ARTICLE

doi:10.1016/j.ymthe.2005.03.038

Role of Clathrin- and Caveolae-Mediated Endocytosis in Gene Transfer Mediated by Lipo- and Polyplexes

Joanna Rejman,[1] Alessandra Bragonzi,[1] and Massimo Conese[1,2,*]

[1]*Institute for Experimental Treatment of Cystic Fibrosis, HSR Scientific Institute, Via Olgettina 58, 20132 Milan, Italy*
[2]*University of Foggia, Foggia, Italy*

**To whom correspondence and reprint requests should be addressed. Fax: +39 02 26434153. E-mail: conese.massimo@hsr.it.*

Available online 16 June 2005

We investigated the effects of inhibitors of clathrin-mediated endocytosis (chlorpromazine and K^+ depletion) and of caveolae-mediated uptake (filipin and genistein) on internalization of FITC–poly-L-lysine-labeled DOTAP/DNA lipoplexes and PEI/DNA polyplexes by A549 pneumocytes and HeLa cells and on the transfection efficiencies of these complexes with the luciferase gene. Uptake of the complexes was assayed by fluorescence-activated cell sorting. Lipoplex internalization was inhibited by chlorpromazine and K^+ depletion but unaffected by filipin and genistein. In contrast, polyplex internalization was inhibited by all four inhibitors. We conclude that lipoplex uptake proceeds only by clathrin-mediated endocytosis, while polyplexes are taken up by two mechanisms, one involving caveolae and the other clathrin-coated pits. Transfection by lipoplexes was entirely abolished by blocking clathrin-mediated endocytosis, whereas inhibition of the caveolae pathway had no effect. By contrast, transfection mediated by polyplexes was completely blocked by genistein and filipin but was unaffected by inhibitors of clathrin-mediated endocytosis. Fluorescence colocalization studies with a lysosomal marker, AlexaFluor–dextran, revealed that polyplexes taken up by clathrin-mediated endocytosis are targeted to the lysosomal compartment for degradation, while the polyplexes internalized via caveolae escape this compartment, permitting efficient transfection.

Key Words: gene therapy, nonviral cationic vectors, internalization, clathrin, caveolae

INTRODUCTION

In recent years, cationic lipid-mediated and cationic polymer-mediated methods of gene transfer have become useful tools for cellular transfection, potentially applicable to gene therapy. Gene transfer mediated by cationic lipid/DNA complexes (lipoplexes) and cationic polymer/DNA complexes (polyplexes) has been accomplished efficiently both *in vitro* and *in vivo,* showing no immunogenicity and low toxicity [1–5]. Nonviral delivery systems need to overcome several cellular barriers to deliver their cargo, plasmid DNA, into the nucleus. Due to its complexity, the precise mechanism by which synthetic lipids and polymers mediate DNA delivery still remains to be elucidated. Eukaryotic cells utilize different endocytic pathways to internalize a variety of substances and to accomplish different tasks. Therefore, it is conceivable that the mode of internalization of DNA complexes might affect the kinetics of their intracellular processing and, in this way, transfection efficiency as such. Different mechanisms have been proposed to be involved in the internalization of cationic lipid or polymer/DNA complexes. Several studies have reported that these complexes enter the cells by means of clathrin-mediated endocytosis [6,7]. Phagocytosis and macropinocytosis are two other pathways that have been described as possible pathways of internalization of complexes of DNA with cationic lipids or polymers [8,9].

It is generally believed that the major obstacle to efficient gene delivery is entrapment of the DNA in the endosomal compartment, since this eventually leads to its degradation in lysosomes. Different escape mechanisms from the endosomal compartment have been proposed for both lipo- and polyplexes. These include destabilization of the endosomal membrane [10–12], an exchange of cationic lipids with anionic phospholipids normally found on the cytoplasm-facing monolayer of the cell membrane [13], and endosomolysis caused by osmotic swelling [14–16].

The purpose of our present work was to establish whether there is any difference in internalization and intracellular trafficking of DOTAP lipoplexes and PEI polyplexes that could determine their intracellular fate and gene transfer efficiency.

1525-0016/$30.00

Results and Discussion

The Mechanism of Polyplex and Lipoplex Internalization

We studied the uptake of DOTAP/DNA lipoplexes and PEI/DNA polyplexes by A549 type II-derived pneumocytes and HeLa cervical carcinoma cells. We labeled the complexes with FITC–poly-L-lysine (FITC–PLL), after having made sure that the presence of FITC–PLL did not cause any change in the transfection efficiency of these complexes [17], and assayed uptake by fluorescence-activated cell sorting (FACS).

