticles. The difference in gene expression could not be attributed to differences in uptake, as R8- and K8-modified liposomes were taken up primarily via macropinocytosis with comparable efficiency. The extent of R8-nanoparticle escape to the cytosol was double that of K8-nanoparticles. Suppression of endosome acidification inhibited R8-nanoparticle endosomal escape, but enhanced that of K8-nanoparticles. Using spectral imaging in live cells, we showed that R8- and K8-liposomes escaped from endocytic vesicles via fusion between the liposomes and the endosomal membrane. R8-liposomes fused efficiently at both acidic and neutral pH, whereas K8-liposomes fused only at neutral pH. Similar behavior was observed during in vitro lipid mixing and calcein-release experiments. Co-incubation of cells with distinctly labeled K8- and R8-modified nanoparticles confirmed a common uptake pathway and different rates of endosomal escape particularly at longer time intervals. Therefore, it was concluded that R8 on the liposome surface stimulates efficient escape from endocytic vesicles via a fusion mechanism that works at both neutral and acidic pH; in contrast, K8 mediates escape mainly at neutral pH.

Since the first reports demonstrating the ability of the HIV-1 TAT protein to translocate across cell membranes, arginine-rich cell-penetrating peptides (AR-CPPs)2 have continued to attract a lot of attention (1, 2). These AR-CPPs, including the short basic segment of the TAT protein and a group of short homoarginine sequences, possess the ability to cross cell membranes either alone or carrying cargo (3–6). From the perspective of cell biology, the mechanisms responsible for this enhanced internalization have also gained considerable attention as possible novel cell-entry pathways. However, despite the controversy surrounding the uptake mechanism that accompanied their discovery, it is now widely accepted that AR-CPPs are internalized via endocytosis (7–10). We, together with others, have suggested that macropinocytosis is one of the predominant AR-CPP uptake pathways (11–15).

After endocytosis, AR-CPPs, either free (16) or linked to cargo, become entrapped in the endocytic vesicle unless they are equipped with an efficient device for escape, such as the pH-sensitive influenza virus hemagglutinin fusogenic peptide (12, 17), or lysosomotropic agents, which are used to disrupt endosome integrity (18). However, Kaplan et al. (13) reported that more than 99% of the TAT fusion proteins remain entrapped in macropinosomes even after fusogenic peptide treatment. We previously reported that plasmid DNA (pDNA) condensed with octaarginine (R8) ends up in lysosomes after endocytic uptake (19). Therefore, endosomal escape is believed to be the rate-limiting step in delivery of the attached cargo to the cytosol. However, when the pDNA core was encapsulated into liposomes whose surface had been modified with a high density of R8, the liposomes were capable of stimulating uptake via macropinocytosis, and co-localization with lysosomes was reduced, indicating improved endosomal escape efficiency; the net result was high transgene expression (14). One explanation for the difference associated with R8 surface modification is the increased control over the density of R8 available to interact with membranes compared with free peptides, fusion proteins, and R8 polyplexes. These results raised the question as to...
whether octaarginine plays a role in liposome escape from endocytic vesicles. And if so, what is the escape mechanism of these R8-liposomes? Does escape depend only on the positive charge associated with the peptide or is endosomal escape dependent on specific biological properties of the arginine amino acids?

Previous reports using liposomes as model membranes showed that oligoarginine peptides induced either membrane rupture (20) or fusion (21). However, these studies mainly focused on free peptides and were performed on model membranes and not on live cells. Therefore, the present study utilized liposomes modified with R8 as a model for AR-CPPs linked to bulky cargo to study the mechanism of R8-linked cargo escape from endocytic vesicles in live cells. To examine whether the cationic nature of octaarginine plays a role in escape, octaarginine-modified liposomes were compared with those modified with octalysine (K8), a cationic peptide with a structure similar to that of octaarginine. Recently, cellular uptake of oligolysines, which are cationic peptides that lack a guanidinium group, was reported to be comparable with that of oligoarginines (22). Therefore, K8 serves as a good model for the study of the role that the guanidinium group of R8 plays in endosomal escape.

The results of the present study demonstrated that R8-modified liposomes (R8-Lip) escaped from endocytic vesicles much more efficiently than K8-modified liposomes (K8-Lip) despite utilizing the same cellular uptake pathway, macropinocytosis. The escape of both R8-Lip and K8-Lip took place mainly through liposomal fusion to the endosomal membrane. R8-Lip fusion was efficient at both acidic and neutral pH, whereas K8-Lip fusion was limited to neutral pH. These results demonstrate that R8 plays an active role in mediating liposome and endosomal membrane fusion, which leads to efficient escape. The function of R8 in fusion is discussed in detail and the difference between R8 and K8 is explained.

**EXPERIMENTAL PROCEDURES**

**Materials**—Egg phosphatidylcholine (EPC), cholesterol (Chol), 1,2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rh-DOPE), 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N-(7-nitro-2,1,3-benzoxadiazol-4-yl) (NBD-DOPE), l-α-phosphatic acid (PA) (from chicken egg, monosodium salt), and dioleoylphosphatidyl ethanolamine (DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Sulforhodamine B was purchased from Molecular Probes (Eugene, OR). 1,2-Dioleoyl-sn-glycerol-3-phospho-1-serine (DOPS), poly-L-lysine, bafilomycin A1, chlorpromazine, and amiloride were purchased from Sigma. Calcein and HEPS were obtained from Dojindo (Japan). Cholic acid was obtained from Nakalai Tesque (Japan). Stearylzed octaarginine (STR-R8) and stearylzed octalysine (STR-K8) were synthesized as described previously (23). Other chemicals were purchased from Wako Pure Chemicals (Japan). Plasmid DNA pCMV-luc encoding luciferase was prepared using an EndoFree Plasmid Mega Kit (Qiagen, Germany). The anti-luciferase siRNA (21-mer, 5′-GCGUCUGCUGUGGCACGCTT-3′, 5′-GGGUGGCACCAGCGTCCT-3′) and the anti-green fluorescent protein siRNA (5′-GCGUACCCUGAAGUUCAU-CTT-3′, 5′-GAUGAACUUCGGGUCAGCTT-3′) were obtained from Thermo Electron GmbH (Germany). NIH 3T3 cells were obtained from the American Type Culture Collection (Manassas, VA).

