

# High Density of Octaarginine Stimulates Macropinocytosis Leading to Efficient Intracellular Trafficking for Gene Expression<sup>\*[5]</sup>

Received for publication, March 23, 2005, and in revised form, October 27, 2005 Published, JBC Papers in Press, December 2, 2005, DOI 10.1074/jbc.M503202200

Ikramy A. Khalil<sup>†§</sup>, Kentaro Kogure<sup>‡§</sup>, Shiroh Futaki<sup>¶||</sup>, and Hideyoshi Harashima<sup>‡§1</sup>

From the <sup>†</sup>Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo, Hokkaido 060-0812, the <sup>‡</sup>Core Research for Evolutional Science and Technology and <sup>¶</sup>Precursory Research for Embryonic Science and Technology, Japan Science and Technology Agency, 3-1-6 Shibuya, Shibuya-ku, Tokyo 150-0002, and the <sup>||</sup>Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan

The mechanism of the arginine-rich peptide-mediated cellular uptake is currently a controversial issue. Several factors, including the type of peptide, the nature of the cargo, and the linker between them, appear to affect uptake. One of the less studied factors, which may affect the uptake mechanism, is the effect of peptide density on the surface of the cargo. Here, we examined the mechanism of cellular uptake and intracellular trafficking of liposomes modified with different densities of the octaarginine (R8) peptide. Liposomes modified with a low R8 density were taken up mainly through clathrin-mediated endocytosis, leading to extensive lysosomal degradation, whereas those modified with a high R8 density were taken up mainly through macropinocytosis and were less subject to lysosomal degradation. Furthermore, the high density R8-liposomes were able to stimulate the macropinocytosis-mediated uptake of other particles. When plasmid DNA was condensed and encapsulated in R8-liposomes, the levels of gene expression were three orders of magnitude higher for the high density liposomes. The enhanced gene expression by the high density R8-liposomes was highly impaired by blocking uptake through macropinocytosis. The different extents of gene expression from different densities of the R8 peptide on the liposomes could be explained principally by the existence of an intracellular trafficking route, but not by the uptake amount, of internalized liposomes. These results show that the density of the R8 peptide on liposomes determines the uptake mechanism and that this is directly linked to intracellular trafficking, resulting in different levels of gene expression.

The human immunodeficiency virus TAT<sup>2</sup>-derived peptide is a small basic peptide that has been shown to successfully mediate the efficient

cellular uptake of a wide variety of cargos, including proteins, peptides, and nucleic acids (1–3). Torchilin *et al.* (4) recently demonstrated that even certain TAT-linked liposomes with a diameter of 200 nm could be efficiently internalized into a variety of cell lines in intact form. The attachment of TAT directly to the liposome surface without a spacer or the presence of high molecular weight polyethylene glycol spacers abolished liposome internalization, indicating the importance of the direct contact of TAT with the cell surface (4). More recently, other groups reported an enhanced cellular uptake of liposomes when modified with TAT peptide (5, 6). Complexes formed between TAT-liposomes and DNA also showed enhanced transfection *in vitro* and *in vivo* (7).

Despite the well demonstrated ability of TAT to internalize different cargos, the internalization mechanism of the peptide itself or its cargo remains a controversial issue. According to early studies, none of the classic receptor-, transporter-, or endocytosis-mediated processes seemed to be involved in the uptake of TAT and other similar peptides (8, 9). Direct penetration- and inverted micelle-driven delivery have been suggested as possible internalization mechanisms (10). However, the mechanism of entry was recently re-evaluated based on possible problems that may occur due to cell fixation prior to microscopy observation, and more evidence appeared to support the involvement of endocytosis (11, 12). Endocytosis-mediated uptake is also a controversial issue, because various findings showing the involvement of different endocytic mechanisms have been reported (11–18).

The TAT sequence, which is critical for translocation, contains several arginine residues (19). Homopolymers of arginine are similar to the TAT peptide in terms of efficiency and uptake mechanism (19, 20), making them a possible candidate for mimicking the TAT peptide. The optimum number of arginine residues for efficient internalization was shown to be ~8 residues (19). The octaarginine (R8) peptide could mediate the efficient intracellular delivery of macromolecules, and similar to the TAT peptide but less studied, the exact uptake mechanism of R8-cargos is still largely unknown.

The diversity of results regarding the uptake mechanism of arginine-rich peptides suggests that some factors may affect the entry mechanism. These factors include the type of peptide, its mode of exposure to the cell surface, the nature of the cargo, and the chemical linkage between the peptide and the cargo (21). For example, we have previously shown that the R8 peptide and its complexes with DNA were taken up by different mechanisms, suggesting that the nature of the interaction between the peptide and the cell surface (*i.e.* the peptide in free or complexed state) affects the uptake mechanism (22). Another possible factor that has been less studied is the effect of peptide density on the

orescence; RLU, relative light units; TMR, tetramethylrhodamine; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline.

<sup>\*</sup> This work was supported by Grants-in-Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by Grants-in-Aid for Scientific Research on Priority Areas from the Japan Society for the Promotion of Science. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>[5]</sup> The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S3.

<sup>1</sup> To whom correspondence should be addressed. Tel.: 81-11-706-3919; Fax: 81-11-706-4879; E-mail: [harashima@pharm.hokudai.ac.jp](mailto:harashima@pharm.hokudai.ac.jp).

<sup>2</sup> The abbreviations used are: TAT, transactivator of transcription; R8, octaarginine peptide; EPC, egg phosphatidylcholine; Chol, cholesterol; Rh-PE, *N*-(1-lissaminerhodamine-B-sulfonyl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; NBD, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl); DOPE, dioleoylphosphatidylethanolamine; CHMS, cholesteryl hemisuccinate; STR-R8, stearyl-octaarginine; R8-Lip, octaarginine-modified liposomes; R8-Lip-HD, R8-Lip modified with a high density of STR-R8 (5.2 mol % of total lipid); R8-Lip-LD, R8-Lip modified with a low peptide density (0.86 mol % of total lipid); R8-Cps, R8-modified DNA-coated particles; HSPG, heparan sulfate proteoglycan; PTD, protein transduction domain; Tf, transferrin; ND, neutral dextran; R8-Lip-LD-RA, R8-Lip-LD containing rhodamine aqueous phase; R8-Lip-HD-E, empty R8-Lip-HD; MF, mean flu-

internalization mechanism. It was previously shown that a single TAT peptide was sufficient to allow the cellular delivery of an unfolded fusion construct of the same protein (23). Other studies showed that several TAT peptides attached to the surface of the cargo were required to permit efficient cellular delivery (24). However, no direct comparison to show the effect of peptide density on the uptake mechanism was conducted. Liposomes are good tools for use in such a comparison, because their surface can be easily modified with different densities of peptide and they can provide localized areas of high peptide density that are available to interact with the cell membrane. Therefore, the main purpose of this study was to investigate the effect of peptide density on the internalization mechanism and intracellular trafficking of cargos modified with arginine-rich peptides. The R8 peptide was chosen as a prototype of arginine-rich peptides and liposomes were chosen as an example of cargos.

