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RESEARCH ARTICLE

Potocytosis and cellular exit of complexes as cellular pathways for gene delivery by polycations

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Abstract

Background Although polycations are among the most efficient nonviral vectors for gene transfer, the gene expression they allow is still too low for *in vivo* applications. To engineer more potent polycationic vectors, the factors governing the intracellular trafficking of a plasmid complexed with current polycations need to be identified.

Methods and results The trafficking of plasmid DNA complexed to glycosylated polylysines or polyethylenimine (PEI) derivatives was studied by electron microscopy of human airway epithelial cells. The cellular processing of complexes varied with their size and the polycation derivative used: large complexes (>200 nm) made with all polycationic vectors studied were internalized by macropinocytosis. In contrast, intermediate (100–200 nm) ligand-coupled polylysine and PEI complexes primarily entered through clathrin-coated pits. Complexes were then found in endosomal vesicles, accumulated in lysosomes or vesicles near the nucleus and their nuclear entry was limited. For the population of small complexes (≤100 nm) obtained with PEI derivatives, they were internalized through caveolae and pursued a traffic pattern of potocytosis to the endoplasmic reticulum where their fate remains unclear. Finally, some complexes exited the cells either by regurgitation when PEI derivatives were used or through an exosome-like pathway for glycosylated-polylysine complexes.

Conclusions The different pathways of complex trafficking observed in relation with complex size imply the development and study of vectors forming complexes with definite size. Moreover, the complex exit we describe may contribute to the well-established short-term efficiency of gene transfer based on synthetic vectors. It favors the engineering of vectors allowing repeated treatment. Copyright © 2005 John Wiley & Sons, Ltd.

Keywords glycosylated polycations; gene transfer; cystic fibrosis; electron microscopy; intracellular trafficking; potocytosis

Introduction

Polycations are among the most efficient nonviral vectors for gene transfer into various cell types. Gene delivery mediated by polycations such as polyethylenimine (PEI) or polylysine is a multistep process that involves DNA condensation, cellular uptake of the complexes by endocytosis, their release from endosomal vesicles before complex degradation in lysosomes, vector unpacking, nuclear uptake, transcription of the plasmid and translation (for reviews, see [1–4]). The most attractive property of PEI for gene transfer is probably its ability to induce destabilization of endosomal membranes

leading to the release of plasmid–PEI complexes into the cytoplasm [5]. This property is not shared by polylysine, for which endosomolytic agents must be added to achieve efficient gene transfer. However, the main cellular barriers to polycation-mediated gene delivery are restricted cytoplasmic diffusion and entry into the nucleus.

To promote specific cellular uptake of complexes and hopefully efficient intracellular trafficking, we and others have substituted polylysine and PEI with sugar residues (for reviews, see [2,6,7]). Because our ultimate goal is cystic fibrosis gene therapy, we studied the efficiency of glycosylated polycations to transfer genes into airway epithelial cells. We showed that lactosylated (Lac–) PEI was more efficient than unsubstituted PEI for gene transfer into immortalized and primary airway epithelial cells. Furthermore, among the various glycosylated PEIs and polylysines tested, Lac – PEI was the most efficient vector [8]. The better gene transfer efficiency obtained with Lac – PEI complexes than unsubstituted PEI complexes was attributed to a higher amount of the former incorporated by airway epithelial cells and to lower cytotoxicity reflecting reduced endosomolytic capacity [9].

Most of the electron microscopy investigations devoted to gene transfer with polycations were restricted to the study of the size and morphology of complexes according to the protocol of complex formation [10–13]. Herein, we used transmission electron microscopy to examine the intracellular trafficking of complexes, made with gold-labeled plasmid DNA and Lac – PEI, unsubstituted PEI or glycosylated polylysines, into immortalized (ΣCFTE29o- cells) and primary human airway epithelial cells. In addition to confirming previous results, which showed that gene transfer into eukaryotic cells based on the use of polycations is a multistep process, the findings obtained in this investigation suggest the existence of previously unsuspected intracellular pathways that may influence the efficiency of gene transfer by using polycation derivatives as vectors. Thus, small complexes ($\leq$100 nm) were taken up through caveolae and pursued a traffic pattern of potocytosis leading to the endoplasmic reticulum (ER), from which their fate remains unclear. Moreover, some complexes were seen to exit the cells after 24 h, either by regurgitation or through an exosome-like pathway. This exit of complexes may contribute to the well-established short-term efficiency of gene transfer based on synthetic vectors.

Materials and methods

Cell culture

The immortalized, human tracheal epithelial ΣCFTE29o- cells, kindly given by D. C. Gruenert (University of Vermont, Burlington, VT, USA), were derived from ciliated cells collected from a cystic fibrosis patient homozygous for the ΔF508 *CFTR* mutation. ΣCFTE29o- cells have no cAMP-dependent chloride transport [14]. They were cultured as previously described [14].

For primary cultures, non-cystic fibrosis bronchial tissue was collected from patients at the time of open thoracotomy for localized lung tumors and immediately immersed in DMEM/F12 medium supplemented with 20 mM HEPES and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Bronchial tissue had a normal architecture at the light microscopy level. The ciliated cell isolation and culture were based on those described by Wu *et al*. for epithelial cell cultures [15] and modified according to Grosse *et al*. [9].

Polycationic vectors

Five percent of the amino groups of PEI (25 kDa, branched polymer; Sigma, St. Louis, MO, USA) were substituted by a lactosylthiocarbamoyl residue, as previously described [8]. Fluorescein-conjugated Lac – or unsubstituted PEI was prepared as previously described [8].

Poly-L-lysine (40 kDa; average degree of polymerization: 190; Bachem Feinchemikalien, Budendorf, Switzerland) was partially substituted with 66 ± 5 lactosyl (Lac) or 76 ± 4 mannosyl (Man) residues as previously described [16–19].

Gold-labeled plasmid DNA

The plasmid pCMV*Luc* (pUT 650, 5.15 kb; Cayla, Toulouse, France) was biotinylated using the Fast-Tag nucleic acid labeling system (Vector Laboratories, Burlingame, CA, USA). A solution of 30 μg of biotinylated plasmid was incubated for 30 min at room temperature with 60 μl of AuroProbe EM streptavidin G10 (Amersham Pharmacia Biotech Europe, Orsay, France). The preparation was purified by gel filtration through a Sephadex® G-50 spin column and concentrated to 1 mg/ml using a Microcon YM-100 centrifugal filter device (Millipore Corp., Bedford, MA, USA).