Fig. 1A represents the internalization of DOTAP lipoplexes by A549 and HeLa cells in the presence of inhibitors of clathrin- or caveolae-mediated uptake. Pretreatment of the cells with filipin or genistein, both reported to block caveolae-mediated uptake processes [37,38], prior to incubation with the lipoplexes had no significant effect on their uptake. By contrast, potassium depletion of the cells, which is known to perturb clathrin-mediated endocytosis [33], caused a more than 50% decrease of the uptake of the lipoplexes. Also chlorpromazine, another inhibitor of clathrin-mediated endocytosis [34], decreased internalization of the lipoplexes. The

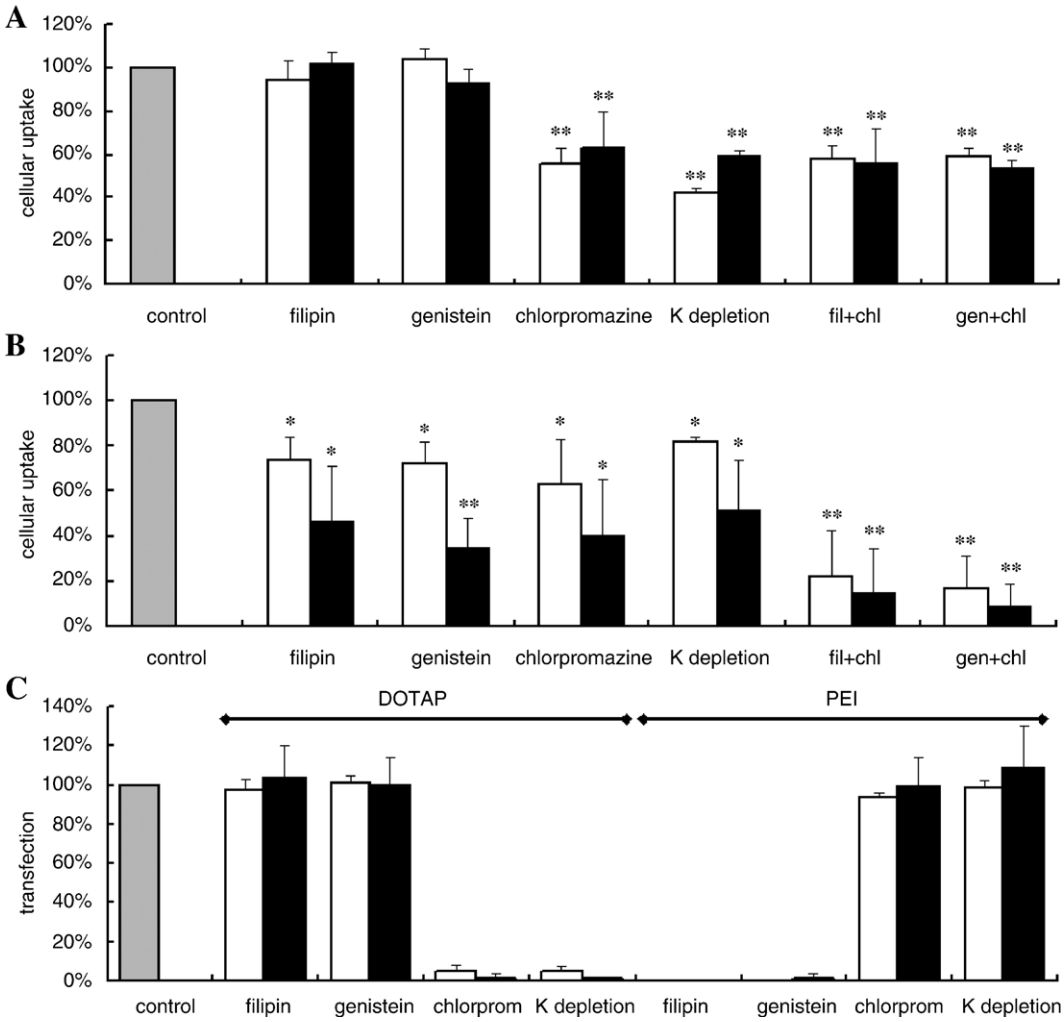

FIG. 1. Effects of inhibitors on internalization and cellular transfection of DOTAP lipoplexes and PEI polyplexes. Internalization (A) of DOTAP lipoplexes and (B) of PEI polyplexes. Cells were pretreated with chlorpromazine, genistein, or filipin or depleted of intracellular potassium (for the details see Materials and Methods). Subsequently, FITC–PLL-labeled lipoplexes were added and after 2 h cells were analyzed by FACS. 10,000 cells were measured in each sample. A549 cells, white bars; HeLa cells, black bars. The fluorescence intensity in control cells (gray bars) was set as 100%. Mean values ± SD were obtained from three separate experiments carried out in duplicate. Student's *t* test was carried out to determine the statistical significance of the data (*$P < 0.05$; **$P < 0.01$). (C) Cellular transfection mediated by polyplexes and lipoplexes. A549 (white bars) and HeLa (black bars) cells, pretreated with different inhibitors, were transfected with lipoplexes or polyplexes and screened for luciferase gene expression. The level of luciferase in control cells (gray bars) was set as 100%.

doi:10.1016/j.ymthe.2005.03.038

combination of chlorpromazine and any inhibitor blocking caveolae-mediated internalization did not cause any further decrease in the uptake of DOTAP lipoplexes. These data are fully compatible with a major role of clathrin-mediated endocytosis in the internalization of DOTAP lipoplexes. The predominant role of this mechanism in lipoplex uptake was earlier reported for lipoplexes with other cationic lipids [7].

