Cytoskeletal involvement in the cellular trafficking of plasmid/PEI derivative complexes

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Abstract

We have studied the cytoskeletal involvement in the cellular trafficking of complexes made with plasmid/PEI or plasmid/lactosylated PEI in cystic fibrosis airway epithelial cells (ΣCFTE29o- cells). Complexes were incubated in the presence of cytoskeletal inhibitors, and the number of transfected cells was determined by flow cytometry. Complexes were also generated with fluorescein-labeled PEI derivatives and the cell fluorescence intensity was determined by flow cytometry. In the presence of cytochalasin D to depolymerize actin filaments or nocodazole to disrupt microtubules, gene transfer efficiency with both PEI derivatives was decreased by 90%. The uptake of fluoresceinylated complexes studied by flow cytometry was decreased by 50% in the presence of cytochalasin D for both types of complexes (p < 0.005) and unchanged in the presence of nocodazole. When cytoskeletal inhibitors were added to the cell culture after the complex uptake had occurred, gene transfer efficiency was decreased by 75% and 50% in the presence of nocodazole and cytochalasin D, respectively. Upon nocodazole-microtubule network disruption, the lysosomal localization of complexes was reduced, as assessed by confocal microscopy. Our results show a major cytoskeletal involvement in the cellular trafficking of complexes made with both PEI derivatives: actin filaments mainly in complex uptake, and microtubules in the trafficking of complexes towards the nucleus, probably through guided transport of complex-containing endosomal vesicles.

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1. Introduction

Gene delivery by nonviral vectors is a complex process and despite significant advances in vector formulation, it still fails to achieve levels of expression high enough to allow their use in medicine. The understanding of the barriers that plasmid/vector complexes encounter inside the cell allows the development of strategies to overcome these rate-limiting steps. Hence, internalization of complexes made with nonviral vectors, escape from endosomes, dissociation of the plasmid DNA from its vector, diffusion of the plasmid in the cytoplasm and entry into the nucleus have been thoroughly studied for both cationic lipid and cationic polymer vectors [1–3].

The cytoskeleton is vital to the function of all eukaryotic cells and is required for multiple cellular events including endocytosis and the transfer of cargo within the endocytic system [4]. Actin plays a role in internalization; microtubules and actin are required for efficient transcytosis and delivery of proteins to late endosomes and lysosomes. Given the major role of the cytoskeleton in intracellular transport, it is likely that it is involved in the cellular trafficking of complexes made with nonviral vectors. However, few studies have focused on the possible role of the cytoskeleton on complex cellular transport, and the effect of cytoskeleton-active agents on gene expression is still a matter of debate. With regards to the cytoplasmic transport of non-complexed plasmids, it was shown that plasmids are able to move along the microtubule network [5] and that the diffusion of DNA is slowed by actin filaments [6]. For complexes made with cationic lipids, microtubule-depolymerizing agents were found to increase dramatically the efficiency of gene transfer in various cell types and this effect was attributed in part to an inhibition of complex transport to
lysosomes [7–9]. For complexes made with the cationic polymer polyethylenimine (PEI), Suh et al. have used live-cell real-time multiple particle tracking in COS-7 cells and shown an efficient transport of complexes on microtubules leading to their perinuclear accumulation [10]. Similarly, Kulkarni et al. have used spatio-temporal image correlation spectroscopy to follow plasmid/PEI complexes in HeLa cells and shown that the complex movement observed was caused by their transport along microtubules [11]. However, in both studies, the effect of microtubule-depolymerizing agents on gene transfer efficiency and their effect on complex trafficking in cell organelles were not evaluated, and the role of actin filaments was not studied.

In order to better understand the involvement of the cytoskeleton in the intracellular trafficking of complexes made with cationic polymers, we have studied the effect of actin- and microtubule-depolymerizing agents on gene transfer efficiency of a plasmid DNA complexed with PEI and a PEI derivative, lactosylated PEI. PEI was chosen because it is one of the most efficient nonviral vectors. We have previously shown that PEI substituted with lactose residues is more efficient for gene transfer and less toxic than PEI [12]. These properties were attributed to a higher amount of lactosylated complexes incorporated by epithelial cells and reduced endosomolytic properties [12]. We have also studied the effect of such cytoskeletal inhibitors on the cellular uptake and intracellular trafficking of plasmid/PEI derivative complexes.