Here, we present results showing that the mechanism of uptake of liposomes modified with a low R8 density shifted from clathrin-mediated endocytosis to macropinocytosis when the density of R8 was increased. The uptake route influenced intracellular trafficking, resulting in a remarkable difference in gene expression when condensed plasmid DNA was encapsulated into each type of liposome. These results highlight important features concerning the mechanism of entry and intracellular fate of R8-modified nanoparticles and demonstrate the role of the peptide density in determining the cellular uptake pathways of cargos. Furthermore, the data provided here indicate that uptake through macropinocytosis is more efficient in terms of avoiding lysosomal degradation resulting in an enhanced gene expression.

## EXPERIMENTAL PROCEDURES

**Materials**—Egg phosphatidylcholine (EPC), cholesterol (Chol), *N*-(lissaminerhodamine-B-sulfonyl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (Rh-PE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE), and dioleoylphosphatidylethanolamine (DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Sulforhodamine B, Syto-24, tetramethylrhodamine-labeled neutral dextran (70 kDa), FITC-labeled transferrin, and LysoSensor Green were purchased from Molecular Probes (Eugene, OR). Cholesteryl hemisuccinate (CHEMS), poly-L-lysine, amiloride, and filipin were purchased from Sigma-Aldrich. Stearyl-octaarginine was synthesized as described previously (25). Other chemicals were purchased from Wako Chemicals (Osaka, Japan). Plasmid DNA Pcmv-luc-encoding luciferase was prepared by using an EndFree Plasmid Mega Kit (Qiagen, Hilden, Germany). NIH3T3 cells were obtained from the American Type Culture Collection (Manassas, VA).

**Preparation of the Octaarginine-modified Liposomes**—Liposomes were basically composed of EPC and Chol (7:3 molar ratio), and stearyl-R8 (STR-R8) was incorporated at 0–10 mol % of the total lipid. To label the lipid phase, Rh-PE or NBD-phosphoethanolamine was incorporated at 1 mol % of the total lipid. Liposomes were prepared by hydration method followed by extrusion with a Mini-Extruder (Avanti Polar Lipids), through polycarbonate membrane filters (Nucleopore) of 400, 200, and 100 nm. Sulforhodamine B was used as an aqueous phase marker, when required. Liposomes were purified on a Bio-Gel A-1.5m column (100–200 mesh). The particle sizes of the liposomes were measured by a quasi-elastic light scattering method, and the zeta potential was determined by means of an electrophoretic light scattering spectrophotometer (ELS-8000, Photol Otsuka Electronics).

**Preparation of the Octaarginine-modified DNA-coated Particles**—Plasmid DNA was condensed with poly-L-lysine as described previously

(26). A condensed DNA solution was then added to the lipid film, formed by the evaporation of a chloroform solution of the lipids: EPC/Chol/STR-R8 (7:3:0.086 or 0.52 molar ratio), or DOPE/CHEMS (9:2 molar ratio) on the bottom of a glass tube, followed by incubation for 10 min to hydrate the lipid film. The glass tube was then sonicated for ~1 min in a bath-type sonicator (125 W, Branson Ultrasonics, Danbury, CT). In the case of DOPE/CHEMS, the particles, after sonication, were incubated with an aqueous solution of STR-R8 (0.86 or 5.2 mol % of total lipids) for 30 min at room temperature. The size and zeta potential of the coated particles were measured as described above.

**Confocal Laser Microscopy**—To investigate the cellular uptake of the R8-modified liposomes (R8-Lip), NIH3T3 cells were treated with double-labeled R8-Lip (NBD-labeled lipid and rhodamine-labeled aqueous phase, final concentration of 0.1 mM lipid) in serum-free medium at 37 °C for 1 h. The cells were then washed 3 times with ice-cold PBS and analyzed by confocal laser microscopy (LSM510, Carl Zeiss). To investigate the mechanism of internalization of R8-Lip, cells were incubated in the absence or presence of sucrose (0.4 M) for 30 min or amiloride (5 mM) for 10 min. Different R8-liposomes, containing a rhodamine aqueous phase, were then added, and the incubation was continued for 1 h. The cells were then washed 3 times with ice-cold PBS supplemented with heparin and observed by confocal microscopy. Nuclei were stained with Syto-24 in the last 20 min of incubation. For the colocalization study, in the case of transferrin, cells were first incubated with R8-Lip containing rhodamine aqueous phase for 30 min, the medium was then removed, a new medium containing FITC-labeled transferrin (5 μM) was added, and incubation was continued for 5 min before observation within 5 min. In the case of neutral dextran, NBD-labeled R8-liposomes were added to cells followed by adding tetramethylrhodamine-labeled neutral dextran (5 μM) and incubation continued for 30 min. The medium was then exchanged with liposome-free medium containing neutral dextran followed by incubation for 10 min. Cells were then washed with ice-cold PBS and observed. To investigate the intracellular fate of the liposomes, NIH3T3 cells were treated with different R8-liposomes containing an aqueous rhodamine phase at 37 °C for 30 min. The medium was then removed; the cells were washed with new medium, incubated in fresh liposome-free medium for a total of 1, 3, or 6 h, and then observed with confocal microscopy. Nuclei were stained with Syto-24 in the last 20 min of incubation. The same experiment was repeated using R8-Lip modified with low density of STR-R8 and containing rhodamine aqueous phase in the absence or presence of non-labeled R8-Lip modified with a high density of STR-R8 (a 5-fold increase, calculated as R8 content). To quantify the intracellular fluorescence, the total pixel areas of the fluorescence were measured using AquaCosmos software (Hamamatsu Photonics, Hamamatsu, Japan). To investigate the colocalization of R8-Lip with lysosomes, cells were incubated with different R8-Lip preparations containing rhodamine aqueous phase at 37 °C for 30 min. The medium was then removed; the cells were washed with new medium and incubated in fresh liposome-free medium for a total of 3 h. Thirty minutes before observation by confocal microscopy, the LysoSensor was applied at a final concentration of 1 μM to stain the acidic compartments.

**Flow Cytometry**—To investigate the cellular uptake of R8-Lip, NIH3T3 cells were incubated in serum-free medium containing different R8-Lip (final concentration, 0.1 mM lipids) for 1 h at 37 °C. At the end of the incubation, the medium was removed, and the cells were washed once with ice-cold PBS with or without heparin (20 units.mL<sup>-1</sup>). The cells were then trypsinized and washed twice by centrifugation at 4 °C (± heparin), suspended in 1 ml of PBS, and, after passing through a nylon mesh, they were analyzed by flow cytometry (BD Biosciences). To