As depicted in Fig. 1B, the uptake of the PEI polyplexes by A549 cells pretreated with filipin or genistein was diminished by about 25%, relative to control values. We observed a slightly higher degree of inhibition (about 40%) for A549 cells treated with chlorpromazine. The uptake of polyplexes by A549 cells depleted of potassium was reduced by only 20%. Interestingly, the combination of chlorpromazine and any inhibitor interfering with caveolae-mediated internalization resulted in a substantial further decrease in the uptake of PEI polyplexes.

Fig. 1B also presents data on the internalization of PEI polyplexes by HeLa cells. Pretreatment with genistein, filipin, or chlorpromazine or depletion of intracellular potassium reduced polyplex uptake by at least 50%. The highest degree of inhibition was observed for genistein (65%). Simultaneous treatment with two inhibitors interfering with two different pathways of internalization almost entirely abolished the uptake of polyplexes by HeLa cells.

The Effects of Endocytosis Inhibitors on Cellular Transfection Mediated by Polyplexes and Lipoplexes

After having established the effects of different endocytosis inhibitors on the internalization of DOTAP lipoplexes and PEI polyplexes, we investigated the effects of these compounds on cellular transfection mediated by these complexes. To this end, we transfected A549 and HeLa cells with lipoplexes or polyplexes containing the luciferase gene and screened them for expression of enzyme activity.

Transfection of both A549 and HeLa cells by DOTAP lipoplexes was unaffected by the caveolae inhibitors filipin and genistein (Fig. 1C). This is fully compatible with the lack of effect of these compounds on DOTAP lipoplex uptake, which apparently does not involve caveolae, as described in the preceding section. In sharp contrast to this, lipoplex-mediated transfection was completely blocked by inhibitors of clathrin-mediated endocytosis (Fig. 1C), in line with the strong inhibitory effect of these inhibitors on lipoplex uptake as also described in the preceding section.

In contrast to our observations of the DOTAP lipoplexes, transfection mediated by PEI polyplexes was completely abolished by inhibition of the caveolae pathway with filipin or genistein, whereas inhibition of clathrin-mediated endocytosis did not have any effect on transfection (Fig. 1C). In Fig. 1B we demonstrated that PEI polyplexes, in contrast to DOTAP lipoplexes, are internalized by two different pathways, one clathrin mediated and the other caveolae mediated. Now it appears (Fig. 1C) that only one of them, i.e., the caveolae-mediated pathway, leads to efficient transfection.

Earlier studies provide indirect evidence of caveolae involvement in polyplex uptake. Kichler and co-workers [18] reported a low level of luciferase expression in HepG2 cells transfected with DNA/PEI complexes and demonstrated that in these cells most of the internalized DNA was degraded in intracellular compartments. For 293 cells they observed high transfection and no intracellular degradation. Since HepG2 cells lack endogenous caveolins [19], the polyplexes cannot be internalized via a caveolae-mediated pathway, which, as demonstrated by our results, is required to achieve efficient gene transfer. Instead, they are taken up by clathrin-mediated endocytosis, which ultimately leads to their degradation in the lysosomal compartment.

Ogris and co-workers [20] observed that large aggregates of DNA and PEI (>500 nm), even though internalized very slowly and to a low extent, were more efficient in gene transfer than small polyplexes, which were taken up very rapidly and almost completely. Since the composition of these polyplexes was identical, the only parameter determining their gene transfer potential was their size. Observations of the uptake of fluorescent latex beads published by Rejman and colleagues [21] revealed that the size of ligand-devoid particles determines the pathway of their entry into the cell and their subsequent intracellular routing. Microspheres with a diameter of <200 nm were internalized via clathrin-mediated endocytosis and were ultimately delivered to the lysosomes, while 500-nm particles entered the cells via caveolae and never reached the lysosomal compartment.

A number of *in vivo* observations showing rapid crossing of small linear PEI22/DNA polyplexes from the blood compartment to lung tissue [3,17,22,23] may seem to contradict caveolae involvement in polyplex uptake. However, later studies showed that these small polyplexes tend to aggregate on the cell surface before being internalized in a transfection-effective way [17,22,23]. More recently this was confirmed under *in vitro* conditions and proposed to be the main reason for the high transfection activity of these polyplexes [24].