**Preparation of the Octaarginine- or Octalysine-modified Liposomes**—Liposomes were mainly composed of DOPE and PA (7:2 molar ratio) or EPC/Chol/STR-R8 or STR-K8 (7:3:1 molar ratio). For spectral imaging experiments, the liposomes were labeled by including 0.5 mol % Rh-DOPE and 1 mol % NBD-DOPE (mol % of the total lipid fraction). Liposomes were prepared using the hydration method followed by sonication for 1 min in a bath-type sonicator (85 W, Aiwa Co., Japan). In the case of DOPE/PA liposomes, after sonication, the liposomes were incubated with an aqueous solution of STR-R8 or STR-K8 (10 mol % of total lipids) for 30 min at room temperature. For flow cytometry experiments, an EPC/Chol/STR-R8 or STR-K8 lipid film was hydrated with sulforhodamine B, which was used as an aqueous phase marker, followed by extrusion through polycarbonate membrane filters (Nucleopore) with pore sizes of 400 and 100 nm using a Mini-Extruder (Avanti Polar Lipids). Liposomes then were purified on a Sephadex G-100 column (Amersham Biosciences AB). The particle size distribution of the prepared liposomes was determined using a dynamic light-scattering method and the ζ-potential was calculated from the electrophoretic mobility as determined using a Zetasizer Nano ZS (ZEN 3600, Malvern Instruments, UK).

**Preparation of Octaarginine- or Octalysine-modified DNA-coated Particles**—Plasmid DNA was condensed with poly-L-lysine as described previously (24). 0.25 ml of the condensed DNA solution was then added to the lipid film, which was formed by the evaporation of a chloroform solution of 137.5 nmol of lipids, DOPE/PA (7:2 molar ratio), on the bottom of a glass tube, followed by a 10-min incubation to hydrate the lipid film. The final lipid concentration was 0.55 mM. Rh-DOPE was included as a lipid-phase marker at 1 mol % of the total lipid when required. The glass tube was then sonicated for 1 min in a bath-type sonicator. When required, the coated particles were incubated with an aqueous solution of STR-R8 or STR-K8 (10 mol % of total lipids) for 30 min at room temperature after sonication. The size and ζ-potential of the coated particles were measured as described above.

**Transfection Assay**—One day before transfection, NIH 3T3 cells were seeded into 24-well plates at 4 × 10^4 cells per well. Cells were incubated for 3 h at 37 °C with 0.25 ml of serum-free Dulbecco’s modified Eagle’s medium (Invitrogen) containing unmodified, R8- or K8-modified DNA-coated particles (Unmod-Cps, R8-Cps, or K8-Cps) encapsulating 0.4 μg of DNA. Next, 1 ml of medium containing 10% fetal calf serum was added and the cells were incubated for a total of 24 h. To investigate the effects of different inhibitors on gene expression, cells were pretreated with bafilomycin A1 (0.5 μm) for 30 min or with chloroquine just before addition of R8-Cps or K8-Cps and the incubation was continued for 3 h. Next, 1 ml of medium containing 10% fetal calf serum was added and the incubation was continued for an additional 21 h. The cells were then washed and lysed using 75 μl of reporter lysis buffer (Promega, Madison, WI). Luciferase activity in the cell lysate then
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was measured using a luminometer (Luminescencer-PSN, ATTO, Japan). The amount of protein in the cell lysate was determined using a BCA protein assay kit (Pierce). Luciferase activities were expressed as relative light units (RLU) per mg of protein.

Flow Cytometry—To investigate the extent of cellular uptake for R8-Cps and K8-Cps, NIH 3T3 cells were incubated in serum-free medium containing increasing concentrations of Rh-DOPA-labeled Cps for 1 h at 37 °C. Following incubation, the medium was removed, and the cells were washed once with ice-cold phosphate-buffered saline (PBS) containing heparin (20 units/ml). The cells were then trypsinized, washed twice using PBS/heparin and centrifugation at 4 °C, suspended in 0.5 ml of PBS, and, after being passed through a nylon mesh, analyzed by flow cytometry (BD Biosciences). Live cells were gated on forward and side scatter, and 10,000 viable cells were analyzed. To check the rate of cellular uptake, the same experiment was repeated at a concentration of DNA-coated particles equal to the pDNA dose used for transfection and incubation with particles was continued for 15, 90, and 180 min at 37 °C. Cells were then washed and analyzed as described above. To examine the mechanism of R8-Lip and K8-Lip internalization, cells were incubated in the absence or presence of amiloride (1.5 mM) or chlorpromazine (5 µg/ml) for 30 min. R8-Lip and K8-Lip containing rhodamine in the aqueous phase were then added (final lipid concentration, 0.1 mM), and the incubation was continued for 1 h. After washing once with PBS supplemented with 4 mM cholic acid and twice with PBS as described above, cells were analyzed by flow cytometry.