2. Methods and materials

2.1. Polyethylenimine vectors and complex formation

Polyethylenimine (PEI) (25 kDa, branched polymer) was purchased from Sigma (St Louis, MO, USA). Five percent of the amino groups of PEI were substituted by a lactosylphenylthiocarbamoyl residue leading to Lac-PEI; fluorescein-labeled PEI or Lac-PEI were prepared as previously described [13]. Because our ultimate goal is cystic fibrosis gene therapy, we studied the immortalized, human tracheal epithelial ΣCFTE29o- cells, kindly given by D.C. Gruenert (California Pacific Medical Center Research Institute, San Francisco, CA, USA). These cells were derived from a CF patient homozygous for the F508del cystic fibrosis transmembrane conductance regulator (CFTR) mutation and have no cAMP-dependent chloride transport [14]. They were cultured as previously described [14]. Two days before transfection, ΣCFTE29o- cells were seeded (2×10^5 cells per well) in a 12-well plate or on coverslips (5×10^4 cells) in a 24-well plate to study either gene transfer efficiency or the intracellular trafficking of complexes, respectively. On the day of transfection, the plasmid DNA (2.5 µg) and the desired amount of PEI for a charge ratio PEI/nitrogen of 10 (from a stock solution of PEI, 30 mM in nitrogen) were separately diluted into 25 µl of 150 mM NaCl and mixed together [12]. Plasmid DNA/PEI or Lac-PEI complexes were diluted into 450 µl of serum-free minimal essential medium (MEM) and after removal of the growth medium, complexes were added to each culture well (500 µl well in the 12-well plate or 250 µl well in the 24-well plate).

2.2. Study of cytoskeletal inhibitors on PEI-mediated gene transfer efficiency and complex uptake

To investigate the role of actin filaments and microtubules on gene transfer, cells were pre-incubated for 1 h at 37 °C in serum-free MEM containing the depolymerizing agents cytochalasin D (25 µM) (Sigma) or nocodazole (25 µM) (Sigma), respectively [15]. Both drugs were dissolved in DMSO and an equal volume of drug-free DMSO (0.05%) was used as a control medium. Experiments were also conducted in DMSO- and drug-free medium and results were similar when experiments were conducted in both mediums (culture medium with 0.05% DMSO or DMSO- and drug-free culture medium). Cells were then incubated in the presence of complexes diluted in the pre-incubation medium. Complexes were made with a plasmid pCMV-GFP (pGFPemd-cmv; Packard, Meriden, CT, USA) which contains a gene encoding the green fluorescent protein (GFP) under the control of the human cytomegalovirus (CMV) promoter. After a 2-h incubation at 37 °C, the transfection medium was replaced with fresh growth medium containing the depolymerizing agents for 5 h and with fresh growth medium containing 10% serum afterwards. Twenty-four hours later, cells were trypsinized, washed, fixed with 3% paraformaldehyde and the percentages of fluorescent cells were determined using an Epics Altra flow cytometer (Beckman Coulter, Fullerton, CA, USA) used with 488-nm excitation and 520-nm emission wavelength filters. To study more precisely the role of cytoskeletal inhibitors on the intracellular trafficking of complexes, the pre-incubation step in the presence of inhibitors was omitted in some experiments.

To investigate the effect of cytoskeletal inhibitors on complex uptake, cells were similarly pre-incubated for 1 h at 37 °C in serum-free MEM containing the depolymerizing agents and then in the presence of complexes made with fluorescein-labeled PEI or Lac-PEI diluted in the same pre-incubation medium. After a 2-h incubation at 37 °C, cells were rinsed twice with sodium citrate buffer, pH 4.6, to remove cell surface-bound complexes [16] and trypsinized. For each experiment, the fluorescence of 5000 single cells was measured by flow cytometry and the means±s.e.m of 4 experiments were calculated.