It is known that uptake kinetics of clathrin-mediated endocytosis is much faster than that of caveolae-mediated uptake. Therefore, we also determined the effect of time of incubation on the level of luciferase expression ("transfection efficiency"). Fig. 2 demonstrates that in A549 cells DOTAP lipoplexes produce nearly maximal levels of gene expression already after a 15-min internalization period. For PEI polyplexes, on the other hand, levels of transfection were still very low at up to 1 h of internalization, maximal levels being attained only after 3 h of internalization.

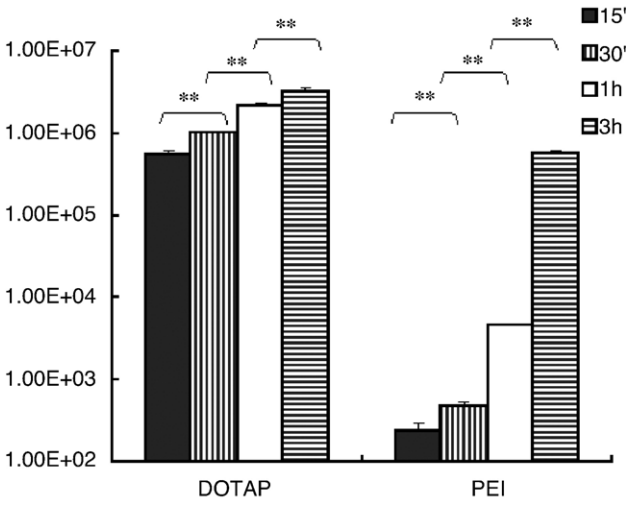

FIG. 2. Kinetics of polyplex- and lipoplex-mediated gene transfer. Cells were incubated with DOTAP/DNA or PEI/DNA complexes for different periods of time, then washed, and further incubated in fresh medium. The reporter gene expression was screened 24 h later. The data are expressed as relative light units per milligram of protein. Mean values ± SD were obtained from three separate experiments carried out in duplicate (**$P < 0.01$, Student's t test).

These data are compatible with a rapid internalization of DOTAP lipoplexes with kinetics distinctive of clathrin-mediated internalization of ligands such as transferrin. This is fully in line with the results of the effects of inhibitors on gene expression, depicted in Fig. 1C, which also point to a clathrin-dependent uptake mechanism for transfection by DOTAP lipoplexes.

In contrast, the uptake of PEI complexes, as reflected by gene expression levels, was substantially slower, maximal expression becoming apparent only after several hours of internalization (Fig. 2). Such slow kinetics are characteristic for internalization via a mechanism involving caveolae, confirming our observations on the effect of inhibitors summarized in Fig. 1C.

The Effects of Internalization Pathway on Intracellular Processing of Lipoplexes and Polyplexes

The preceding sections demonstrate that PEI polyplexes are internalized by two different endocytic pathways, only one of which leads to efficient transfection of the cells. This would imply that the polyplexes taken up by these two pathways are processed by the cells in different ways. To examine that, we incubated A549 cells first with a lysosomal compartment marker, i.e., a fluorescently labeled dextran, for the time required to complete its accumulation in the lysosomal compartment and subsequently with FITC-labeled polyplexes following prior treatment with inhibitors of caveolae- or clathrin-mediated uptake. Then we determined the localization of the two types of fluorescence by fluorescence microscopy. In cells treated with chlorpromazine, which blocks clathrin-mediated endocytosis while leaving caveolae-mediated uptake unaffected, dextran and polyplexes did not colocalize (Fig. 3A). In cells treated with filipin, which blocks caveolae-mediated uptake while leaving clathrin-mediated uptake unaffected, the polyplexes colocalized with the dextran in the lysosomes (Fig. 3B). Apparently, while PEI polyplexes internalized by the clathrin-mediated endocytosis pathway are targeted to the lysosomal compartment, they do not reach this compartment when taken up exclusively via caveolae.

Up until now, there has been no consensus as to whether PEI/DNA complexes reach the lysosomal compartment. Some studies indicate that PEI polyplexes do reach this compartment [25,26], whereas others present evidence against a lysosomal localization [27]. Bieber and colleagues [28] reported a partial colocalization of a lysosomal marker and PEI, indicating that only a fraction of the polyplexes reaches the lysosomal compartment. Our present data demonstrating that PEI complexes are taken up by two mechanisms, which further determine their intracellular fate, may help to explain these contradictory results.

Evidence that the caveolar pathway avoids the lysosomal compartment abundantly emerges from studies on a variety of pathogens. As a matter of fact, some pathogens (viruses and bacteria) that use caveolae as their portal of entry escape delivery to and digestion in lysosomes [29]. It is thought that caveosomes lack the proper signal molecules required for interaction with other cellular compartments [30].