investigate the cellular association in the presence of heparin, NIH3T3 cells were incubated in serum-free medium containing increasing concentrations of heparin for 5 min at 37 °C. Different R8-Lip preparations containing rhodamine aqueous phase were then added, and the incubation was continued for 1 h. At the end of the incubation, the medium was removed, and the cells were washed once with ice-cold PBS, trypsinized, washed twice by centrifugation at 4 °C, suspended in 1 ml of PBS, passed through a nylon mesh, and analyzed by flow cytometry. To examine the mechanism of internalization of R8-Lip, cells were incubated in the absence or presence of a mixture of metabolic inhibitors (sodium azide, 0.1%, sodium fluoride, 10 mM, and antimycin A, 1  $\mu\text{g}\cdot\text{mL}^{-1}$ ) for 30 min, sucrose (0.4 M) for 30 min, amiloride (5 mM) for 10 min, or filipin (1  $\mu\text{g}\cdot\text{mL}^{-1}$ ) for 1 h. Different R8-Lip preparations, containing rhodamine aqueous phase, were then added, and the incubation was continued for 1 h. Then cells were analyzed by flow cytometry after washing 3 times with PBS supplemented with heparin, as described above. To investigate the uptake of neutral dextran, cells were incubated with tetramethylrhodamine-labeled neutral dextran (5  $\mu\text{M}$ ) in the presence or absence of empty (non-labeled) R8-Lip modified with a high density of STR-R8 for 30 min at 37 °C then analyzed. To investigate the stimulation of macropinocytosis, cells were incubated with R8-Lip modified with low density of STR-R8 and containing a rhodamine aqueous phase mixed with increasing concentrations of empty (non-labeled) R8-Lip modified with a high density of STR-R8 for 1 h at 37 °C. The experiment was performed in the presence or absence of amiloride (5 mM) to inhibit macropinocytic uptake.

**Transfection Assay**—One day before transfection, NIH3T3 cells were seeded into 24-well plates at  $4 \times 10^4$  cells per well. Cells were incubated for 1 h at 37 °C with 0.25 ml of serum-free medium containing different R8-modified DNA-coated particles containing 0.4  $\mu\text{g}$  of DNA. Next, 1 ml of medium containing 10% fetal calf serum was added, and the incubation continued for an additional 23 h. The cells were then washed and solubilized with reporter lysis buffer (Promega, Madison, WI). Luciferase activity in the cell lysate was then measured by means of a luminometer (Luminescencer-PSN, ATTO, Japan). The amount of protein in the cell lysate was determined using a BCA protein assay kit (Pierce). To investigate the contribution of different uptake pathways in gene expression, cells were pretreated with or without sucrose (0.4 M) or amiloride (2.5 mM) for 10 min and R8-modified DNA-coated particles (lipid composition DOPE/CHEMS) containing 5.2 mol % STR-R8 were then added, and the incubation was continued for 1 h. The medium was then removed, and the cells were washed 3 times with PBS containing 20 units. $\text{mL}^{-1}$  heparin and once with PBS. The cells were then incubated in the presence of serum-free medium for 70 min followed by further incubation in the presence of 1 ml of medium containing 10% FBS for periods of up to 12 h. To control the effects of sucrose or amiloride on intracellular events, cells were first loaded with R8-modified DNA-coated particles for 1 h, then washed and incubated for 70 min in the presence of these reagents, after which, 1 ml of medium containing 10% fetal bovine serum was added, and the cells were further incubated for a total of 12 h.

## RESULTS

**Preparation and Characterization of Octaarginine-modified Liposomes**—A series of R8-Lip containing various concentrations of STR-R8 peptide was prepared. In this preparation, the stearyl moiety acts as an anchor to the lipid membrane leaving the R8 peptide freely attached to the surface. The zeta potential of the prepared liposomes was determined as a measure of their net charge (Fig. 1A). Increasing the concentration of STR-R8 peptide caused an initial rapid increase in zeta

potential followed by a slower increase for concentrations up to  $\sim 5$  mol %. No further increase in zeta potential was observed  $>5$  mol % STR-R8, indicating that the liposomal surface was saturated with the cationic peptide. In the next experiments, we chose liposomes modified with a low density (0.86 mol %) of STR-R8 (R8-Lip-LD), which had a low positive charge, and those modified with a high density (5.2 mol %) of STR-R8 (R8-Lip-HD), which had the highest possible positive charge. Data related to the characterization of the different liposomes used in this study are shown in Table 1.

**Cellular Uptake of R8-liposomes**—First, we quantitatively compared the cellular uptake of R8-Lip-LD and R8-Lip-HD containing an aqueous rhodamine phase by flow cytometry. We confirmed that the surface-bound liposomes could be removed by means of heparin wash (data not shown). The measured intracellular fluorescence in the case of R8-Lip-HD was higher than that for R8-Lip-LD by less than one order of magnitude (Fig. 1B). When we measured the total fluorescence (surface-bound plus internalized liposomes) by excluding the heparin washes, we found that the fluorescence in the case of R8-Lip-HD was about one order of magnitude higher than that in the case of R8-Lip-LD (data not shown). Liposomes that were devoid of STR-R8 did not show any intracellular or surface-bound fluorescence (data not shown). The cellular uptake of both liposomes was further confirmed using confocal laser microscopy of living cells (supplemental Fig. S1). Surface-bound fluorescence was higher in the case of R8-Lip-HD. Taken together; these results indicate that both R8-Lip-LD and R8-Lip-HD can bind to the cell surface, especially R8-Lip-HD, and that they are efficiently internalized.

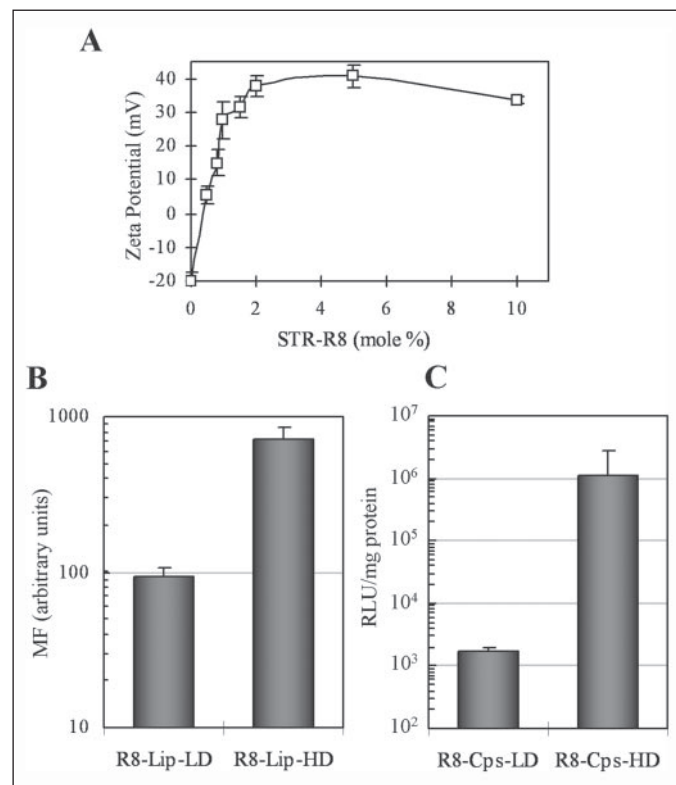
**Transfection Activities of R8-Lip Containing Plasmid DNA**—Because R8-Lip showed a high potential for the intracellular delivery of macromolecules encapsulated in their cores, we investigated the cellular uptake of R8-Lip-containing plasmid DNA, for use in gene delivery. We prepared condensed DNA particles coated with a lipid envelope consisting of EPC and Chol and modified with the R8 peptide (R8-modified coated particles, R8-Cps) as described under “Experimental Procedures.” We incubated the cells for 1 h with R8-Cps containing FITC-labeled DNA modified with 0.86 or 5.2 mol % STR-R8 (R8-Cps-LD and R8-Cps-HD) and then observed the cells by confocal microscopy. In both cases, the intracellular fluorescence was similar to the pattern of distribution obtained earlier with R8-Lip (data not shown), indicating the efficient cellular uptake of plasmid DNA encapsulated in the R8-Lip. We next investigated the transfection efficiency of R8-Cps containing plasmid DNA encoding a luciferase reporter gene. R8-Cps-LD did not show a significant gene expression, whereas the R8-Cps-HD produced gene expression levels about 3 orders of magnitude higher (Fig. 1C). This difference in gene expression is not correlated with the difference in cellular uptake as indicated by flow cytometry and confocal microscopy. Therefore, the superiority of the R8-Cps-HD regarding gene expression cannot be explained by differences in the amount of DNA internalized, but that there are intracellular events responsible for this difference.