Gene transfer procedure

Complexes were prepared as previously described [9,20]. Briefly, plasmid DNA (5 μg of pCMV*Luc* or pCMV*GFP*:pGFPemd-cmv; 4.80 kb; Packard, Meriden, CT, USA) and the desired amount of Lac – or unsubstituted PEI to obtain a PEI nitrogen/DNA phosphorus (N/P) charge ratio of 10 (from a stock solution of PEI, 30 mM in nitrogen) were separately diluted into 45 μl of 150 mM NaCl and mixed together. Plasmid DNA/Lac – or unsubstituted PEI complexes were diluted in 900 μl of serum-free minimal essential medium (MEM) and added to each culture well after removal of the growth medium. After a 1-h incubation at 37 °C, the transfection medium was replaced with fresh growth medium.

Similarly, plasmid DNA (5 μg in 0.7 ml of serum-free MEM) was mixed with glycosylated poly-L-lysine (10 μg of Man–poly-L-lysine or 15 μg of Lac – poly-L-lysine in 0.3 ml of serum-free MEM) and incubated for 30 min

at 37 °C. Then, 1 ml of MEM containing the complexes was supplemented with 100 μM chloroquine and added to each culture well after removal of the growth medium. After a 4-h incubation at 37 °C, the transfection medium was replaced with fresh growth medium.

Uptake of complexes

To investigate the uptake of complexes by the cells, ΣCFTE29o- cells (2×10^5 cells/well in a 12-well plate) were preincubated in MEM containing nystatin (Sigma, 50 μg/ml) or filipin (Sigma, 5 μg/ml) for 45 min at 37 °C. Transfection was then performed in the same medium for 1 h with 500 μl of fluorescein-conjugated Lac – or unsubstituted PEI complexes. After transfection, cells were rinsed twice with sodium citrate buffer, pH 4.6, to remove cell-surface-bound complexes and trypsinized. The fluorescence of 5000 cells was measured with an EPICS Elite flow cytometer (Beckman Coulter, Fullerton, CA USA) used with 488-nm excitation and 520-nm emission wavelength filters.

Transmission electron microscopy

ΣCFTE29o- cells or primary bronchial epithelial cells (5×10^5 cells/well in a 6-well plate) were transfected with 1 ml of complexes made with gold-labeled DNA. At various times, from 1 to 24 h, the cells were trypsinized and fixed in suspension with 2.5% glutaraldehyde (TAAB, Aldermaston, UK) in 0.1 M phosphate buffer (pH 7.4) for 10 min at 4 °C and as a pellet for 2 h. After two washes in 0.1 M cacodylate buffer supplemented with 0.15 M sucrose (pH 7.3), the pellets were post-fixed with 2% osmic acid in 0.1 M cacodylate buffer for 30 min at room temperature, stained with 2.5% aqueous uranyl acetate for 30 min at room temperature, then washed and dehydrated in serially graded ethanol before being embedded in Araldite/Epon (resin kit, TAAB). Ultrathin sections (70 nm) were cut with a diamond knife using a Diatome Ultramicrotome and placed on 300×75 mesh copper/rhodium grids to be examined under a Philips BioTwin CM 120 transmission electron microscope (FEI, Cambridge, UK) operated at 80 kV. A total of 170 grids with an average of 1500 sections of cells per grid were observed.

Confocal microscopy

ΣCFTE29o- cells (5×10^4 cells/well seeded on coverslips in a 24-well plate) were incubated with 500 μl of biotinylated DNA complexes for 1 h at 4 °C to allow complex binding to the cell membrane, but not their uptake. Then, the complexes remaining free in the supernatant were withdrawn and cells were incubated in growth medium at 37 °C for 15 min to 8 h. At the indicated times, cells were processed as previously described [20]. Briefly, cells were fixed in 3% paraformaldehyde for 15 min, incubated for 10 min with 0.1 M glycine in phosphate-buffered saline (PBS) and then for 15 min with 0.2% bovine serum albumin and 0.05% saponin in PBS. Afterwards, the cells were incubated for 1 h with the primary rabbit polyclonal antibodies (Ab), either directed against a peptide mapping to the amino terminus of caveolin-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted 1/200) as a marker of caveolae; or a peptide mapping to the carboxyl terminus of calnexin (StressGen, Biotechnology, Victoria, Canada; diluted 1/500) as a marker of ER. Cells were then washed and incubated for 45 min with fluorescein-conjugated goat anti-rabbit Ab (Molecular Probes, Eugene, OR, USA; diluted 1/200). Finally, the biotinylated plasmid DNA was labeled with rhodamine-coupled streptavidin (Molecular Probes; diluted 1/200), coverslips were washed, mounted in Vectashield solution (Vector Laboratories) and examined with an MRC-1024 BioRad confocal system (Hercules, CA, USA) mounted on a Diaphot 300 inverted microscope. The krypton/argon laser was tuned to generate 488-nm and 568-nm excitation wavelengths. Serial sections, 0.1-μm thick, were used to determine the intracellular localizations of complexes. In quantification experiments, cells from adjacent fields were analyzed: all complexes within a given cell were counted and their localizations inside and outside a given organelle were determined. Percentages represent the number of colocalized complexes among all the complexes present inside a cell. Images were obtained with a Kalman acquisition device and processed with Adobe Photoshop 7.0 software.

Statistical analysis

For gene transfer studies, comparisons were made using the non-parametric Kruskall-Wallis test. For confocal studies, data are expressed as means ± SEM of the analysis of 45 cells, from three independent experiments. Comparisons were made using the non parametric Mann–Whitney U test. A value of $p \leq 0.05$ was considered to be statistically significant.

Results

Gene transfer into ΣCFTE29o- cells using PEI and polylysine derivatives as vectors

Twenty-four hours after the gene transfer procedure, the number of transfected cells was analyzed by flow cytometry. Glycosylated polylysines allowed less than 10% of the cells to be transfected (Figure 1). In contrast, Lac – PEI and PEI allowed 34% and 24% of the cells to be transfected, respectively ($p < 0.05$, when all vectors were compared).