An important question to be answered is why DNA apparently is released on time from DOTAP lipoplexes internalized via clathrin-mediated endocytosis to become transfection effective, while DNA in PEI polyplexes, taken up by the same internalization pathway, does not become transfection effective but probably ends up in the lysosomal compartment to be degraded. One possible explanation may be the difference in mechanisms postulated for DNA release from lipoplexes and polyplexes, respectively. The model proposed by Xu and Szoka [13] and further extended by Hafez [31] assumes that anionic phospholipids in the endosomal membrane form neutral charge pairs with cationic lipids from the lipoplexes, allowing DNA to dissociate from the complex. This model is supported by the observation that DOPE strongly increases gene transfer mediated by lipoplexes [11,12], which is attributed to DOPE's potential to promote the formation of an inverted hexagonal phase (H_{II}). This in turn would destabilize the lipoplex structure and ultimately the endosomal membrane, thus facilitating the release of the plasmid DNA from the lipoplexes into the cytosol. Obviously, such a mechanism cannot apply to the polyplexes, which, by their nature, lack the proper lipid constituent required for the proposed release mechanism.

doi:10.1016/j.ymthe.2005.03.038

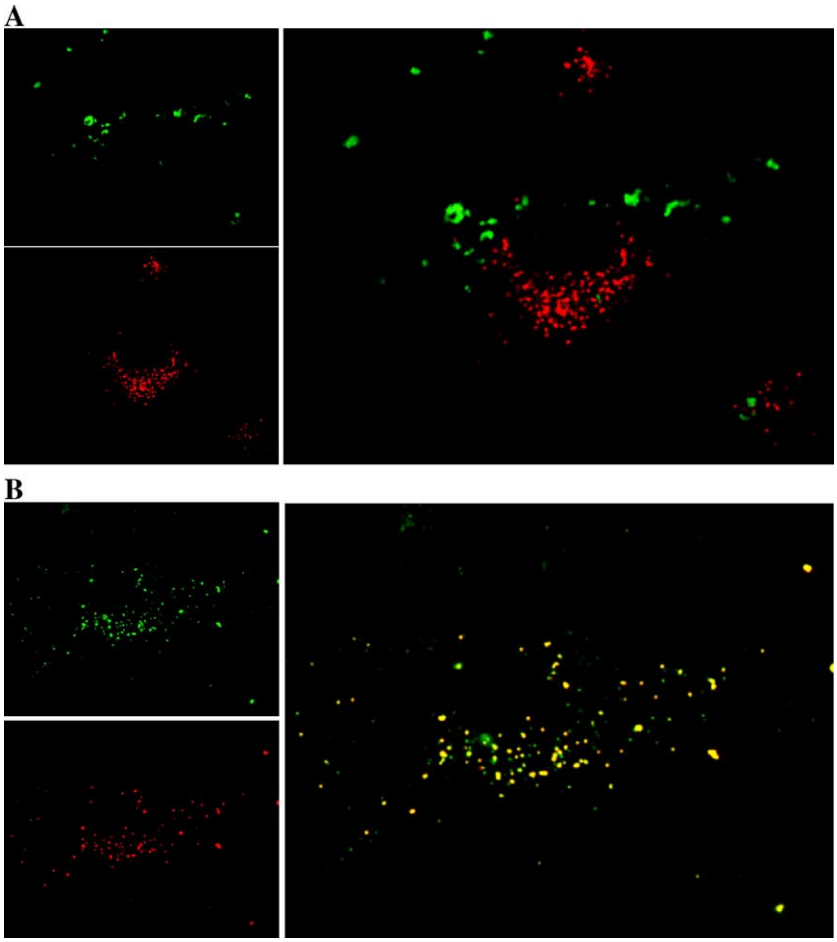

FIG. 3. The effect of internalization pathway on intracellular processing of lipoplexes and polyplexes. Cells were preincubated with fluorescent dextran (Dextran, AlexaFluor 594) to label the lysosomal compartment. Then the cells were treated with (A) chlorpromazine or (B) filipin for 30 min. The cells were incubated with FITC-labeled PEI polyplexes for a subsequent 2 h. After treatment with trypan blue the cells were further cultured for 2 h and then examined by means of fluorescence microscopy. Each micrograph represents a single cell. Original magnification: ×1000. Upper left, AlexaFluor–dextran (red); lower left, FITC-labeled PEI polyplexes (green); right panel, merged picture.

The mode of DNA release from polyplexes is described as the "proton sponge" mechanism [14–16]. This implies that PEI sequesters the protons that are transported into the endosomal compartment, thus preventing its acidification. Subsequently, the increased osmolarity in this compartment causes the influx of water that eventually leads to endosome lysis and release of contents into the cytoplasm.

Other reports question the role of endosomal acidification in the proton sponge hypothesis. Bieber and coworkers [28] claim that release of the gene from the complex occurs from the lysosomal compartment, while Forrest and Pack [32] presented data indicating that PEI does not preclude exposure of polyplexes to an acidic environment. Furthermore, it has been demonstrated that PEI is incapable of destabilizing membranes even under acidic conditions [18].