**Mechanism of Uptake of Different R8-liposomes**—Because the gene expression levels of the R8-Cps were not correlated with the amount of liposomes internalized, we investigated the mechanism of uptake of different R8-Lip as a candidate to explain the difference in the intracellular fate of the particles. It has previously been shown that the cell-surface heparan sulfate proteoglycans (HSPGs) act as nonspecific receptors in the cellular binding of different protein transduction domain (PTD) peptides (27, 28). As mentioned above, several washings with a buffer containing heparin were sufficient to remove the surface-bound liposomes. Furthermore, the addition of heparin to the medium prior to the addition of R8-Lip dramatically inhibited the cellular bind-



ing and internalization of R8-Lip especially in the case of R8-Lip-LD (supplemental Fig. S1). In both cases, concentrations as low as  $1 \text{ unit} \cdot \text{mL}^{-1}$  were sufficient to completely block the interaction of the liposomes with the cell surface. Taken together, these data probably show the involvement of cell-surface HSPGs in the uptake of R8-Lip, similar to other PTD-liposomes (6).

To investigate the contribution of the endocytic pathway in the internalization of R8-Lip, we incubated cells with R8-Lip-LD or R8-Lip-HD in the presence or absence of a mixture of metabolic inhibitors that inhibit all types of endocytosis through energy depletion (29) and then measured the internalized liposomes using flow cytometry. As shown in Fig. 2, the metabolic inhibitors strongly inhibited the uptake of both types of liposomes, indicating that the uptake process is highly energy-



**FIGURE 1. Characterization, cellular uptake, and transfection activities of different octaarginine-modified liposomes or DNA-coated particles.** A, zeta potentials for different R8-liposomes (R8-Lip) containing increasing concentrations of stearyl octaarginine (STR-R8). Each point represents the mean  $\pm$  S.D. of at least two different preparations measured in triplicates. B, flow cytometric analysis of cells incubated for 1 h with liposomes modified with low density (R8-Lip-LD) or high density (R8-Lip-HD) of R8 and containing rhodamine aqueous phase. The mean fluorescence (MF) of 10,000 cells is shown. Error bars represent the  $\pm$  S.D. for three different experiments performed in duplicate. C, transfection activities of DNA-coated particles modified with low density (R8-Cps-LD) or high density (R8-Cps-HD) of the R8 peptide and prepared with a lipid envelope containing EPC/Chol. Luciferase activities were measured as described under "Experimental Procedures" 24 h after transfection and are expressed as the relative light unit (RLU) per milligram of protein. Data represent the mean  $\pm$  S.D. of three different experiments performed in triplicate.

**TABLE 1**  
Characterization of R8-modified liposomes

	Lipid composition (molar ratio)	Diameter <sup>a</sup>	Zeta potential <sup>a</sup>
		nm	mV
R8-Lip-LD-RA <sup>b</sup>	EPC:Chol:STR-R8 (7:3:0.086)	152 $\pm$ 18	13 $\pm$ 11
R8-Lip-HD-RA <sup>b</sup>	EPC:Chol:STR-R8 (7:3:0.52)	102 $\pm$ 10	35 $\pm$ 4
R8-Lip-LD-FL <sup>c</sup>	EPC:Chol:NBD-PE:STR-R8 (7:3:0.1:0.085)	170 $\pm$ 2	7 $\pm$ 1
R8-Lip-HD-FL <sup>c</sup>	EPC:Chol:NBD-PE:STR-R8 (7:3:0.1:0.52)	149 $\pm$ 3	40 $\pm$ 1

<sup>a</sup> Data represent means  $\pm$  S.D. of at least two different determinations.

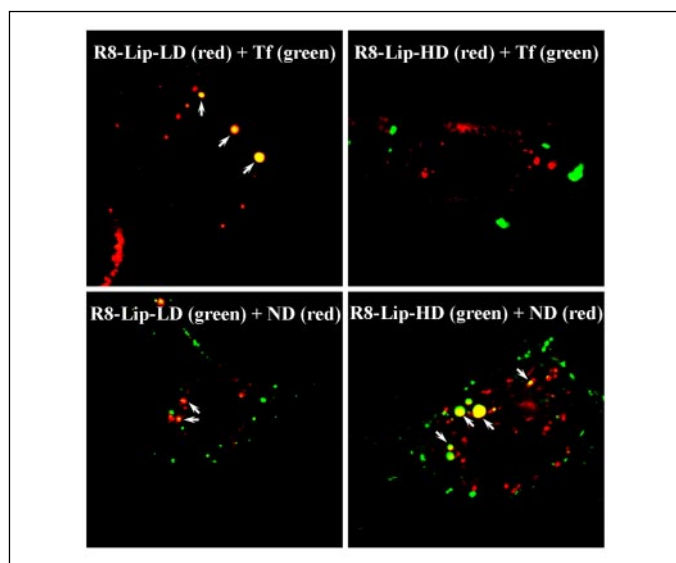
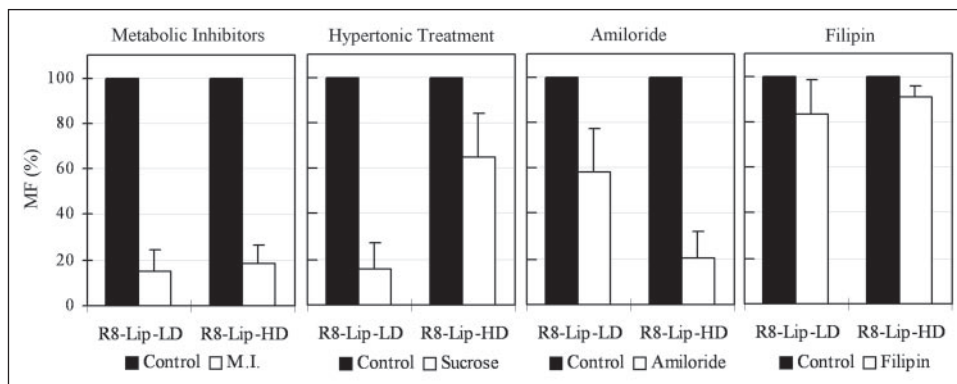
<sup>b</sup> RA, liposomes containing rhodamine-labeled aqueous phase.