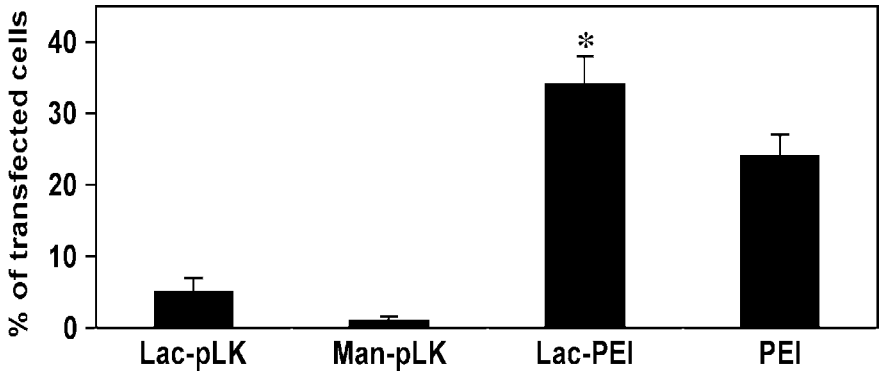

Figure 1. Gene transfer into ΣCFTE29o- cells using PEI and polylysine derivatives as vectors. A plasmid encoding the *gfp* gene (pCMV*GFP*, 5 μg/ml) was complexed with lactosylated polylysine (Lac – pLK), mannosylated polylysine (Man–pLK), Lac – PEI or unsubstituted PEI and incubated with ΣCFTE29o- cells. Cells were subsequently cultured in MEM and, 24 h later, the percentage of fluorescent cells was determined by flow cytometry (*$p < 0.05$)

Intracellular trafficking of plasmid DNA/Lac – or unsubstituted PEI complexes in airway epithelial cells

To identify the plasmid DNA by transmission electron microscopy, it was labeled with gold particles (10 nm in diameter) before complexation with the vector. However, results obtained with unlabeled plasmid DNA/Lac – or unsubstituted PEI complexes were similar to those described below. After a 1-h incubation of ΣCFTE29o- cells and complexes, free complexes were removed and the cells were examined at various times, between 1 and 24 h. Complexes of both types, made with Lac – (Figures 2A–2C) or unsubstituted PEI (Figures 3A and 3B), formed very heterogeneous populations, with complex size ranging from 30 to 700 nm in diameter. Images of complex uptake by the cells were obtained from 1 to 3 h after excess complex removal and showed that various internalization pathways could be identified as a function of complex size. Large (>200 nm) Lac – (Figure 2A) or unsubstituted PEI (Figure 3A) complexes were engulfed by the cells through membrane ruffles, suggesting an endocytic process of macropinocytosis. For Lac – PEI complexes, clathrin-coated pits were sometimes located at the cell surface near the macropinosome-like vesicle (Figure 2A). Intermediate Lac – PEI complexes (100–200 nm) were seen inside or in close proximity to clathrin-coated pits, suggestive of cell entry through clathrin-dependent endocytosis (Figure 2B). Clathrin-coated pits were never observed near unsubstituted PEI complexes. Small complexes (≤100 nm) were usually found inside or near caveolae, evocative of an entry via potocytosis (Figures 2C and 3B). Caveolae were more often observed with unsubstituted PEI complexes.

Once internalized, between 3 and 6 h, most of the complexes were seen in endosomal vesicles, which were partially filled with the complexes (Figure 2D), or complexes were seen in large macropinosome-like vesicles (Figure 3C). Complexes were also observed in vesicles with loose-fitting membrane and internal vesicular images characteristic of late endosomes (Figure 2E). Many complexes were also observed in lysosomes, as shown by the presence of myelin-like concentric layers of membranes in the vesicles (Figure 3D). In these lysosomal vesicles, complexes often appeared to be degraded (Figure 2F). Analysis of many images suggested that the major intracellular route for Lac – PEI complexes was passage from early endosomes to late endosomes and delivery to lysosomes. In contrast, for unsubstituted PEI complexes, very few images of late endosomes were observed and the major intracellular route appeared to be passage from macropinosomes to lysosomes. In addition to this trafficking from endosomes to lysosomes that was observed in almost all cells containing complexes of intermediate and large size between 3 and 6 h, small complexes (≤100 nm) took another intracellular route. They were seen in the ER (Figures 2G and 3E), which is consistent with the endocytic pathway of potocytosis suggested by the images we saw. At later times, between 6 and 24 h, intermediate and large complexes were gathered in vesicles localized near the nucleus (Figure 2H). Sometimes, the vesicle membrane was discontinuous (Figure 3F), but this might reflect the cutting plane of the section and not disruption of the endosomal membrane. However, free gold-labeled DNA was seen in the cytosol (Figure 2I), attesting to its escape from endosomes. No complexes were ever observed in the nucleus but, very rarely, free gold-labeled DNA was seen there (Figure 2J). After 24 h, some aggregated complexes that appeared degraded were again observed in the extracellular space and some were seen being regurgitated by the cells (Figures 2K and 3G). At each time, cells incubated with unsubstituted PEI complexes were severely damaged (Figure 3H), whereas this was not the case with cells incubated with Lac – PEI complexes.

The intracellular trafficking of Lac – or unsubstituted PEI complexes was similarly studied in primary human bronchial epithelial cells. Although the analysis was difficult because few complexes are internalized into these cells [9], the trafficking was similar to that observed in ΣCFTE29o- cells. The main features were a gathering of Lac – PEI complexes in vesicles near the nucleus