Preparation of Octaarginine- or Octalysine-modified Particles Encapsulating siRNA—siRNA was conditioned with STR-R8 at a nitrogen:phosphate ratio of 2.9 as described previously (25). A lipid film, formed by evaporation of a chloroform solution of lipids, DOPE/PA (7:2 molar ratio) on the bottom of a glass tube, was hydrated with RNase-free water during a 10-min incubation; the final lipid concentration was 0.55 mM. The glass tube was then sonicated for 1 min in a bath-type sonicator followed by sonication for 10 min using a probe-type sonicator (Branson Digital sonifier 250, Branson Ultrasonics Corporation, Danbury, CT) while cooling on ice. After sonication, 500 µl of small unilamellar vesicles were added to 250 µl of conditioned siRNA, followed by vortexing for 1 min. The resulting siRNA-coated nanoparticles then were incubated with an aqueous solution of STR-R8 or STR-K8 (10 mol % of total lipids) for 30 min at room temperature. The size and ζ-potential of the coated particles were measured as described above.

Luciferase Gene Silencing in Stably Transfected HeLa Cells—HeLa cells stably expressing luciferase were seeded in 24-well plates at a density of 4 × 10⁴ cells/well. After 24 h, the cells were incubated for 3 h at 37 °C with 0.25 ml of serum-free OptiMEM medium (Invitrogen) containing different R8- or K8-modified siRNA-coated nanoparticles (siRNA-R8-Cps or siRNA-K8-Cps) equivalent to 0.1 and 0.4 µg of siRNA. Next, 1 ml of Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum was added to the cells, followed by a 21-h incubation. The cells were then washed with PBS and lysed using 75 µl of reporter lysis buffer (Promega). Luciferase activity in the cell lysate then was measured as described above for the “Transfection Assay.”

Confocal Laser Scanning Microscopy (CLSM)—To investigate the intracellular trafficking of R8-Cps and K8-Cps, NIH 3T3 cell plasma membranes were first stained with the PKH67 green fluorescent cell linker (Sigma) according to the manufacturer’s protocol. Cells then were treated with modified DNA-coated particles encapsulating rhodamine-labeled pDNA, at a final concentration of 0.01 mM lipid, in serum-free medium at 37 °C for 30 min. Cells were washed once with medium and the incubation was continued for an additional 2.5 h in serum-free medium. Cells were then washed and examined using a confocal laser microscope (LSM510, Carl Zeiss Co. Ltd., Jena, Germany) equipped with an oil-immersion objective lens (Plan-Apochromat ×63/NA = 1.4). To follow the endosomal escape of K8- and R8-Cps simultaneously incubated with the cells at different time intervals, NIH 3T3 cell plasma membranes were first stained with PKH67, as mentioned above. Cells then were pulsed with K8-Cps encapsulating Cy5-labeled pDNA together with R8-Cps encapsulating rhodamine-labeled pDNA, each at a final concentration of 0.01 mM lipid, in serum-free medium at 37 °C for 5 min. Cells were then washed once with medium and examined until 30 min (short time interval) using a confocal laser microscope as described above. When checking the escape for a long time interval, the same procedure was repeated, except for a pulse time of 15 min, and the examination was conducted after 3 h.

Evaluation of Membrane Fusion between Liposomes and Endocytic Vesicles Using Live Cell Spectral Imaging—For following liposome-membrane fusion in live cells we used a modification of the method that was reported earlier (26). Briefly, R8- and K8-modified liposomes were prepared with an additional 1 mol % NBD-DOPA (excitation wavelength, 460 nm; emission wavelength, 534 nm) and 0.5 mol % rhodamine-DOPA (excitation wavelength, 550 nm; emission wavelength, 590 nm) as a donor and acceptor of fluorescence resonance energy transfer (FRET), respectively. The dual-labeled liposomes were then incubated with NIH 3T3 cells (final lipid concentration, 0.08 mM) for 1 h in serum-free Dulbecco’s modified Eagle’s medium. Cells were washed and supplied with fresh medium and incubated for an additional 1 h. 10 min before the end of incubation, Hoechst 33342 dye was added to the medium at a final concentration of 5 µg/ml to stain the nucleus. After incubation, the cells were washed and then observed by confocal laser scanning microscopy (LSM510 META; Carl Zeiss Co. Ltd.) to localize liposomes within the cell. To spectrometrically evaluate membrane fusion between the liposomes and the endocytic vesicles during escape, NBD was excited by light (488 nm) from an argon laser. Emitted light filtered through a dichroic mirror (HFT488) was spectrometrically analyzed using META equipment over a range of 510 to 630 nm. Spectral data are presented as the relative fluorescence intensity compared with maximal intensity (586 nm).

Studying Fusion of R8- and K8-modified Liposomes to Negatively Charged Liposomes—Fusion of vesicles detected as mixing of membrane lipids was monitored using a modified probe dilution assay that is based on FRET (21, 27). In brief, large unilamellar vesicles comprised of DOPC/DOPS/Chol in the molar ratio 4.75:
4.75:0.5 and containing 1 mol % NBD-DOPE and 0.5 mol % rhodamine-DOPE as a donor and acceptor of FRET, respectively, were prepared using the film hydration method described elsewhere (21). Fusion was monitored by adding the double-labeled liposomes (final concentration is 0.25 mM of total lipids) to unlabelled K8- or R8-Lip (final concentration is 2.25 mM of total lipids) in both acidic and neutral aqueous media (10 mM HEPES, 1 mM EDTA, 0.1 mM NaCl, adjusted to pH 5.5 or 7.4 with NaOH). The emission fluorescence spectra were monitored between 500 and 600 nm mV (excitation wavelength, 490 nm) before (EPC:Chol/K8-Lip-RA) and after (EPC:Chol/R8-Lip-RA) double-labeled K8- or R8-Lip (final concentration is 2.25 mM of total lipids) treatments.