<sup>c</sup> FL, liposomes containing NBD-labeled lipid phase.

dependent and supporting the contribution of endocytosis as a major uptake pathway in the case of R8-Lip. We next investigated the contribution of different endocytic uptake pathways that have been characterized to date, using specific inhibitors of each type (30–34). Clathrin-mediated endocytosis is the major and best characterized endocytic pathway (30). Macropinocytosis and caveolae represent other types of clathrin-independent endocytosis (31–34). We examined the effects of the following inhibitors: a hypertonic medium to specifically inhibit clathrin-mediated endocytosis through dissociation of the clathrin lattice (30), amiloride to specifically inhibit macropinocytosis by inhibiting the  $\text{Na}^+/\text{H}^+$  exchange required for macropinocytosis (14, 30), and filipin to specifically inhibit caveolar uptake through cholesterol depletion (17, 30). The use of a hypertonic medium strongly inhibited the uptake of R8-Lip-LD, whereas it inhibited the uptake of R8-Lip-HD by  $\sim 35\%$ . This indicated that clathrin-mediated endocytosis is the major uptake pathway of R8-Lip-LD, whereas the uptake of R8-Lip-HD probably involves different pathways as a major entrance route. Surprisingly, in the presence of the macropinocytosis inhibitor amiloride,  $>50\%$  of the particles were internalized in the case of R8-Lip-LD, whereas only 20% were internalized in the case of R8-Lip-HD. Taken together, these results indicate that only the R8-Lip modified with high amounts of R8 peptide uses macropinocytosis as the major entrance route, whereas those modified with a low density of R8 mainly use the classic clathrin-mediated endocytosis. The caveolar inhibitor filipin inhibited the uptake of both liposomes only slightly, indicating the minor contribution of caveolae in the uptake process. The effects of sucrose and amiloride were confirmed by confocal microscopy observations of living cells (supplemental Fig. S2). Cytochalasin D and nystatin, both of which can inhibit macropinocytosis and caveolae (30), inhibited the uptake of R8-Lip-HD (data not shown). Because the presence of the specific caveolae inhibitor, filipin, caused only a minor inhibition of uptake, these results further confirm the involvement of macropinocytosis in the uptake.

**Colocalization between R8-Lip and Different Endocytosis Markers—**Transferrin (Tf) is a well known marker for the internalization through clathrin-mediated endocytosis (35). Neutral dextran, 70 kDa (ND), is a fluid phase marker that can be used to trace the internalization via macropinocytosis (14). We performed a colocalization study between R8-Lip and these markers using confocal microscopy (Fig. 3). Intracellular R8-Lip-LD was highly colocalized with Tf, whereas R8-Lip-HD did not show any significant colocalization with Tf under the same conditions. In the case of the fluid phase marker ND, most of the intracellular liposomes were colocalized with the marker in large intracellular vesicles in the case of R8-Lip-HD, whereas only a partial colocalization was observed in the case of R8-Lip-LD. The partial colocalization in the case of R8-Lip-LD is consistent with the result shown in Fig. 2, which shows that amiloride partially inhibited uptake. These results show that R8-Lip-HD and Tf mainly use distinctive internalization pathways, whereas there is a high contribution of the clathrin-mediated endocytosis in the case of R8-Lip-LD. Bodipy-LacCer, a marker of caveolae

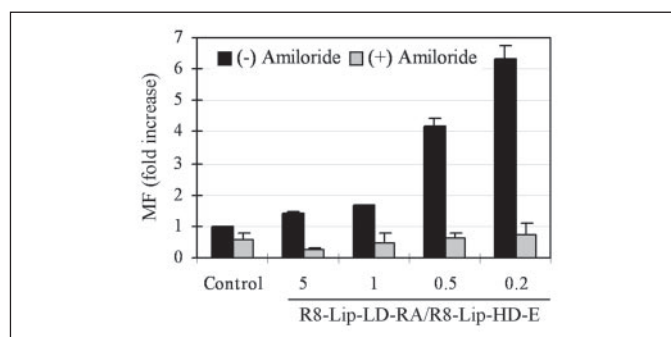
**FIGURE 2. Mechanism of cellular uptake of different R8-liposomes.** NIH3T3 cells were incubated for 1 h with liposomes modified with low or high density of STR-R8 (R8-Lip-LD or R8-Lip-HD) and containing rhodamine aqueous phase in the absence or the presence of a mixture of metabolic inhibitors (M.I.) (sodium azide, 0.1%, sodium fluoride, 10 mM, and antimycin A,  $1 \mu\text{g}\cdot\text{ml}^{-1}$ ), a hypertonic medium (sucrose, 0.4 M), the macropinocytosis inhibitor amiloride (5 mM), or the caveolar inhibitor filipin ( $1 \mu\text{g}\cdot\text{ml}^{-1}$ ). The mean fluorescence (MF) of 10,000 cells was measured by flow cytometry and is expressed as the percentage of the fluorescence measured in the absence of the inhibitors. Error bars represent the  $\pm$ S.D. for three different experiments performed in duplicate.



**FIGURE 3. Colocalization between different R8-liposomes and different endocytosis markers.** NIH3T3 cells were incubated with R8-Lip-LD or R8-Lip-HD containing an aqueous rhodamine phase for 30 min and FITC-labeled transferrin (Tf) was then added, and the incubation continued for 5 min before observation by confocal microscopy. In the case of neutral dextran 70 kDa (ND), cells were incubated with R8-Lip-LD or R8-Lip-HD containing NBD-lipid phase then ND labeled with tetramethylrhodamine (TMR) was added, and incubation continued for 30 min followed by another incubation for 10 min in the presence of liposome-free medium containing ND. Arrows indicate intracellular liposomes colocalized with the endocytosis markers.

(36), did not show significant colocalization with both liposomes (data not shown). These results are consistent with the results of the inhibition study explained above, confirming the change of the cellular entry port upon changing the peptide density on the liposomal surface.