CONCLUSIONS

Our study provides strong evidence that DOTAP lipoplexes are internalized by cells solely by means of clathrin-mediated endocytosis, while PEI polyplexes are internalized both by clathrin-mediated and by caveolae-mediated endocytosis. While lipoplexes internalized via the clathrin-mediated route are fully transfection effective, for the polyplexes only the caveolae-dependent route leads to effective transfection. These observations are explained as follows. While the DNA complexed in the DOTAP lipoplexes is efficiently released from the acidic endosomal compartment prior to endosome/lysosome fusion, due to the presence of the cationic lipids, and thus escapes intralysosomal degradation, the DNA in polyplexes internalized by clathrin-mediated endocytosis cannot be released and is taken with the complex into the lysosomal compartment to be degraded. Only the DNA

that enters the cell by caveolae-mediated uptake of the polyplexes escapes the lysosomal destination and can become transfection effective.

Our observations may contribute to the rational design of novel strategies to overcome intracellular barriers in PEI-mediated gene transfer.

MATERIALS AND METHODS

Cell cultures. A549 pneumocytes and HeLa cells were from ATCC. A549 cells were cultured in Dulbecco's modified Eagle's medium and HeLa cells in Iscove's modified Dulbecco's medium. The media were supplemented with 10% FCS, 2 mM L-glutamine, and 100 μg/ml penicillin/streptomycin (Gibco BRL, Life Technology, Merelbeke, Belgium) at 37°C (5% CO_2).

Plasmid preparation. The plasmid pCLuc carries the *Photinus pyralis* luciferase coding region under the control of the cytomegalovirus immediate-early enhancer/promoter region [5]. It was isolated from *Escherichia coli* with the Maxi-Prep Kit from Qiagen (Hilden, Germany). Isolated DNA was stored in TE buffer (100 mM NaCl, 10 mM Tris–HCl) at a concentration of 1 mg/ml after its purity was verified by determining the ratio of absorbance at 260/280 nm and gel electrophoresis.

Complex labeling. FITC–PLL-labeled complexes were prepared as previously described [17]. Briefly, 3.6 μl of 1 mM FITC–PLL was added to 2 μg of DNA and incubated 15 min at room temperature, which was followed by addition of either PEI or DOTAP. The presence of FITC–PLL did not cause any decrease in transfection efficiency of these complexes.

FACS analysis. After incubation with lipoplexes or polyplexes, the cells were washed and detached by trypsinization. After centrifugation, the cells were resuspended and analyzed by flow cytometry (Becton–Dickinson FACScan). Ten thousand cells were measured in each sample.

Trypan blue quenching. Trypan blue (TB) solution in PBS (0.04%) was employed to quench extracellular fluorescence. To explore the feasibility of using TB as a quenching dye in our system we performed a control experiment. Briefly, cells were incubated with fluorescently labeled complexes on ice and subsequently washed twice with ice-cold PBS. Trypan blue-treated and untreated samples were further analyzed by FACS. The fluorescence of TB-treated cells was quenched by 90–100%.

Potassium depletion. Here we adopted a procedure described by Larkin and co-workers [33], which results in rapid depletion of cellular potassium. Briefly, A549 cells were washed once with potassium-free buffer containing 140 mM NaCl, 20 mM Hepes, pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/ml D-glucose. Subsequently, the cells were washed with hypotonic buffer (potassium-free buffer diluted with water 1:1), followed by washing with potassium-free buffer (three times). Control cells were treated with buffer containing 140 mM NaCl, 20 mM Hepes, pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/ml D-glucose, and 10 mM KCl. Lipoplexes or polyplexes were incubated with the cells in potassium-free or potassium-containing buffer for 90 min. Subsequently, the cells were analyzed by FACS.

Treatment with inhibitors. Cells were treated with chlorpromazine (10 μg/ml) [34] or genistein (200 μM) [35,36] or filipin (5 μg/ml) [37,38] (all from Sigma) in normal culture medium for 30 min at 37°C. Subsequently, lipoplexes or polyplexes were added and incubation was continued for 2 h. Subsequently, the cells were analyzed by FACS.

The concentration of chlorpromazine and the conditions for potassium depletion employed in the study were such that uptake of fluorescently labeled transferrin, which is widely recognized as a ligand exclusively internalized via clathrin-mediated endocytosis, was inhibited by 90–100%. To optimize the concentrations of genistein and filipin we employed BODIPY–lactosylceramide (BODIPY-LacCer; Molecular Probes), since it has been reported that this sphingolipid is exclusively internalized via a caveolae-mediated mechanism [39]. The concentration of genistein and filipin used in the study blocked the uptake of BODIPY-LacCer by 80–100%. The uptake of fluorescently labeled transferrin and lactosylceramide was assayed by FACS.