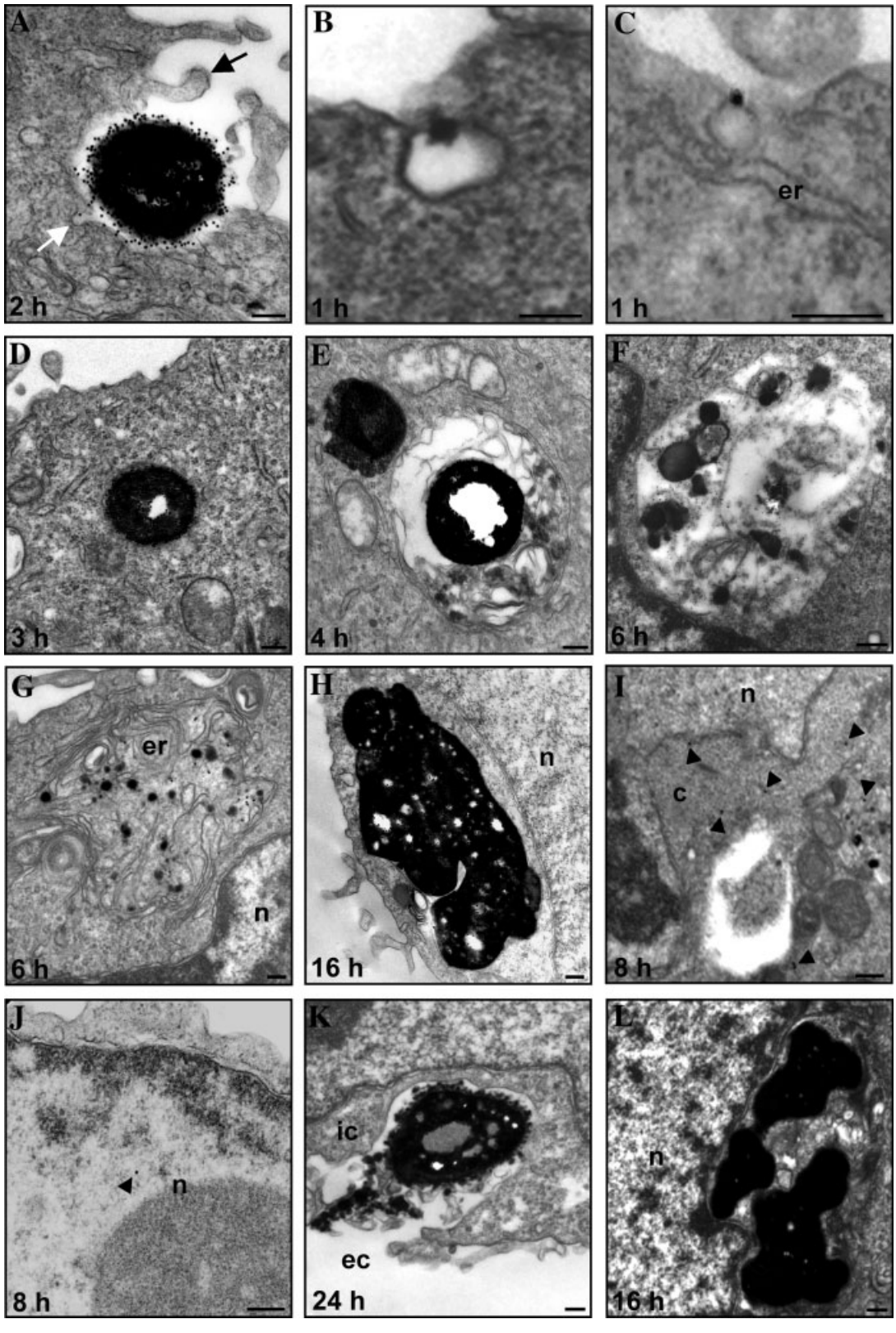

Figure 2. Transmission electron micrographs of ΣCFTE29o- cells (A–K) and primary human bronchial epithelial cells (L) transfected with gold-labeled plasmid DNA/Lac – PEI complexes. (A) Cellular uptake of a large complex (>200 nm) involving both membrane ruffles (black arrow) and clathrin-coated pits (white arrow). (B) Uptake of an intermediate complex (100–200 nm) via a clathrin-coated pit. (C) Uptake of a small complex (≤100 nm) via a caveola (er: endoplasmic reticulum). Complexes in (D) an endosomal vesicle, (E) a late endosome and (F) a lysosome in which the complex appears to be degraded. (G) Small complexes (≤100 nm) in the endoplasmic reticulum (er) (n: nucleus). (H) Accumulation of complexes in a vesicle near the nucleus. Free gold-labeled DNA (arrowheads) (I) in the cytosol (c) and (J) in the nucleus. (K) Regurgitation of complexes into the extracellular space (ec) (ic: intracellular space). (L) Accumulation of complexes in vesicles near the nucleus of a primary airway epithelial cell. Bars = 200 nm