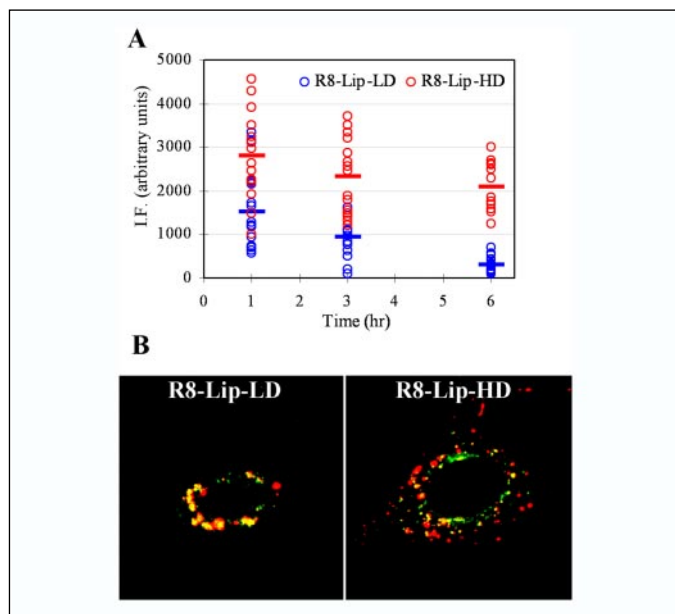
**Stimulation of Macropinocytosis by R8-Lip**—During the colocalization study between R8-Lip-HD and ND, we observed that the uptake of ND is increased significantly compared with cells treated with ND alone (data not shown). Using flow cytometry, we quantitatively confirmed the increase in the uptake of ND in the presence of R8-Lip-HD (supplemental Fig. S3). These results indicate that the R8-Lip-HD may have the ability to stimulate uptake through macropinocytosis, and this might be the reason for the change in the major entrance route by the change in peptide density on the liposomal surface. To investigate this hypothesis, we incubated cells with fixed concentrations of R8-Lip-LD-containing rhodamine aqueous phase (R8-Lip-LD-RA) together with increasing amounts of empty R8-Lip-HD (R8-Lip-HD-E) and then measured the internalized R8-Lip-LD-RA using flow cytometry (Fig. 4). The presence of R8-Lip-HD-E increased the internalization of R8-Lip-LD-RA in a concentration-dependent manner. When a 5-fold excess of the R8-Lip-



**FIGURE 4. Stimulation of macropinocytosis.** NIH3T3 cells were incubated for 1 h with R8-Lip-LD containing rhodamine aqueous phase (R8-Lip-LD-RA) in the presence of increasing concentrations of empty (non-labeled) R8-Lip-HD (R8-Lip-HD-E) in the absence or the presence of the macropinocytosis inhibitor amiloride. The mean fluorescence (MF) of 10,000 cells was measured by flow cytometry and is expressed as the -fold increase in fluorescence measured for cells incubated with only R8-Lip-LD-RA. Error bars represent the  $\pm$ S.D. for two different experiments performed in duplicate.

HD-E was used, a 6-fold increase in uptake of the R8-Lip-LD-RA was observed. Parallel experiments were performed in the presence of amiloride to inhibit internalization through macropinocytosis. The presence of amiloride almost completely inhibited the increase in uptake of R8-Lip-LD-RA at all concentrations used. These results indicate that R8-Lip-HD increased the uptake of R8-Lip-LD mainly through stimulating macropinocytosis, which suggests that a certain peptide concentration is required to stimulate uptake pathways that are different from the clathrin-mediated endocytosis.

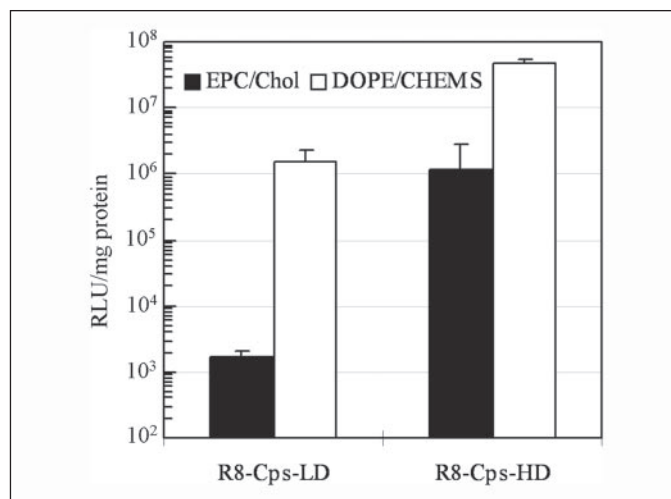
**Intracellular Fate of Different R8-Lip**—Because R8-Lip modified with different densities of R8 peptide follow different uptake pathways as suggested earlier, we investigated the intracellular fate of the liposomes in both cases using confocal laser scanning microscopy. We incubated cells with R8-Lip-LD or R8-Lip-HD, both of which contained an aqueous rhodamine phase, for 30 min, after which, the medium was exchanged with liposome-free medium, and the incubation was continued for a total of 1, 3, or 6 h. At the end of the incubation, the cells were examined by confocal microscopy, in which cells were scanned from bottom to top, and the section with the highest intracellular fluorescence possible was recorded for each cell (representative images are shown in supplemental Fig. S3). The pixel areas of the intracellular fluorescence in images from at least 15 different cells were quantified as described earlier. As shown in Fig. 5A, total intracellular fluorescence detected in the case of R8-Lip-LD after 1 h was reduced by >80% after 6 h, whereas it was only reduced by 25% in the case of R8-Lip-HD. Intracellular liposomes in the case of R8-Lip-LD are probably subjected to extensive degradation in the lysosomal compartment leading to the disappearance of most of the internalized fluorescence. Uptake by mac-



**FIGURE 5. Intracellular fate of different R8-Lip and colocalization with lysosomes.** *A*, a quantitative analysis of intracellular fluorescence. Cells were incubated with R8-Lip-LD (blue circles) or R8-Lip-HD (red circles) for 30 min, and then the medium was changed to liposome-free medium and incubation continued for a total of 1, 3, or 6 h before observation with confocal microscopy. Pixel areas of intracellular fluorescence (I.F.) from 15 cells were determined in each case as described under "Experimental Procedures." Blue and red short lines indicate the average fluorescence in each case. *B*, representative images of NIH3T3 cells treated with R8-Lip-LD or R8-Lip-HD containing rhodamine aqueous phase at 37 °C for 30 min then incubated in fresh liposome-free medium for a total of 3 h and subsequently observed by confocal microscopy. The lysosomes were stained green with LysoSensor before observation.

ropinocytosis involves the formation of large heterogeneous macropinosomes (>1  $\mu\text{m}$ ), which have been proposed to be leaky (33). The intracellular fate of macropinosomes differs depending on the cell type (32). In macrophages, they tend to completely merge with lysosomes, whereas in other cell types, they fuse only with other macropinosomes and tend to be recycled and their contents are not targeted for lysosomal degradation (32). To investigate the colocalization with lysosomes, we incubated cells with different R8-liposomes for 30 min followed by incubation in liposome-free medium for 2.5 h, and then we stained the lysosomal compartment with FITC LysoSensor before observation by confocal microscopy (Fig. 5*B*). A high degree of colocalization between R8-Lip and lysosomal compartment was found in the case of R8-Lip-LD that were internalized mainly by clathrin-mediated endocytosis, whereas only a partial colocalization could be found in the case of R8-Lip-HD. This indicates that internalization through macropinocytosis may represent a good tool for avoiding lysosomal degradation. Therefore, the R8-Lip-HD is less subject to lysosomal degradation, which may explain why high levels of intracellular fluorescence could be observed in this case even after 6 h.