**RESULTS**

Characterization of Prepared Liposomes and DNA- or siRNA-coated Particles—During preparation of the liposomes or coated particles, the stearyl moiety acted as an anchor to the lipid bilayer leaving K8 or R8 peptides freely attached to the surface. The size and ζ potential of prepared liposomes and coated particles are summarized in Table 1. K8- and R8-modified liposomes had comparable sizes and ζ-potential. Similar tendencies were observed for other coated particles between K8 and R8 given here, indicating that there were no significant differences in the physicochemical properties between K8- and R8-modified particles.

Transfection Activity of Unmodified Cps Versus K8- or R8-modified Cps—We studied the effect of surface modification of DOPE/PA-coated particles encapsulating plasmid DNA encoding the luciferase reporter gene with either K8 or R8 peptides (10 mol % of total lipid) on transfection efficiency. Modification of the surface with the K8 peptide increased gene expression levels ~20-fold compared with unmodified particles. Interestingly, modification with R8 enhanced gene expression ~320-fold compared with Unmod-Cps and 17-fold compared with K8-Cps (Fig. 1). The negative ζ-potential of Unmod-Cps may be related to low cellular uptake, explaining the inferior transfection activity of the unmodified particles; however, the physicochemical characteristics of K8-Cps and R8-Cps could not explain the differences in gene expression levels (Table 1). These results indicate that the enhanced gene expression observed with R8-modified particles compared with K8-modified particles is not due to the positive charge of R8, even though the pKₐ of the guanidino moiety in arginine is higher than that of the amino group in lysine. Therefore, the superiority of R8 may be explained by enhanced cellular uptake, improved endosomal escape, and/or improved nuclear delivery.

**Cellular Uptake of K8- and R8-modified Particles**—First, the cellular uptake of Rh-DOPE-labeled K8-Cps and R8-Cps was quantified using flow cytometry. Previously, it was confirmed that cell surface-bound Cps can be effectively removed using a heparin wash (14, 29). Four different concentrations of DNA-coated particles were tested, two of which were lower, and one of which was higher than the pDNA dose used for transfection, as shown in Fig. 1. No significant difference (p > 0.05) was found between the measured intracellular fluorescence of...
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K8-Cps and R8-Cps at the four pDNA concentrations examined (Fig. 2A). This result excluded the amount of cellular uptake as an explanation for the difference in the gene expression levels of K8-Cps and R8-Cps. Next, to establish if K8- and R8-Cps have different rates of uptake, the uptakes of K8-Cps and R8-Cps were examined at three time points: 15, 90, and 180 min. Fig. 2B shows that the uptake of both K8-Cps and R8-Cps increased gradually for a period of 3 h. No significant difference ($p > 0.05$) was found between the measured intracellular fluorescence of K8-Cps and R8-Cps at any of the three time points. This result indicates that the rate of uptake of K8- and R8-Cps is not a factor in their difference in gene expression level. Next, to determine whether K8- and R8-modified particles have different mechanisms of uptake, the uptake of K8-Lip and R8-Lip encapsulating a rhodamine-labeled aqueous phase were examined with or without co-treatment with one of two drugs: amiloride, an inhibitor of the Na$^+$/H$^+$ exchange required for macroinocytosis (12, 30); or, chlorpromazine, an amphiphilic drug, which disrupts clathrin-mediated internalization by inhibiting the relocation of clathrin and AP2 from the cell surface (31, 32). In the current study, the removal of surface-bound particles using a cholic acid wash that solubilized the liposomes on the cell surface, releasing the entrapped aqueous phase marker, was confirmed (data not shown). The concentrations of amiloride and chlorpromazine used in our experimental conditions were determined to be non-toxic to NIH 3T3 cells based on the MTT assay using Tetra color ONE (Seikagaku-gyo, Japan) (25) and on viable cell counts using trypan blue exclusion (data not shown). Amiloride strongly inhibited the uptake of both K8-Lip and R8-Lip, whereas chlorpromazine had no effect (Fig. 2C). We previously confirmed that liposomes modified with a high density of R8, similar to those used in the current study, were primarily taken up by macroinocytosis (14). The results of the present study showed that cellular uptake of K8-Lip also occurs via this endocytic pathway and the efficiency of K8-Lip cellular uptake is almost the same as that of R8-Lip. Therefore, the difference in gene expression levels between K8-Cps and R8-Cps cannot be attributed to differences in particle uptake.

**Extent of K8-Cps and R8-Cps Endosomal Escape**—To examine whether K8-Cps and R8-Cps differ in their capacity to escape endocytic vesicles, co-localization of coated particles encapsulating rhodamine-labeled pDNA, and of endocytic vesicles, was quantified using CLSM. The size and $\zeta$-potential of the coated particles encapsulating rhodamine-labeled pDNA were similar to the values obtained for unlabeled particles (Table 1). The plasma membrane label PKH67 was used to label different endocytic vesicles. PKH67 stably incorporates a green fluorescent dye into the lipid region of cell membranes (29); thus, most internalized vesicles should be labeled regardless of the internalization pathway. Co-localization was quantified by counting free particles and particles co-localized with vesicles, including macroinocosomes, in live cells after a 3-h incubation. A total of 150–160 particles were counted in at least three independent experiments. Fig. 3A shows that, for the R8-Cps, most of the pDNA signals were evident as red dots that were not co-localized with the endocytic vesicles’ green marker. In contrast, for K8-Cps, the majority of pDNA signals were co-localized with endocytic vesicles. It is worth mentioning that the red signals may represent free pDNA polyplexes or whole coated particles. Quantitative data revealed that the extent of R8-Cps escape from endocytic vesicles was higher than that of K8-Cps, ~62% compared with only ~26% for K8-Cps (Fig. 3B). To establish whether acidification of endocytic vesicles plays a role in particle escape, bafilomycin A1, a selective vacuolar proton pump inhibitor, was used to inhibit acidification of different endocytic vesicles (33, 34). At less acidic endosomal pH, including that of macroinocosomes (in the presence of bafilomycin A1), K8-Cps escape was enhanced, whereas that of R8-Cps was inhibited, as judged by greater co-localization of R8-Cps with endocytic vesicles (Fig. 3A). Fig. 3B shows that, in the presence of bafilomycin A1, the extent of K8-Cps and R8-Cps escape was comparable, at ~43% and 40%, respectively.