2.3. Intracellular localization of plasmid DNA/PEI or Lac-PEI complexes

The plasmid pCMV Luc (pUT 650; Cayla, Toulouse, France) was biotinylated using the FastTag nucleic acid-labeling system (Vector Laboratories, Burlingame, CA, USA) and complexed to PEI or Lac-PEI. ΣCFTE29o- cells on coverslips were incubated with complexes for 1 h at 4 °C to allow complex binding to the cell membrane, but not their cellular uptake. Then, the complexes remaining free in the supernatant were removed and cells were incubated at 37 °C in growth medium from 10 min up to 8 h. Some experiments were also conducted in the presence of depolymerizing agents. At the indicated times, cells were washed, fixed with 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. Finally, the biotinylated plasmid DNA was labeled with rhodamine-coupled streptavidin (Molecular Probes, Eugene, OR, USA; diluted 1/200) and some intracellular organelles were labeled by immunocytochemistry. Cells were then washed, mounted in Vectashield solution (Vector Laboratories) and examined with an MRC-1024 Biorad confocal system (Hercules, CA, USA) mounted on a Nikon Diaphot 300 inverted microscope. The krypton/argon laser was tuned to generate 488-nm and 568-nm excitation wavelengths. Serial sections, 0.5-μm thick, were used to determine the intracellular localizations of complexes. In quantification experiments, 15 cells from adjacent fields were analyzed: all complexes within a given cell were counted and their localization inside or outside a given organelle was determined. The reported percentages represent the number of colocalized complexes among all the complexes present inside a cell. Images were obtained with a Kalman acquisition device, colocalization was determined by using a pixel-based analysis and processed with Adobe Photoshop, 7.0 software.

2.4. Labeling of cell organelles

The following primary antibodies were used: the mouse monoclonal antibody (mAb) directed against the α-tubulin protein, clone B-5-1-2 (Sigma; diluted 1:2000) as a marker of microtubules; the mouse mAb directed against the human transferrin receptor, clone H68.4 (Zymed Laboratories, San Francisco, CA, USA; diluted 1:200) as a marker of early endosomes and the mouse mAb directed against the lysosomal-associated membrane protein 1 (LAMP1), clone H4A3 (PharMingen, San Diego, CA, USA; diluted 1:500) as a marker of lysosomes. The fluorescein-conjugated goat anti-mouse Ab (Molecular Probes; diluted 1:200) was used as a secondary antibody. For the labeling of actin filaments, cells were permeabilized with 0.2% Triton-X-100 after cell fixation and incubated for 30 min at room temperature with 0.5 μg/ml fluorescein-coupled phalloidin (Sigma), a toxin that binds polymeric F actin [18].

2.5. Statistical analysis

Data are expressed as means±s.e.m. of at least three independent experiments or of the analysis of 15 cells for confocal studies. For each vector, comparisons were made by a one way analysis of variance (ANOVA) or a non-parametric Mann–Whitney U-test for flow cytometry and confocal studies, respectively. Values of p ≤ 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Effect of cytoskeletal inhibitors on PEI- or Lac-PEI-mediated gene transfer efficiency

To determine the role of cytoskeleton on PEI- or Lac-PEI-mediated gene transfer into ΣCFTE29o- cells, cytochalasin D was used to disrupt actin filaments [19] and nocodazole, to disrupt microtubules [20]. In the control medium (containing DMSO, the solvent of both drugs, but no drug), PEI and Lac-PEI complexes allowed the expression of GFP in 22±3 and 29±3% of cells, respectively (Fig. 1). In the presence of either cytochalasin D or nocodazole, the percentages of cells expressing GFP decreased by 88% for both types of complexes (for PEI, in the presence of cytochalasin D: 3±1% of cells, p < 0.05, and in the presence of nocodazole: 6±1, p < 0.001; for Lac-PEI: in the presence of cytochalasin D: 2±1, p < 0.001, and in the presence of nocodazole: 3±1%, p < 0.001). The flow cytometry analysis allowed the determination of cellular mortality and it was always low and similar for all the conditions studied (DMSO, nocodazole or cytochalasin D).