To further confirm these findings, we examined the fate of R8-Lip-LD-RA that had been internalized in the absence or the presence of R8-Lip-HD-E to stimulate macropinocytosis. In the absence of R8-Lip-HD-E (*i.e.* clathrin-mediated endocytosis being the major entrance pathway), the intracellular fluorescence observed after 1 h mostly disappeared after 6 h (data not shown). Under the same conditions but in the presence of R8-Lip-HD-E (*i.e.* macropinocytosis is the major entrance pathway), high levels of intracellular fluorescence were found, even after 6 h (representative images are shown in supplemental Fig. S3). This confirms that particles internalized by macropinocytosis are less subject to lysosomal degradation and that the presence of R8-Lip-HD changes the intracellular trafficking and the fate of the R8-Lip-LD.

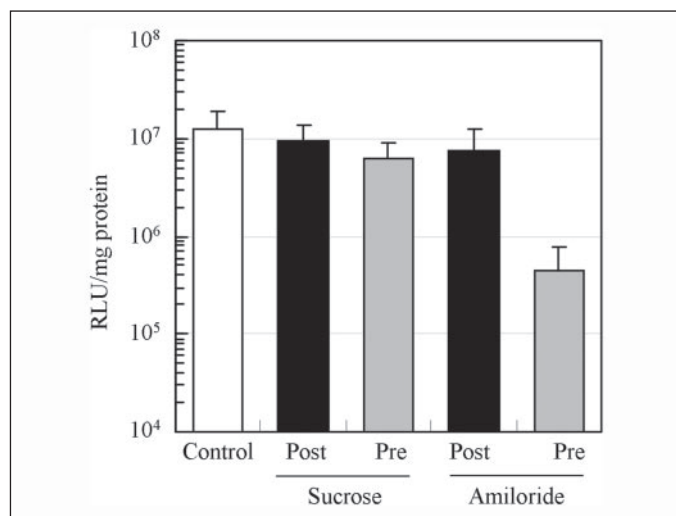


**FIGURE 6. Comparison of transfection activities of different DNA-coated particles.** *A* luciferase coding DNA-coated particles modified with low density (R8-Cps-LD) or high density (R8-Cps-HD) of R8 were prepared with a lipid envelope containing EPC/Chol or DOPE/CHEMS and used to transfect cells. Luciferase activities were measured as described under "Experimental Procedures" 24 h after transfection and are expressed as the relative light unit (RLU) per milligram of protein. Data represent the mean and  $\pm$  S.D. of three different experiments performed in triplicate.

**Enhanced Transfection Activities of R8-Cps Using Fusogenic Lipids**—The data presented above suggest that particles internalized by macropinocytosis (*i.e.* R8-Cps-HD) are not highly subject to lysosomal degradation compared with those internalized through clathrin-mediated endocytosis (*i.e.* R8-Cps-LD). Therefore, the difference in gene expression observed in Fig. 1*C* seems to arise mainly from the ability of both particles to avoid lysosomal degradation. To further assess the ability of different R8-Cps to escape from endosomes or lysosomes, we examined the effect of the fusogenic lipid dioleoylphosphatidylethanolamine (DOPE), which has the ability to enhance the endosomal escape of particles trapped in endosomal vesicles through fusion with the endosomal membrane (37). We prepared and tested the transfection activities of R8-Cps consisting of a lipid envelope prepared with the non-fusogenic lipids EPC and Chol or with the fusogenic lipid DOPE and cholesteryl hemisuccinate (CHEMS) (37, 38). In the presence of DOPE, the transfection activities of R8-Cps-LD were increased by about 3 orders of magnitude, whereas transfection activities of R8-Cps-HD were increased by less than 2 orders of magnitude (Fig. 6). This indicates that the low transfection activities in the case of non-fusogenic R8-Cps-LD is not due to the low cellular uptake, but mainly due to the low endosomal escape ability. The enhanced activities of the fusogenic R8-Cps-HD may occur due to the enhanced endosomal escape of the fraction of the particles, which was internalized by clathrin-mediated endocytosis. Another possibility is that the fusogenic lipid can further enhance the release of the particles from macropinosomes. In either case, the presence of devices that can enhance endosomal escape appears to be useful in terms of increasing the transfection activities of particles modified with the R8 peptide. Using confocal microscopy, we confirmed that macropinocytosis is the major entrance pathway for R8-Cps-HD and that the lipid composition had no effect on the internalization mechanism (data not shown).

**Contribution of Different Uptake Pathways in Gene Expression**—Because macropinocytosis is the major contributor to the uptake of R8-Cps-HD, whereas clathrin-mediated uptake is a relatively minor one, we investigated the contribution of both pathways in the gene expression process mediated by R8-Cps-HD (DOPE/CHEMS) (Fig. 7). A minor inhibition of gene expression was observed after blocking





**FIGURE 7. Contribution of different uptake pathways in gene expression.** Cells were transfected with R8-Cps-HD (lipid envelope consisting of DOPE/CHEMS) in the absence (Control) or the presence (Pre) of sucrose or amiloride as described under "Experimental Procedures." To control the effects of sucrose and amiloride on intracellular events, cells were first loaded with R8-Cps-HD then treated with these reagents (Post). Luciferase activities were measured 12 h after transfection and are expressed as the relative light unit (RLU) per milligram of protein. Data represent the mean and  $\pm$ S.D. of three different experiments performed in triplicate.

internalization through clathrin-mediated endocytosis using a hypertonic medium. This shows that the minor fraction that is internalized through this pathway does not actually lead to efficient gene expression. Meanwhile, blocking internalization through macropinocytosis caused a strong inhibition in gene expression ( $\sim$ 95%), confirming that this pathway is the major contributor to efficient gene expression, in accordance with the above findings. Therefore, the role of R8 in increasing gene expression levels is not only increasing the binding to the cell surface, but extends to changing the uptake mechanism to more efficient pathways and the subsequent control of the intracellular trafficking of the particles.

## DISCUSSION

PTDs like TAT and other arginine-rich peptides have been used to promote the cellular delivery of many types of cargos (1–3). Similarly, the R8 peptide was shown to be efficiently internalized into cells and to be able to mediate the cellular delivery of macromolecules, including peptides, proteins, and plasmid DNA (19, 39). However, the uptake mechanism of the arginine-rich peptides and their cargos is a matter of intense discussion in the literature. Different reports have demonstrated the involvement of different endocytic pathways in the uptake of these peptides or their cargos (11–18). This discrepancy of results suggests that some factors may affect the internalization mechanism, such as the properties of the peptide and cargos and the linkage between them. One factor that can possibly affect the internalization mechanism is the peptide concentration on the cargo surface. In this study, we investigated the uptake mechanisms of liposomes modified with the R8 peptide, and we focused on how the peptide density on the liposome surface affects the internalization mechanism. Furthermore, we followed the intracellular trafficking of internalized liposomes to understand the environment they are in and to rationally improve the system to achieve maximum activity.