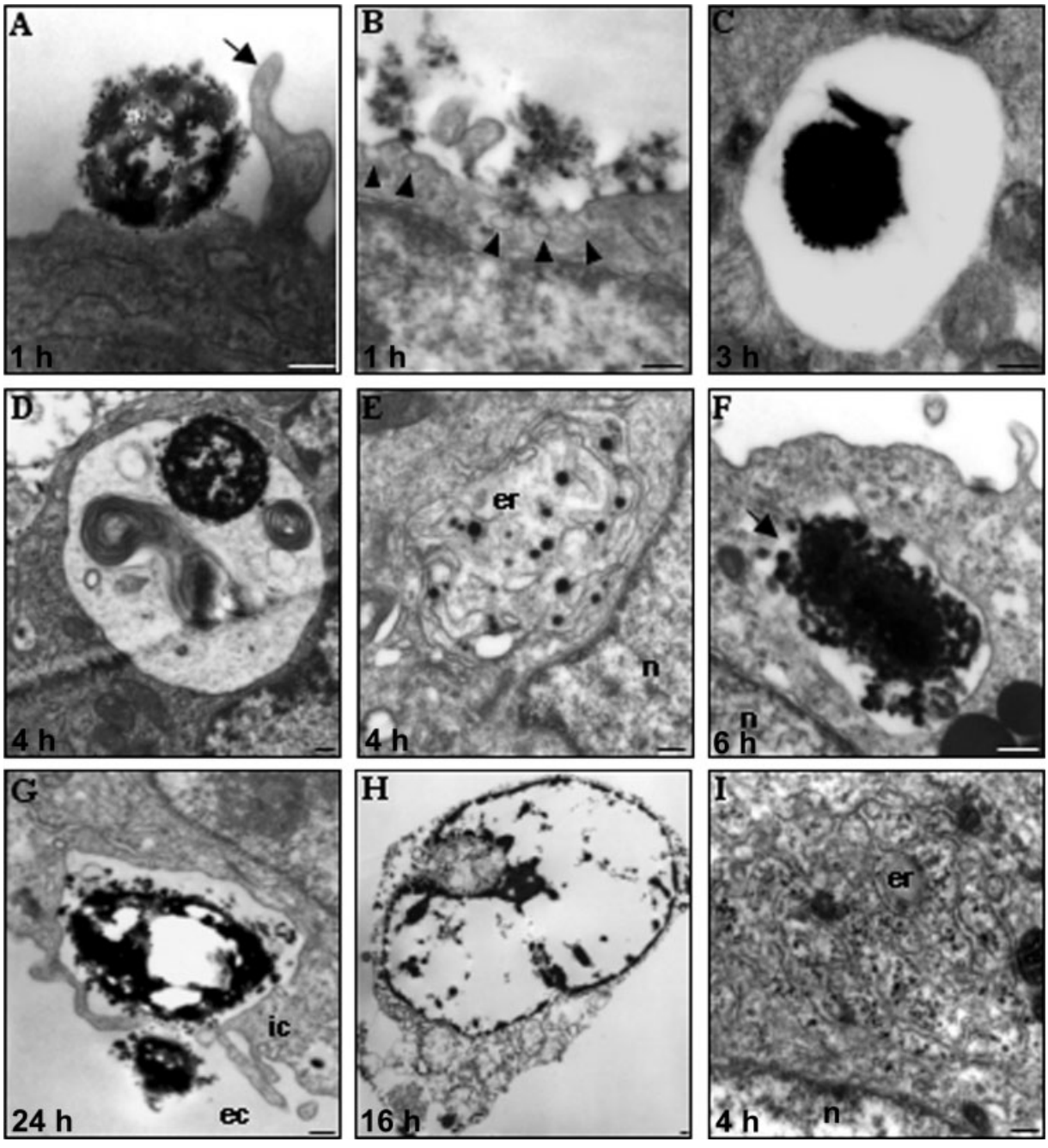

Figure 3. Transmission electron micrographs of ΣCFTE29o- cells (A–H) and primary human bronchial epithelial cells (I) transfected with gold-labeled plasmid DNA/unsubstituted PEI complexes. (A) Cellular uptake of a large complex (>200 nm) involving membrane ruffles (arrow). (B) Uptake of small complexes (≤100 nm) via caveolae (arrowheads). Complexes in (C) a large macropinosome-like vesicle and (D) a lysosome. (E) Small complexes (≤100 nm) in the endoplasmic reticulum (er) (n: nucleus). (F) Accumulation of complexes in a vesicle with a disrupted membrane (arrow) near the nucleus. (G) Regurgitation of complexes into the extracellular space (ec) (ic: intracellular space). (H) Death of cells having taken up unsubstituted PEI complexes. (I) Small complexes (≤100 nm) in the ER in a primary airway epithelial cell. Bars = 200 nm

(Figure 2L) and an accumulation of small unsubstituted PEI complexes in the ER (Figure 3I).

Potocytosis as an intracellular route for Lac – or unsubstituted PEI complexes in airway epithelial cells

Because transmission electron microscopy images suggested that some complexes might enter the cells by caveolar endocytosis, the internalization of Lac – or unsubstituted PEI complexes via caveolae was investigated by using nystatin and filipin which inhibit uptake via caveolae [21]. In the presence of nystatin, the uptake of Lac – or unsubstituted PEI complexes was decreased by 35% ($p > 0.05$) and 45% ($p < 0.05$), respectively, as compared with the uptake observed in control medium (Figure 4A). The same results were observed in the presence of filipin (data not shown). The caveolar localization

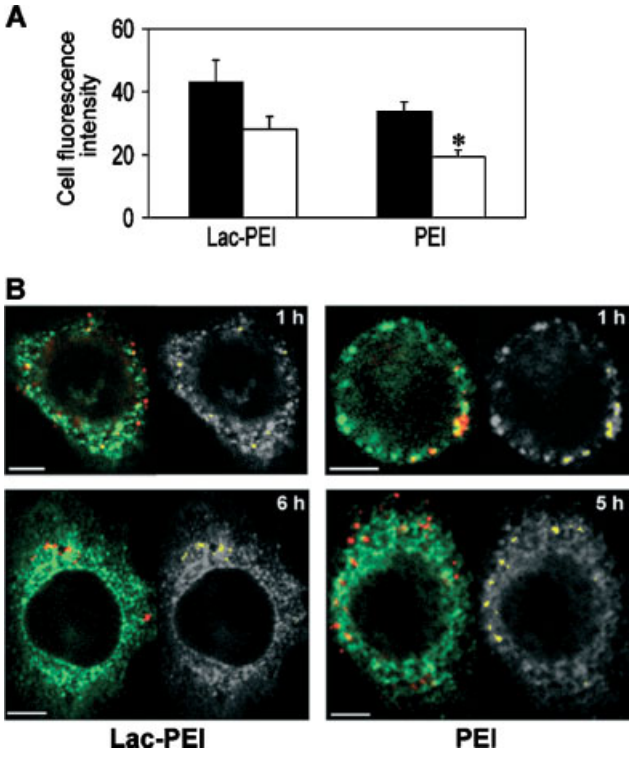

Figure 4. (A) Uptake of Lac – PEI or unsubstituted PEI complexes via caveolae and (B) localization of complexes in caveolae (top) and ER (bottom). (A) After a 45-min preincubation in MEM (■) or in MEM containing nystatin (50 μg/ml, □), ΣCFTE29o- cells were incubated for 1 h in the same medium in the presence of fluorescein-conjugated Lac – or unsubstituted PEI complexes. Cell fluorescence intensity was measured by flow cytometry (expressed as relative light units) (*$p < 0.05$). (B) ΣCFTE29o- cells were incubated with biotinylated plasmid DNA/Lac – PEI or DNA/unsubstituted PEI complexes for 1 h at 4 °C, then washed, incubated at 37 °C for the indicated times, fixed and examined by confocal microscopy. In the left-hand panels, biotinylated DNA labeled with rhodamine-coupled streptavidin appears red; caveolae and ER immunolabeled with anti-caveolin-1 antibodies and with anti-calnexin antibodies, respectively, followed by fluorescein-labeled anti-rabbit antibodies, appear green. In the right-hand panels, pixel analyses of colocalizations of labeled plasmid DNA and organelle appear yellow. Bars = 5 μm

of Lac – or unsubstituted PEI complexes was also studied in ΣCFTE29o- cells. Caveolae were labeled with antibodies recognizing caveolin-1, a protein component of the filamentous coat that lines the inside surface of each caveola membrane [22]. Both types of complexes were localized in vesicles expressing caveolin-1 between 30 min and 2 h, reaching maximal levels at 1 h and involving $19 \pm 2\%$ of the Lac – PEI and $31 \pm 5\%$ of the unsubstituted PEI complexes ($p < 0.05$; Figure 4B, top). As molecules internalized by caveolae may be destined for delivery to the ER (for reviews, see [23,24]), localization of complexes in the ER was investigated. The ER was labeled with an antibody recognizing calnexin, an ER transmembrane protein. Lac – and unsubstituted PEI complexes were found in the ER between 4 and 7 h. For Lac – PEI complexes, maximal localization was seen at 6 h and involved $18 \pm 3\%$ of complexes. In contrast, for unsubstituted PEI complexes, maximal localization was seen at 5 h and involved $35 \pm 5\%$ of complexes ($p < 0.05$; Figure 4B, bottom). Therefore, these observations suggest that unsubstituted and Lac – PEI complexes can be transported intracellularly via potocytosis, but this pathway seems to be used preferentially by unsubstituted PEI complexes.