Our results show that the cytoskeleton plays a major role in PEI derivative-mediated gene transfer. Using technologies to characterize the individual behavior of subcellular particles, plasmid DNA/PEI complexes were shown to achieve rapid perinuclear accumulation due to their active transport along microtubules [10,11,21,22]. Although microtubule depolymerization with nocodazole inhibited this perinuclear complex accumulation [10], the effect of microtubule-depolymerizing agents on gene transfer efficiency was not reported. We here show that cytoskeleton depolymerization dramatically decreases gene transfer efficiency mediated by PEI derivatives.

In contrast, the cationic lipid-mediated gene transfer increased in the presence of microtubule-depolymerizing agents [7–9,23] and was unchanged with actin filament-depolymerizing agents [9]. Taken together, these results suggest quite a different cytoskeletal involvement in lipofection and polyfection processes. We therefore studied the putative involvement of the cytoskeleton in the uptake and subsequent intracellular trafficking of complexes made with PEI derivatives.

3.2. Cytoskeletal involvement in the cellular uptake of complexes made of PEI or Lac-PEI

To evaluate the contribution of actin filaments and microtubules on the cellular uptake of PEI or Lac-PEI complexes,
CFTE29o- cells were pre-incubated for 1 h in the presence of cytochalasin D or nocodazole and subsequently incubated for 2 h in the presence of the same medium and of complexes made with fluorescein-labeled PEI or Lac-PEI. The cell fluorescence intensity was then measured by flow cytometry. With both types of complexes, in the presence of nocodazole, the cell fluorescence intensity was similar to that observed when using control medium (p>0.05) (Fig. 2). In contrast, with both types of complexes, in the presence of cytochalasin D, the cell fluorescence intensity decreased by 50%, as compared with that observed in control medium (p<0.01 for PEI and p<0.001 for Lac-PEI) (Fig. 2). These results show that the cellular uptake of complexes made with PEI derivatives is strongly dependent on actin filaments.

The role of actin filaments on complex uptake was further investigated by studying the distribution of PEI or Lac-PEI complexes in relation with actin filaments by confocal microscopy. As early as 10 min after the beginning of complex incubation at 37 °C, Lac-PEI complexes were observed as punctuated structures localized on actin filaments near the plasma membrane. This colocalization of actin and complexes was observed up to 1 h and was absent afterwards (Fig. 3). The same results were obtained with PEI complexes. These results confirm an involvement of actin filaments in the cellular uptake of PEI derivative complexes and suggest a role in the early steps of their intracellular trafficking.

The mechanisms of uptake of lipoplexes and polyplexes have been thoroughly studied [1,24–27]. Mostly depending on their size, PEI complexes were shown to be taken up by the cells through various pathways, receptor-mediated endocytosis, macropinocytosis or potocytosis. However, the role of actin filaments or microtubules in complex uptake has not been thoroughly investigated. Actin is coupled to receptor-mediated endocytosis and is likely to be involved in other pathways of internalization [4]. In contrast, microtubule depolymerization does not generally affect endocytosis [4]. Whatever the pathway of uptake for PEI derivative complexes, our results showing that actin depolymerization decreased complex uptake, while microtubule depolymerization had no effect, are consistent with established cell biology data and with recent findings by Kopatz et al. [28].

### 3.3. Cytoskeletal involvement in the intracellular trafficking of plasmid DNA/PEI or /Lac-PEI complexes

To evaluate the contribution of actin filaments and microtubules in the intracellular trafficking of PEI or Lac-PEI complexes, CFTE29o- cells were incubated for 1 h at 37 °C in the presence of plasmid pCMV-GFP/PEI or /Lac-PEI complexes to allow an efficient complex internalization. Then, cells were post-incubated for 5 h at 37 °C in the presence of cytochalasin D or nocodazole. Twenty-four hours later, the percentage of cells expressing GFP was measured by flow cytometry. In control medium (DMSO), PEI and Lac-PEI allowed 36±2 and 38±2% of cells to express GFP, respectively. With both types of complexes, in the presence of cytochalasin D, the percentages of cells expressing GFP decreased by 60% as compared with that observed in control medium (14±2 and 14±1% of cells for PEI and Lac-PEI complexes, respectively; p<0.001) (Fig. 4). Similarly, with both types of complexes, in the presence of nocodazole, the percentages of cells expressing GFP decreased by 75% as compared with that observed in control medium (9±1 and 10±1% of cells for PEI and Lac-PEI complexes, respectively; p<0.001) (Fig. 4). These results suggest that once the PEI derivative complexes have been taken up by the cells, their cellular transport is strongly dependent on actin filaments and microtubules.
The contribution of microtubules on the cellular trafficking of PEI or Lac-PEI complexes was further investigated by confocal microscopy. As early as 10 min after the beginning of complex incubation at 37 °C, Lac-PEI complexes were observed as punctuated structures localized on microtubules (Fig. 5). A colocalization of complexes with microtubules was mostly seen between 30 min and 2 h and was absent after 4 h (Fig. 5). At that time, the majority of complexes were observed at the periphery of the nucleus. With PEI complexes, similar results were obtained with a colocalization between 10 min and 3 h.