We prepared R8-modified liposomes using a simple procedure in which the peptide is attached to a stearyl moiety that acts as an anchor to the lipid membrane of the liposomes. To examine the integrity of the liposomes during internalization, we examined the uptake of double-

labeled R8-liposomes modified with different densities of the peptide (R8-Lip-LD and R8-Lip-HD). Cells treated with different R8-Lip for 1 h showed a high intracellular fluorescence, and both liposomal labels were colocalized on the plasma membrane or in the cytosol of cells in both cases (supplemental Fig. S1). The intracellular colocalization of the markers excludes the possibility of fusion between the liposomal membrane and the cell membrane. In addition, the intracellular fluorescence in both cases appeared as punctuated signals in the cytosol indicating a possible contribution of endocytosis, a finding that is further supported by the strong inhibition of uptake after energy depletion (Fig. 2). This clearly supports recently reported data suggesting that endocytic uptake is essential for cellular uptake of particles modified with arginine-rich peptides (11–18). Similar results showing efficient cellular uptake of intact PTD-modified liposomes have been reported previously (4–6).

We initially obtained evidence that the role of R8 extends beyond just increasing the binding of the particles to the cell surface to changing the fate of internalized liposomes. R8-Cps modified with low R8 density were taken up by cells, however, did not produce significant gene expression. The R8-Cps modified with high R8 density produced a 3 orders of magnitude higher gene expression, whereas the internalized particles were less than one order of magnitude higher than the low density particles. This indicates that the intracellular fate of the particles is improved by increasing the peptide density. It is generally accepted that different uptake mechanisms lead to different intracellular trafficking of internalized particles, because the intracellular fate of particles is closely linked to the entry mode. We hypothesized that the uptake mechanism is a strong candidate for explaining this difference in the intracellular fate of the particles depending on the peptide density. Therefore, we investigated the contribution of different endocytic pathways to the internalization of the R8-Lip using flow cytometry after confirming that the measured fluorescence represented only internalized liposomes by removing the surface-bound liposomes with a heparin wash (12), and the results were confirmed using confocal microscopy of living cells. It was recently shown that the internalization of the TAT-Cre fusion protein as well as that of R8 and TAT peptides occurs mainly through macropinocytosis (14–16). Here, we show that large drug carriers, such as liposomes that are  $\sim$ 100 nm in diameter, can also be internalized by macropinocytosis. Evidence for this includes the strong inhibition of uptake in the presence of the macropinocytosis inhibitor, amiloride (Fig. 2), and the high colocalization with the macropinocytosis marker, neutral dextran (Fig. 3). Furthermore, we found that a high peptide density on the liposomal surface is required for internalization by this pathway, because the low density R8-Lip were internalized mainly by clathrin-mediated endocytosis. In addition, the presence of high density R8-Lip stimulated the macropinocytosis-mediated uptake of neutral dextran and the low density R8-Lip. Surprisingly, the presence of the same concentration of peptide, but in the free form, failed to stimulate the macropinocytosis-mediated uptake of low density R8-Lip and only slightly increased the uptake of the macropinocytosis marker (data not shown). Therefore, the extent of stimulation of macropinocytosis is significantly higher in the case of R8-Lip-HD than in the case of the free peptide. This suggests that the liposomal surface can provide regions in which R8 is highly condensed, thus enhancing interaction with the cell membrane, and this localized interaction might be required to stimulate macropinocytosis. It was previously shown that arginine-rich peptides are able to induce a significant rearrangement in the cell cytoskeleton similar to that seen during macropinocytosis (16). This rearrangement may be responsible for the stimulation of uptake through macropinocytosis, which is shown here.

The uptake of condensed DNA particles coated with a lipid envelope

modified with high R8 density occurs also by macropinocytosis (data not shown). However, we previously reported that the uptake of complexes formed directly between R8 and DNA are mainly internalized by clathrin-mediated endocytosis (22) and are mainly trapped in the endosomes (40). This difference between direct complexation and lipid coating indicates that the nature of the interaction between the peptide and the cell surface is important in stimulating macropinocytosis. This also demonstrates the importance of the lipid envelope in controlling the topology of the peptide to determine the entrance route, because the ability to stimulate macropinocytosis is higher for the R8 peptide on the liposome surface compared with the R8 complexed with DNA.

One of the important issues is the difference in intracellular trafficking after clathrin-mediated endocytosis and macropinocytosis that can be stimulated by a high density of the R8 peptide on liposomal surface. The normal endosomes formed after clathrin-mediated endocytosis eventually fuse with lysosomes where degradation of the internalized material occurs, whereas the fate of the macropinosomes depends on the cell type. For example, in macrophages, they fuse with lysosomes while they are mainly recycled and are not efficiently targeted for lysosomal degradation in some other cell types (32). The low extent of colocalization between R8-Lip-HD and lysosomes (Fig. 5B) suggests that macropinosomes in the cell line used here are not targeted for lysosomal degradation. We show here that macropinocytosis is more favorable than the clathrin-mediated endocytosis in terms of gene expression, because blocking internalization through macropinocytosis caused a ~95% inhibition in gene expression. The data presented here suggest that the superiority of macropinocytosis is mainly due to the low level of lysosomal degradation of internalized particles, indicating the significance of macropinocytosis as an entrance pathway. In addition, the internalized particles may be easily released to the cytosol, because macropinosomes are assumed to be leaky compared with other endocytic vesicles (33). This ability appears to be enhanced by the use of pH-sensitive fusogenic lipids, such as DOPE (Fig. 6).

Similar to previous reports using other PTDs (27, 28), the presence of heparin in the medium dramatically inhibited the cellular binding and uptake of R8-modified particles, suggesting that the presence of cell-surface HSPGs may be important for cellular binding. Based on the previous finding that arginine-rich peptides induce cytoskeleton rearrangement (16), and our finding that a high peptide density is required to stimulate macropinocytosis, we speculate that a strong and multiple binding between the R8 peptide and the cell-surface HSPGs may be required to affect the latter in a way that would induce a cytoskeleton rearrangement resulting in the initiation of ruffle formation followed by uptake via macropinocytosis. Such a proposed multiple binding is available only in the case of the high density R8-Lip. Although the exact role of cell-surface HSPGs in cellular uptake is not clear, their cytoplasmic domains are known to interact with the actin cytoskeleton (41), which suggests that surface proteoglycans may be involved in stimulating endocytic uptake. A significant role for proteoglycans in the phagocytosis of cationic vectors was previously reported (42).

In conclusion, our results show that the density of the R8 peptide determines the internalization mechanism, which is directly linked to intracellular trafficking. The remarkable difference in gene expression between DNA-coated particles modified with different R8 densities can be explained principally by differences in the intracellular trafficking of DNA as well as the carriers. Our results indicate that cells have the ability to recognize differences in the nature of nanoparticles and to respond by internalizing them via different mechanisms.

**Acknowledgment**—We thank Dr. M. S. Feather for his helpful advice in writing the English manuscript.

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## High Density of Octaarginine Stimulates Macropinocytosis Leading to Efficient Intracellular Trafficking for Gene Expression

Ikramy A. Khalil, Kentaro Kogure, Shiroh Futaki and Hideyoshi Harashima

*J. Biol. Chem.* 2006, 281:3544-3551.

doi: 10.1074/jbc.M503202200 originally published online December 2, 2005

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