**Silencing Activity of K8-Cps and R8-Cps Encapsulating siRNA**—To confirm our prior observation that R8-Cps endosomal escape is superior to that of K8-Cps, particles encapsulating synthetic double-stranded anti-luciferase siRNA (21 base pairs) were tested for their ability to deliver siRNA to the cytosol and to elicit an RNA interference effect. The purpose of this experiment was to compare the escape efficiency of the coated particles without the interference resulting from nuclear delivery events that take place in pDNA transfection events. Prepared particles were ~100 nm in diameter and had $\zeta$-potential in the range of 36–37 mV (Table 1). Fig. 4 shows that siRNA-K8-Cps equivalent to 0.1 $\mu$g of siRNA resulted in ~46% silencing of luciferase activity, compared with ~75% in the case of siRNA-R8-Cps. Increasing the siRNA dose to 0.4 $\mu$g
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FIGURE 2. Extent, rate, and mechanism of cellular uptake of K8- and R8-liposomes and DNA-coated particles. A, flow cytometric analysis of cells incubated for 1 h with pDNA-coated particles modified with octalysine (K8-Cps) or octaarginine (R8-Cps) and labeled with rhodamine-DOPE, a lipid-phase marker. The mean fluorescence (MF) of 10,000 cells is shown. No significant difference was observed between K8-Cps and R8-Cps uptake at the four doses tested (p > 0.05, t test). B, the same experiment was repeated at a concentration of DNA-coated particles equal to the pDNA dose used for transfection (in Fig. 1) and incubation with particles was continued for 15, 90, and 180 min at 37 °C. Cells were then washed and analyzed as mentioned above. No significant difference was observed between K8-Cps and R8-Cps uptake at the three time points tested (p > 0.05, t test). C, mechanism of cellular uptake of different K8- and R8-liposomes. NIH 3T3 cells were incubated for 1 h with liposomes modified with octalysine (K8-liposomes) or octaarginine (R8-liposomes) containing a rhodamine aqueous phase in the absence or presence of the clathrin-mediated endocytosis inhibitor, chlorpromazine (5 μg/ml), or the macropinocytosis inhibitor, amiloride (1.5 mM). The mean fluorescence of 10,000 cells was measured by flow cytometry and is expressed as a percent of the fluorescence measured in the absence of inhibitors. Error bars represent the mean ± S.E. for three different experiments performed in duplicate. Mean fluorescence values in the presence of amiloride were significantly different (*, p < 0.05, unpaired t test) relative to untreated control cells; whereas, there were no differences in MF in the presence of chlorpromazine (p > 0.05, unpaired t test).

Increased the gene silencing activity of siRNA-K8-Cps to 63%, whereas it only slightly improved siRNA-R8-Cps silencing to 79%. Silencing activity was calculated relative to PBS-treated cells because the luciferase activity of cells treated with particles encapsulating non-selective green fluorescent protein siRNA was not significantly different from that of untreated cells (supplementary Fig. S1). These results further confirmed that endosomal escape of R8-Cps encapsulates is superior to that of K8-Cps.

Effect of Chloroquine and Bafilomycin A1 on the Transfection Activities of K8- and R8-Cps—To further confirm the results of confocal microscopy, which demonstrated the superior ability of R8-Cps to escape from endocytic vesicles, the transfection activities of K8-Cps and R8-Cps encapsulating plasmid DNA encoding a luciferase reporter gene were examined in the presence of the membrane-disrupting agent, chloroquine. Chloroquine enhances transfection activity by releasing particles trapped in endosomes and lysosomes (18, 35). As shown in Fig. 5, chloroquine enhanced K8-Cps gene expression ~4-fold, whereas it had only a negligible effect on that of R8-Cps. To determine the involvement of endosome/macropinosome acidification on escape, the effect of bafilomycin A1 on gene expression was studied. The acidification inhibitor resulted in ~90% inhibition of R8-Cps gene expression, whereas it enhanced that of K8-Cps by ~315%. These results agreed with those obtained from confocal microscopy (Fig. 3) and provided additional confirmation that R8-Cps have a greater capacity for endosomal escape than K8-Cps.