Transfections. Formation of DOTAP/DNA and PEI 25 K/DNA complexes was achieved as previously described [5,40]. Briefly, the following amounts of each vector were used per microgram of DNA: 6 μl of 1 mg/ml DOTAP solution (Roche) or 0.3 μl of 100 mM PEI solution (Sigma). Cells were seeded 1 day prior to transfection in 6- or 24-well plates. The cells were incubated with the complexes in FCS-supplemented medium. Luciferase expression was evaluated 24 h later, unless indicated otherwise.

Lysosomal staining. A549 cells were preincubated with fluorescent dextran (Dextran, AlexaFluor 594 10,000 MW; Molecular Probes) for 12 h at 37°C to allow the marker to accumulate in the endosomal/lysosomal compartment. After extensive washing the cells were cultured in a standard cell culture medium for 2 h. The cells were then treated with chlorpromazine (10 μg/ml) or filipin (5 μg/ml) in normal culture medium for 30 min at 37°C. Subsequently, fluorescently labeled polyplexes were added and incubation was continued for 2 h. The cells were extensively washed with PBS and treated with trypan blue (0.04%) to quench extracellular fluorescence. After further incubation for 2 h (under standard conditions) samples were examined by means of fluorescence microscopy (Zeiss Axioplan 2; Germany).

Statistical analysis. Statistical significance of differences was evaluated by a two-tailed unpaired Student *t* test. A value of $P < 0.05$ was considered significant.

ACKNOWLEDGMENT

This work was supported by the Associazione Lombarda Fibrosi Cistica and the European Community (Contract 2002-02119).

RECEIVED FOR PUBLICATION OCTOBER 5, 2004; ACCEPTED MARCH 30, 2005.

REFERENCES

1. Gao, X., and Huang, L. (1995). Cationic liposome-mediated gene transfer. *Gene Ther.* **2:** 710–722.
2. Li, S., and Huang, L. (2000). Nonviral gene therapy: promises and challenges. *Gene Ther.* **7:** 31–34.
3. Goula, D., *et al.* (1998). Polyethylenimine-based intravenous delivery of transgenes to mouse lung. *Gene Ther.* **5:** 1291–1295.
4. Ferrari, S., *et al.* (1997). ExGen 500 is an efficient vector for gene delivery to lung epithelial cells in vitro and in vivo. *Gene Ther.* **4:** 1100–1106.
5. Bragonzi, A., *et al.* (1999). Comparison between cationic polymers and lipids in mediating systemic gene delivery to the lungs. *Gene Ther.* **6:** 1995–2004.
6. Friend, D. S., Papahadjopoulos, D., and Debs, R. J. (1996). Endocytosis and intracellular processing accompanying transfection mediated by cationic liposomes. *Biochim. Biophys. Acta* **1278:** 41–50.
7. Zuhorn, I. S., Kalicharan, R., and Hoekstra, D. (2002). Lipoplex-mediated transfection of mammalian cells occurs through the cholesterol-dependent clathrin-mediated pathway of endocytosis. *J. Biol. Chem.* **277:** 18021–18028.
8. Labat-Moleur, F., *et al.* (1996). An electron microscopy study into the mechanism of gene transfer with lipopolyamines. *Gene Ther.* **3:** 1010–1017.
9. Matsui, H., Johnson, L. G., Randell, S. H., and Boucher, R. C. (1997). Loss of binding and entry of liposome–DNA complexes decreases transfection efficiency in differentiated airway epithelial cells. *J. Biol. Chem.* **272:** 1117–1126.
10. Farhood, H., Serbina, N., and Huang, L. (1995). The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochim. Biophys. Acta* **1235:** 289–295.
11. Koltover, I., Salditt, T., Radler, J. O., and Safinya, C. R. (1998). An inverted hexagonal phase of cationic liposome–DNA complexes related to DNA release and delivery. *Science* **281:** 78–81.
12. Smisterova, J., *et al.* (2001). Molecular shape of the cationic lipid controls the structure of cationic lipid/dioleylphosphatidylethanolamine–DNA complexes and the efficiency of gene delivery. *J. Biol. Chem.* **276:** 47615–47622.
13. Xu, Y., and Szoka, F. C., Jr. (1996). Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry* **35:** 5616–5623.
14. Haensler, J., and Szoka, F. C., Jr. (1993). Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconjugate Chem.* **4:** 372–379.
15. Boussif, O., *et al.* (1995). A versatile vector for gene and oligonucleotide transfer