Intracellular trafficking of Lac – or Man–polylysine complexes in airway epithelial cells

To determine whether this caveolar pathway could be taken by other cationic polymers, a similar transmission electron microscopy study was undertaken with Lac – and Man–polylysine complexes incubated with ΣCFTE29o- cells. Unsubstituted polylysine complexes are poorly taken up by ΣCFTE29o- cells [25], and thus were not studied. Because no differences were observed in the size or the intracellular trafficking between Lac – and Man–polylysine complexes, both types are henceforth referred to as glycosylated–polylysine complexes. Complexes made with gold-labeled or unlabeled plasmid DNA pursued similar intracellular trafficking. Glycosylated–polylysine complexes formed large particles with a mean diameter of 700 ± 200 nm (Figure 5A). Images of complex entry into cells were obtained between 1 and 4 h. Complexes were enveloped by membrane ruffles and in proximity to clathrin-coated pits spread over the cell membrane (Figure 5B), suggesting that macropinocytosis and clathrin-dependent endocytosis are involved in the internalization of these complexes.

Once internalized, complexes were observed in large endosomal vesicles whose membranes frequently bore clathrin-coated pits at their surface (Figure 5C). Between 4 and 8 h, complexes were in vesicles with small inwardly budding vesicles characteristic of multivesicular bodies (MVBs; Figure 5D), in vesicles with loose-fitting membrane generating internal vesicular images characteristic of late endosomes (Figure 5E), or in lysosomes with a multilamellar appearance (Figure 5F). In the latter, complexes were often degraded (Figure 5G). Between 4 and 8 h, some complexes were observed in vesicles localized near the nucleus (Figure 5H) or free in the cytosol (Figure 5I). However, between 1 and 24 h, no complex was ever seen inside the nucleus. Between 8 and 24 h, images of MVB fusion with the plasma membrane were observed (Figure 5J) and some free gold-labeled DNA was seen in the extracellular space in close contact with small membrane vesicles that looked like exosomes (Figure 5K).

The intracellular trafficking of glycosylated–polylysine complexes was similarly studied in primary human bronchial epithelial cells. As for PEI, few complexes were present inside the cells. However, the trafficking was similar to that observed in ΣCFTE29o- cells, with the main feature being the presence of complexes in MVBs (Figure 5L). In contrast to what has been observed with PEI complexes, glycosylated–polylysine complexes were

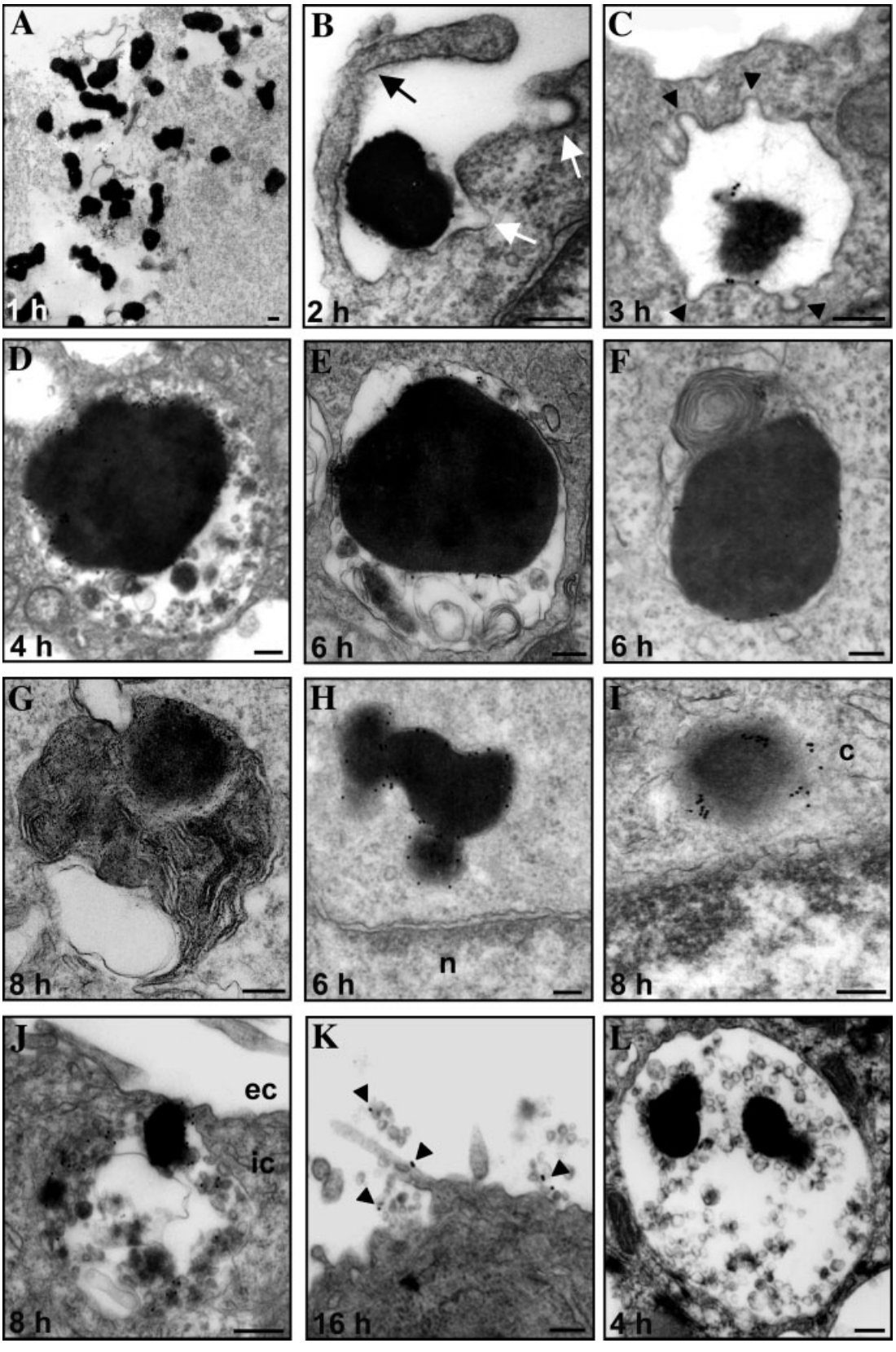

Figure 5. Transmission electron micrographs of ΣCFTE29o- cells (A–K) and primary human bronchial epithelial cells (L) transfected with gold-labeled plasmid DNA/glycosylated-polylysine complexes. (A) Complexes formed a homogeneous population in terms of size. (B) Cellular uptake of a complex involving both membrane ruffles (black arrow) and clathrin-coated pits (white arrows). Complexes in (C) an endosomal vesicle bearing clathrin-coated pits (arrowheads), (D) a multivesicular body (MVB), (E) a late endosome, and (F) a lysosome in which (G) the complex appears to be degraded. (H) Complexes in a vesicle near the nucleus (n) or (I) free in the cytosol (c). (J) Fusion of a MVB with the plasma membrane (ic: intracellular space; ec: extracellular space) and (K) free gold-labeled DNA in the extracellular space in close proximity to exosome-like vesicles (arrowheads). (L) Presence of complexes in an MVB in a primary airway epithelial cell. Bars = 200 nm

never seen near caveolae or in the ER. Moreover, in a confocal microscopy study similar to that described for PEI, glycosylated–polylysine complexes were not found in either caveolin-1-expressing vesicles, or the ER (data not shown).