Involvement of Fusion in Escape from Endocytic Vesicles and the Effects of Endosome Acidification—The data presented above suggest that acidification of macropinosomal endosomes positively affects the escape of R8-Cps, but negatively affects K8-Cps escape. Due to the presence of the fusogenic lipid DOPE, it was hypothesized that acidification affects fusion between the liposomal membrane and endosome during escape. To test this possibility, liposomes were labeled with both lipid phase markers, Rh-DOPE and NBD-DOPE. When these markers are in close proximity to each other, as is the case when both are present on the liposomal membrane, FRET is observed between these markers. When the liposomal membrane fuses with other membranes, the markers separate, resulting in a decrease in FRET (supplementary Fig. S2). After preparing the liposomes, spectrofluorometry was used to confirm FRET and its dissociation in the presence of Triton X-100 (data not shown). Intracellular FRET associated with these liposomes then was followed in live cells using spectral imaging fluorescence microscopy. Only particles in the cytosol were selected for examination, and the fluorescence intensity ratio (R-value) of NBD-DOPE (521 nm) to Rh-DOPE (586 nm) was taken as a measure of fusion (supplementary Fig. S3). To check the effect of an acidification inhibitor on fusion, three independent experiments were performed and the mean R-values were calculated in the absence and presence of bafilomycin A1. Fig. 6 shows that both K8-Lip and R8-Lip fused to endosomes during escape, as judged from the high R-value (i.e. >4). The
The $R$-value for control of non-fusogenic liposomes composed of EPC/Chol (molar ratio 7:3) and modified with 10% K8 or R8 did not exceed 0.2 under similar conditions. When the same experiment was performed in the presence of bafilomycin A1, the acidification inhibitor failed to inhibit fusion of K8-Lip, and the $R$-value remained at $R \sim 0.4$. In contrast, R8-Lip fusion was partially inhibited in the presence of bafilomycin A1, leading to $R$-values in the range of 0.2–0.4.

Of note, the $R$-value of R8-Lip in the presence of bafilomycin A1 remained significantly higher ($p < 0.01$, analysis of variance with Dunnett multiple comparisons post-test) than that of the control non-fusogenic liposomes. It is worth mentioning that the $R$-value is a qualitative measure of fusion and cannot be used to quantify the number of particles involved in the fusion event or the extent of escape.

The Ability of K8-Lip Versus R8-Lip to Fuse to Artificial Membranes

Results of spectral imaging fluorescence microscopy showed that K8- and R8-Lip may fuse to membranes differently depending on the pH. To confirm this observation, K8- and R8-Lip fusion to liposomes composed of negatively charged lipid components, simulating those present in the endosomal membrane, was monitored using a lipid-mixing assay based on FRET. Dual-labeled liposomes used to simulate endosomes were incubated with unlabeled K8-Lip or R8-Lip at neutral pH (7.4) or acidic pH (5.5). The $R$-values calculated at different time points are summarized in Fig. 7A. K8-Lip resulted in high $R$-values just after 5 min of incuba-
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The Ability of K8-Lip Versus R8-Lip to Destabilize Artificial Membranes—Because there was a clear difference between K8-Lip and R8-Lip in the response to endosome acidification, the ability of these liposomes to destabilize an artificial liposomal membrane and release entrapped components at different pH values was examined. Liposomes composed of negatively charged lipid components, simulating those present in the endosomal membrane, and encapsulating the fluorescent marker, calcein, were prepared. At the concentration used in these experiments, calcein is self-quenched inside the liposomes; however, if liposomes rupture, calcein is released and diluted, resulting in a strong fluorescent signal. The liposomes used to simulate endosomes were incubated at either neutral pH (7.4) or acidic pH (5.5). The effects of different concentrations of K8-Lip and R8-Lip on calcein release are summarized in Fig. 7B. K8-Lip released calcein efficiently at neutral pH in a concentration-dependent manner, whereas the amount of calcein released at acidic pH was much lower. In contrast, R8-Lip released calcein at neutral and acidic pH with equal efficiency.

Evaluation of the Endosomal Escape of Simultaneously Incubated K8-Cps and R8-Cps at Different Time Points—To follow the escape of K8- and R8-Cps incubated together with cells, co-localization of K8-Cps encapsulating Cy5-labeled pDNA and R8-Cps encapsulating rhodamine-labeled pDNA with endocytic vesicles was followed at different time points using CLSM. The plasma membrane label PKH67 was used to label different endocytic vesicles. To check the initial escape, cells were pulsed for 5 min with particles, then the particles were chased for 30 min. Fig. 8A shows that at 25 min the majority of K8- and R8-Cps co-localized with the endocytic vesicles, with many cases of K8 and R8-Cps co-localizing in the same vesicle (white signals, Fig. 8). The number of particles that managed to escape was equally distributed between K8- and R8-Cps. Other photos at other short time points showed the same tendency (supplementary Fig. S4A). For observing escape at longer time points, cells were pulsed for 15 min with particles, and then the cells were examined at 3 h. Interestingly, at 3 h the escape of pDNA encapsulated in R8-Cps was more prominent than that in K8-Cps (Fig. 8B). It was also evident that in many cases the

tion at neutral pH indicating efficient fusion, whereas very low R-values were observed at acidic pH indicating no fusion. On the other hand, R8-Lip efficiently fused to the negatively charged liposomes at both neutral and acidic pH.
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escape of pDNA encapsulated in R8-Cps stimulated the escape of pDNA in K8-Cps that might have been co-localized in the same endocytic vesicle, so after escape they appeared co-localized in cytosol. It is worth mentioning that in Fig. 8B many of K8-Cps appeared co-localized with the endocytic vesicles even after 3 h. Other photos in supplementary Fig. S4B showed a similar tendency.

**DISCUSSION**

The present study investigated the role of surface modification of nanoparticles with R8 at high density on the ability of the nanoparticles to escape endocytic vesicles after uptake. K8 was used to determine whether positive charge is sufficient to stimulate efficient uptake and endosomal escape, or if the unique structure of the amino acid, arginine, is required. The transfection activity of R8-Cps was higher relative to that of K8-Cps (Fig. 1). This result may be explained by differences in the extent or pathway of uptake, in the capacity to escape from endocytic vesicles, and/or by differences in nuclear delivery.