doi:10.1016/j.ymthe.2005.03.038

into cells in culture and in vivo: polyethylenimine. *Proc. Natl. Acad. Sci. USA* **92:** 7297–7301.
16. Behr, J. P. (1997). The proton sponge: a trick to enter cells the viruses did not exploit. *Chimia* **51:** 34–36.
17. Bragonzi, A., *et al.* (2000). Biodistribution and transgene expression with nonviral cationic vector/DNA complexes in the lungs. *Gene Ther.* **7:** 1753–1760.
18. Kichler, A., Leborgne, C., Coetynaux, E., and Danos, O. (2001). Polyethylenimine-mediated gene delivery: a mechanistic study. *J. Gene Med.* **3:** 135–144.
19. Fujimoto, T., Kogo, H., Nomura, R., and Une, T. (2000). Isoforms of caveolin-1 and caveolar structure. *J. Cell Sci.* **113**(Pt 19)**:** 3509–3517.
20. Ogris, M., Steinlein, P., Carotta, S., Brunner, S., and Wagner, E. (2001). DNA/polyethylenimine transfection particles: influence of ligands, polymer size, and PEGylation on internalization and gene expression. *AAPS PharmSci.* **3:** E21.
21. Rejman, J., Oberle, V., Zuhorn, I. S., and Hoekstra, D. (2004). Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. *Biochem. J.* **377:** 159–169.
22. Zou, S. M., Erbacher, P., Remy, J. S., and Behr, J. P. (2000). Systemic linear polyethylenimine (L-PEI)-mediated gene delivery in the mouse. *J. Gene Med.* **2:** 128–134.
23. Goula, D., *et al.* (2000). Rapid crossing of the pulmonary endothelial barrier by polyethylenimine/DNA complexes. *Gene Ther.* **7:** 499–504.
24. Wightman, L., *et al.* (2001). Different behaviour of branched and linear polyethylenimine for gene delivery in vitro and in vivo. *J. Gene Med.* **3:** 362–372.
25. Klemm, A. R., Young, D., and Lloyd, J. B. (1998). Effects of polyethyleneimine on endocytosis and lysosome stability. *Biochem. Pharmacol.* **56:** 41–46.
26. Lecocq, M., Wattiaux-De Coninck, S., Laurent, N., Wattiaux, R., and Jadot, M. (2000). Uptake and intracellular fate of polyethylenimine in vivo. *Biochem. Biophys. Res. Commun.* **278:** 414–418.
27. Godbey, W. T., Barry, M. A., Saggau, P., Wu, K. K., and Mikos, A. G. (2000). Poly(ethylenimine)-mediated transfection: a new paradigm for gene delivery. *J. Biomed. Mater. Res.* **51:** 321–328.
28. Bieber, T., Meissner, W., Kostin, S., Niemann, A., and Elsasser, H. P. (2002). Intracellular route and transcriptional competence of polyethylenimine–DNA complexes. *J. Controlled Release* **82:** 441–454.
29. Shin, J. S., and Abraham, S. N. (2001). Caveolae as portals of entry for microbes. *Microbes Infect.* **3:** 755–761.
30. Joiner, K. A., Fuhrman, S. A., Miettinen, H. M., Kasper, L. H., and Mellman, I. (1990). Toxoplasma gondii: fusion competence of parasitophorous vacuoles in Fc receptor-transfected fibroblasts. *Science* **249:** 641–646.
31. Hafez, I. M., Maurer, N., and Cullis, P. R. (2001). On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. *Gene Ther.* **8:** 1188–1196.
32. Forrest, M. L., and Pack, D. W. (2002). On the kinetics of polyplex endocytic trafficking: implications for gene delivery vector design. *Mol. Ther.* **6:** 57–66.
33. Larkin, J. M., Brown, M. S., Goldstein, J. L., and Anderson, R. G. (1983). Depletion of intracellular potassium arrests coated pit formation and receptor-mediated endocytosis in fibroblasts. *Cell* **33:** 273–285.
34. Wang, L. H., Rothberg, K. G., and Anderson, R. G. (1993). Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. *J. Cell Biol.* **123:** 1107–1117.
35. Aoki, T., Nomura, R., and Fujimoto, T. (1999). Tyrosine phosphorylation of caveolin-1 in the endothelium. *Exp. Cell Res.* **253:** 629–636.
36. Liu, P., and Anderson, R. G. (1999). Spatial organization of EGF receptor transmodulation by PDGF. *Biochem. Biophys. Res. Commun.* **261:** 695–700.
37. Schnitzer, J. E., Oh, P., Pinney, E., and Allard, J. (1994). Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J. Cell Biol.* **127:** 1217–1232.
38. Orlandi, P. A., and Fishman, P. H. (1998). Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. *J. Cell Biol.* **141:** 905–915.
39. Puri, V., *et al.* (2001). Clathrin-dependent and -independent internalization of plasma membrane sphingolipids initiates two Golgi targeting pathways. *J. Cell Biol.* **154:** 535–547.
40. Biffi, A., *et al.* (1999). Restoration of bacterial killing activity of human respiratory cystic fibrosis cells through cationic vector-mediated CFTR gene transfer. *Hum. Gene Ther.* **10:** 1923–1930.