Discussion

In addition to confirming previous findings showing that gene transfer into eukaryotic cells based on the use of polycations is a multistep process, this ultrastructural study demonstrated that the intracellular trafficking of plasmid–polycation complexes is dependent on the size of the complexes and the polycation derivative used. Moreover, complex exit from the cells may contribute to the well-established short-term efficiency of gene transfer obtained with synthetic vectors.

The size of latex beads which are inert and devoid of ligands [26] and of complexes made with cationic lipids [27,28] was previously shown to greatly influence the internalization and intracellular routing of such particles. The size of plasmid–polycation complexes is a well-known key factor for transfection efficiency (for reviews, see [29,30]). Our ultrastructural study shows that particles made with polycations engage in different intracellular pathways, depending on their size, which is very heterogeneous, as assessed by our transmission electron microscopy images and by complex size measurements by dynamic light scattering (personal unpublished data). Complexes with a diameter >100 nm were taken up through macropinocytosis or receptor-mediated endocytosis and roughly followed the well-described pathway leading from endosomes to lysosomes or exited the endosomes (for reviews, see [1–3,31]; Figures 6 and 7). In contrast, small complexes (≤ 100 nm) were taken up by caveolae. This is consistent with the fact that caveolae are rounded plasma membrane invaginations of 50–80 nm [23,24]. This uptake via caveolae suggests that small PEI complexes might mainly traffic through the potocytosis pathway (Figure 6). Potocytosis is the mechanism by which molecules are internalized by caveolae and can then be delivered to various locations, the cytoplasm, the ER or other organelles, or even the opposite side of the cell (for reviews, see [23,24]). The results of our ultrastructural and confocal studies suggest that small complexes are mainly delivered to the ER, but their ultimate fate remains unclear. This pathway, from caveolae to the ER, is the one described for simian virus 40 and the viral genome is obviously transported from the ER to the nucleus, although by an as yet unknown mechanism [32]. However, the use of polycations to mimic viruses is still in its very early stages and trafficking of small complexes to the ER might well be a dead end.

In addition to this particle-size-dependent uptake and trafficking, our findings show that complex uptake varies with the polycation derivative used. When PEI or polylysine was glycosylated, clathrin-coated pits were involved in their internalization. Macropinocytosis was another way for complexes with a diameter >200 nm to enter the cells (Figures 6 and 7). In contrast, clathrin-coated pits were never observed in proximity to unsubstituted PEI complexes. These results confirm data obtained in our previous studies conducted with pharmacological inhibitors of endocytosis pathways [9,20] and strengthen the validity of using ligands to obtain cell-specific uptake of complexes and enhanced cell transfer. The subsequent intracellular steps also differed markedly according to the polycation used and the sugar moiety present. Lac – PEI complexes were delivered from early endosomes to late endosomes and lysosomes, whereas the major intracellular route for unsubstituted PEI complexes appeared to be direct delivery from macropinosomes to lysosomes (Figure 6). In contrast, glycosylated–polylysine complexes were seen in late endosomes and lysosomes, but also in MVBs (Figure 7). Complex escape from MVBs is doubtful and their accumulation in MVBs may account for the low gene transfer efficiency observed with glycosylated polylysines, as compared with PEI derivatives (this study and [9,25]). However, for all the vectors studied, passage to the cytosol and entry into the nucleus were very low. Most of the complexes were seen gathered in aggregates at the periphery of the nucleus, as described by Mishra *et al*. [33]. Moreover, unsubstituted PEI complexes were highly cytotoxic. Our observations indicate that a minor modification of the vector may result in a very different intracellular route for the complexes, leading to different outcomes and probably different gene transfer efficiencies. Hence, the intracellular pathway of complexes made with a new nonviral vector cannot be assumed based on previous studies with similar vectors, but requires thorough investigation.

One important finding of this study was the images of complex exit. In a previous confocal microscopy study, we observed that, after 48 h, few glycosylated–polylysine complexes remained inside the cells and that most of them were located in the extracellular space [20]. That observation was confirmed and expanded herein. All polycation complexes studied exited the cells, but the mechanism appeared to vary according to the polycation derivative. For complexes made with PEI derivatives, most images of their exit suggested regurgitation that may correspond to the recycling pathway of macropinosomes [34,35] (Figure 6). For glycosylated–polylysine complexes, the secretion pathway probably involved MVBs, many of which contained numerous complexes. Different fates have been described for the internal vesicles of MVBs: they might transport molecules to lysosomes for degradation; they might serve as temporary storage compartments; or they might be emptied into the extracellular space when the MVB membrane fuses with the plasma membrane. This latter process, in which the vesicles are called exosomes, has been described for different cell types, including alveolar lung cells [36]. The images that we obtained of glycosylated–polylysine complexes exiting airway epithelial cells were highly suggestive of exosomes