**Uptake of K8- and R8-modified Particles**—We reported previously that liposomes modified with a high density of R8 were capable of stimulating uptake through macropinocytosis (14). The present study demonstrates that macropinocytosis was the predominant uptake pathway for liposomes modified with a high density of K8. In addition, the K8-Cps and R8-Cps rate and extent of uptake were similar (p > 0.05) at the concentrations tested (Fig. 2). This agrees with previous findings that both lysine and arginine homopolymers require intact glycosaminoglycans for efficient internalization (22). Although earlier studies using free peptides linked to fluorescent probes showed that arginine homopolymers were taken up more efficiently than lysine homopolymers by the cells (36), a later study using peptides linked to bulky cargo (500 kDa) showed that the efficacy of octalysine to transduce various cell types was equal to or greater than that of octaarginine (22). Recent studies using peptides linked to bulky cargo showed that octalysine conjugates were taken up efficiently by cells (37, 38). The results of the present study show that cellular uptake is not the primary contributor to differences in gene expression between K8-Cps and R8-Cps. These results also emphasize the importance of the high positive-charge density in the stimulation of macropinocytosis.

**Differential Endosomal Escape Capacity between K8- and R8-modified Particles**—After confirming that K8-Cps and R8-Cps enter the cell using the same pathway, results of CLSM demonstrated that co-localization of pDNA encapsulated in R8-Cps with endocytic vesicles was less than that of K8-Cps (Fig. 3). This result indicates that the main difference between K8-Cps and R8-Cps lies in their ability to escape from endocytic vesicles. The results of the silencing study also confirmed that the main difference between K8-Cps and R8-Cps lies in escape and not in nuclear events, as the silencing effect is independent of nuclear delivery (Fig. 4). The effect of chloroquine on transfection activity proved that a significant fraction of K8-Cps remains entrapped in endocytic vesicles, whereas escape of R8-Cps was more efficient (Fig. 5). The efficient R8-Cps escape observed in the present study is consistent with our previous reports on R8-Lip escape after endocytic uptake (14, 29). Cryan et al. (39) also reported rapid and efficient delivery of encapsulated dextrans to the cytoplasm of Calu-3 cells using R8-Lip. Moreover, the relatively inefficient escape of K8-Lip agrees with the report that K8-conjugated peptide nucleic acid showed endosomal trapping after uptake and that splicing correction was achieved only in the presence of endosome-disrupting agents such as chloroquine or 0.5 M sucrose (38).

Because earlier reports showed that acidification of endocytic vesicles is important for escape of entrapped AR-CPPs (40–42), the effect of bafilomycin A1 on particle escape was investigated using CLSM. Consistent with previous reports, bafilomycin A1 inhibited the escape of pDNA encapsulated in R8-Cps from 62 to 42% (Fig. 3). This was accompanied by a 90% inhibition of gene expression (Fig. 5). Surprisingly, bafilomycin A1 failed to inhibit K8-Cps endosomal escape, but rather enhanced it from 26 to 43% (Fig. 3). This was accompanied by a 3-fold increase in gene expression (Fig. 5). The different response of K8- and R8-Cps to endosomal acidification suggested that the mechanism of endosomal escape of K8-Cps and R8-Cps may differ depending on endosome acidification.

**Mechanism of Endosomal Escape of K8- and R8-modified Particles**—Because the lipid composition of particles tested included the fusogenic lipid DOPE, fusion to endosome was postulated as the main mechanism of escape. Studying the fusion of K8-Lip and R8-Lip to endosome using live cell spectral imaging showed that both K8-Lip and R8-Lip fuse to the endosomal membrane (Fig. 6). R8-Lip fusion was partially inhibited in the presence of bafilomycin A1, indicating that fusion of R8-Lip was sensitive to acidification; however, the R-value remained significantly higher (p < 0.01) than that of control nonfusogenic liposomes. This result suggested that R8-Lip can fuse to membranes at acidic and neutral pH. K8-Lip fusion was not inhibited by bafilomycin A1, indicating that K8-Lip fusion was not dependent on endosome acidification. This implied that K8-Lip fusion occurred mainly in the pH-neutral environment of the endocytic vesicle directly after uptake. This hypothesis was supported by results obtained from the lipid mixing experiment, which showed that R8-Lip could fuse efficiently to an artificial membrane simulating the endosomal membrane at both acidic and neutral pH (Fig. 7A). On the contrary, K8-Lip could fuse only to a membrane at neutral pH. Finally, the hypothesis was further supported with results of the calcine-release experiment, which showed that R8-Lip could destabilize membranes at both acidic and neutral pH, whereas K8-Lip destabilized membranes only at neutral pH (Fig. 7B). Based on these results, it was assumed that R8-Lip escape can take place via membrane fusion at both acidic and neutral pH, whereas K8-Lip can escape through fusion only at neutral pH.

To explain the difference between K8-Cps and R8-Cps at the molecular level, the possible changes in the endocytosed particles and endosomal membrane that may occur upon acidification are summarized in Table 2. For fusion to take place between different membranes, three steps are required: 1) specific recognition of the site of fusion; 2) close apposition of membranes; and 3) fusion or coalescence of membranes (43). At neutral pH, both R8-Lip and K8-Lip fuse efficiently, as they are positively charged and the endosomal membrane is negatively charged, so there is a strong interaction between the lipo-
some and endosome. However, at acidic pH, the total positive charge on K8-Lip may decrease due to deprotonation of some adjacent amino groups as a result of electrostatic repulsion (44) and the total negativity of the endosomal membrane is also expected to decrease (Table 2); therefore, fusion is less likely to occur. Lysine polymers, higher than 5 moieties, bind efficiently to membranes rich in anionic phospholipids (45–47); however, their binding is inhibited when the membrane phospholipids are zwitterionic (47). On the other hand, at decreasing endosomal pH, the total positive charge on R8-Lip remains high due to full protonation of all arginine moieties (44). In addition, R8-Lip interact efficiently with amphoteric components embedded in the endosomal membrane even at acidic pH, not only through electrostatic interactions, but more importantly through bidentate hydrogen bonding. Using R8 derivatives with alkylated guanidinium groups that maintain the positive charge but inhibit the hydrogen bonding, Rothbard et al. (48, 49) showed that hydrogen bonding is as important as positive charge in the interaction between octaarginine and membranes; the inability to form hydrogen bonds strongly inhibited the interaction between R8 and cell membranes. It was reported also that the electrostatic contribution to the total free energy of binding between oligoarginines and negatively charged membranes accounts only for 25–30% of total energy. The remaining ∼70–75% is nonelectrostatic, corresponding to hydrogen bonding and/or hydrophobic interactions (20). R8 seems to play an additional role in enhancing fusion through insertion into the endosomal membrane after being neutralized by negatively charged membrane components. The formed ion pair may insert into the membrane and move inwards, driven by the transmembrane potential. This is expected to bring the lipid film of the liposome into intimate contact with the endosomal membrane, resulting in their fusion. This action is thought to increase at acidic pH when the potential across the endosomal membrane is high (50). Oligo- and polyarginines, but not polylysine or oligornithine, were reported to bind to anionic membrane-bound fatty acid salts, cholesterol derivatives, or phosphatidylglycerol to form hydrophobic ion pairs that are soluble in chloroform or octanol (44, 48). For these ion pairs to move across membranes, a membrane potential is required (48, 51). Taken together these results suggest that R8 contributes effectively to the three fusion steps described above.

Model for Endosomal Escape of Octaarginine-modified Particles—After considering the data, a model for endosomal escape was formulated as follows: R8-Cps are capable of escape at both acidic and neutral pH, unlike K8-Cps, which can escape only at neutral pH. Therefore, just after uptake, when endosomal pH is still neutral, both K8- and R8-Cps can escape from endosome at comparable rates. Upon acidification of endosome, the escape of K8-Cps is suppressed, whereas R8-Cps escape continues (Fig. 9). According to the model, escape of R8-modified particles should be more efficient than that of K8-modified particles. This corollary was supported by the CLSM results (Fig. 3). In addition, the prominent silencing effect of siRNA-R8-Cps, which used a lower dose of siRNA than siRNA-K8-Cps, also supported this hypothesis (Fig. 4). The model was also confirmed by results of simultaneous incubation of K8- and R8-Cps with

| Change that takes place upon changes in pH | K8-Lip | R8-Lip |
|-----------------------------------------|--------|--------|
| **Neutral pH** | **Acidic pH** | **Neutral pH** | **Acidic pH** |
| Charge on liposomes | High positive charge | Less positive charge | High positive charge | High positive charge |
| Endosomal membrane charge and membrane structure | | | | |
| Upon acidification of endosome the following changes take place: | | | | |
| 1. Decrease in total membrane negative charge is expected due to unionization of some carboxylic groups in phospholipids rendering them to zwitterionic form. | | | | |
| 2. The transmembrane potential increases by the action of vacuolar H⁺-ATPase | | | | |
| 3. Degradation of heparan sulfate proteoglycans bound to membrane by heparanases. | | | | |
| 4. Maturation of endosome and fusion to other organelles, which may change membrane structure. | | | | |
| Ion pair formation between peptide and endosomal membrane components | High | Lower | High | High |
| Bidentate hydrogen bonding | Absent | Absent | Present | Present |
| Endosomal escape | High | Low | High | High |

TABLE 2
Changes expected to take place to K8-Lip, R8-Lip, and endosomal membrane upon changes in pH

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the cells (Fig. 8 and supplementary Fig. S4). Initially both K8- and R8-Cps escaped from endocytic vesicles with comparable rates; at later time points, R8-Cps escape became more prominent in agreement with the proposed model. The model also agreed with enhanced K8-Cps transfection activity in the presence of bafilomycin A1, which extends the duration of the macropinosomal neutral pH (Fig. 5). The lower transfection activity of R8-Cps observed in the presence of bafilomycin A may be explained by the effect of endosomal acidification not only on R8-Cps fusion to membranes, but also on the endosomal membrane structure as summarized in Table 2. Inhibition of endosomal acidification may inhibit fusion of endosome to other organelles that may be needed to change the nature of endosomal membrane and make it more liable to fusion. Acidification also plays a role in hydrolytic degradation of heparan sulfates bound to the particles by heparanases en route to the lysosomes (52). Magzoub et al. (53) showed that the dissociation of CPPs from heparan sulfates may be a prerequisite for endosomal escape. This suggests that transfection activity of K8-Cps does not reach the initial high gene expression levels of R8-Cps when bafilomycin is used (supplementary Fig. S5).

In conclusion, the results of the present study show that the modification of nanoparticles with a high density of R8 allows their escape from endocytic vesicles via membrane fusion at both acidic and neutral pH. The remarkable difference in gene expression between DNA-coated particles modified with R8 or K8 can be explained by a difference in their ability to escape from endocytic vesicles. The results presented in this report show that AR-CPPs play a role in membrane fusion and emphasize the synergistic effect between R8 and the liposomal lipid bilayer in endosomal escape. In addition, these results show that the guanidinium group of the amino acid arginine, and not only the positive charge, is important for efficient escape.

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