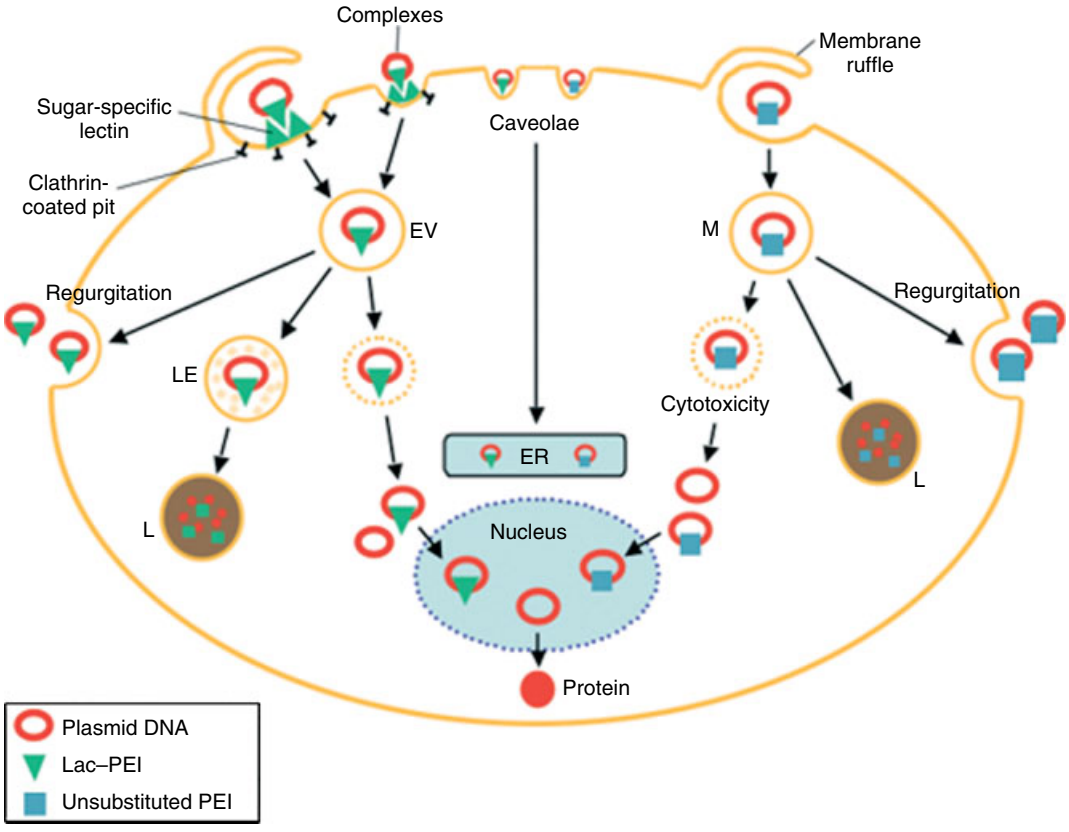

Figure 6. Schematic representation of the intracellular trafficking of plasmid DNA/Lac – or unsubstituted PEI complexes in airway epithelial cells. Small complexes (<100 nm) made with Lac – PEI or PEI follow an intracellular pathway of potocytosis: they are taken up by caveolae and are then transported to the ER. Lac – PEI complexes (100–200 nm) are mostly internalized by the cells through clathrin-coated pits. For larger Lac – or unsubstituted PEI complexes, macropinocytosis is the main means of uptake, while clathrin-coated pits are also involved for Lac – PEI complexes. Once internalized, the major intracellular route for Lac – PEI complexes is passage from endosomal vesicles (EV) to late endosomes (LE) and delivery to lysosomes (L). In contrast, unsubstituted PEI complexes are very cytotoxic and their major intracellular route is passage from macropinosomes (M) to lysosomes. For both types of complexes, passage to the cytosol and entry into the nucleus are limited. Regurgitation of the complexes contributes to the short-term efficiency of gene transfer with PEI derivatives

(Figure 7). Whether by regurgitation or via exosomes, some of the complexes made with all the polycations studied were seen to exit the cells. This is a new and important observation, since it suggests that nonsecretory airway epithelial cells are able to rid themselves of exogenous material. In addition to the absence of plasmid integration into the cell DNA, this complex exit might contribute to the short-term duration of gene transfer based on synthetic vectors.

Our transmission electron microscopy findings confirm previous results, obtained by us and others (for reviews, see [1–4,7,37], that gene transfer into eukaryotic cells using polycations as vectors is a multistep process in

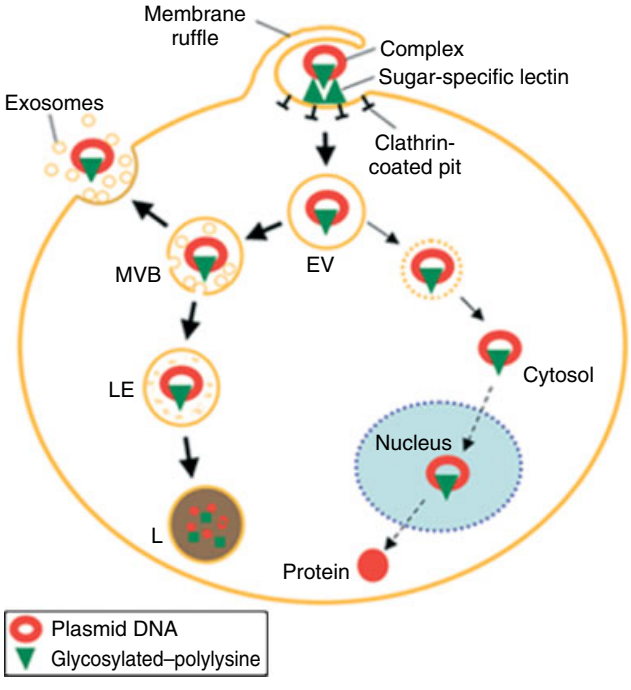

Figure 7. Schematic representation of the intracellular trafficking of plasmid DNA/glycosylated–polylysine complexes in airway epithelial cells. Plasmid/glycosylated–polylysine complexes are taken up by macropinocytosis involving clathrin-coated pits. Complexes in endosomal vesicles (EV) mainly follow a pathway from multivesicular bodies (MVB), to late endosomes (LE) and lysosomes (L). Another route from MVB is plasmid secretion into the extracellular space via exosomes. A few complexes are released from endosomes and reach the cytosol and the nucleus. Steps with bold arrows represent the major pathway

which entry into the nucleus remains one of the most limiting steps. To draw definitive conclusions about the relationships between the efficiency of gene transfer and the various cellular routes we describe, gene transfer of homogeneous populations of complexes of well-defined sizes should be studied. However, it is virtually impossible to generate those sorts of complexes. Nevertheless, our observations suggest the existence of previously unsuspected cellular pathways likely to influence the efficiency of gene transfer with polycation derivatives. Pertinently, small complexes were internalized through caveolae and engaged in a traffic pattern of potocytosis, which led to the ER where their fate remains unclear. Finally, complexes were seen to exit the cells after 24 h and this exit of complexes may contribute to the well-established short-term efficiency of gene transfer based on synthetic vectors. To better analyze these new pathways, complexes with a homogeneous and definite size should be generated. Complexes should also be developed that would allow a repeated treatment, as usually done with classical medicine.

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