Our results show a major role for microtubules and actin filaments in the intracellular trafficking of PEI derivative complexes. More precisely, our confocal studies show that actin filaments are mainly involved in the early steps of complex intracellular trafficking, while microtubules are involved for a longer time. For lipoplexes, little involvement of actin filaments in their intracellular trafficking has been reported [9]. In contrast, for uncomplexed plasmids and mostly in an acellular model, actin was found to be the principal determinant of size-dependent DNA mobility in the cytoplasm [6]. The strong microtubule involvement in the intracellular trafficking of PEI derivative complexes we found in CF airway epithelial cells could be argued to be due to a very specific behavior of complexes in these cells bearing a mutation of a chloride channel. However, our results are consistent with and clarify previous studies using different technical approaches in different cells [10,11]. These data are also consistent with studies showing an involvement of microtubules in the intracellular trafficking of complexes made with cationic lipids [7–9] and of uncomplexed plasmids [5]. However, this involvement of microtubules in polyplex- and lipoplex-mediated gene transfer was radically different, since depolymerization of microtubules either decreased gene transfer efficiency in the case of polyplexes, or increased it in the case of lipoplexes.
of lipoplexes. This suggests an involvement of microtubules at different steps of the intracellular trafficking of polyplexes or lipoplexes. Whatever the intracellular steps followed by polyplexes and lipoplexes, it should be underlined that in all studies, it is still unclear whether this involvement of microtubules in the intracellular trafficking of polyplexes or lipoplexes occurs while complexes are inside or outside endocytic vesicles. We therefore studied the endosomal and lysosomal localizations of polyplexes in the presence of cytoskeleton-depolymerizing agents.

3.4. Involvement of the cytoskeleton in the endosomal and lysosomal localizations of plasmid DNA/PEI or /Lac-PEI complexes

It has been shown that after their cellular uptake, complexes made with PEI derivatives are enclosed in endosomal compartments that ultimately deliver their content to lysosomes for degradation [12,29] and that PEI induces destabilization of endosomal membranes limiting lysosomal degradation [30]. In ΣCFTE29o- cells, we have previously shown that complexes made with PEI and Lac-PEI have a maximal endosomal localization at 30 min and 1 h, respectively, and a maximal lysosomal localization at 1 and 2 h, respectively [12,29]. To study the involvement of the cytoskeleton in the endosomal and lysosomal localizations of plasmid DNA/PEI or /Lac-PEI complexes, ΣCFTE29o- cells were pre-incubated in the presence of either cytochalasin D or nocodazole. The depolymerization of actin filaments or microtubules in such conditions was verified by confocal microscopy (data not shown). Then, cells were incubated in the presence of the same medium and the complexes, up to the previously determined times corresponding to maximal endosomal and lysosomal localizations. Finally, the intracellular localization of complexes was analyzed by confocal microscopy. Cytochalasin D was the only inhibitor causing a marked decrease in the number of complexes per cell: 50% with PEI and 60 to 65% with Lac-PEI of the number of complexes observed in control medium (p<0.05) (Table 1). These results indicate once more that actin is involved in the uptake of complexes. Cytochalasin D did not modify much the percentages of complexes localized in endosomes and lysosomes, as compared with control medium (p>0.05) (Table 1). These results show that once the complexes have been taken up by the cells, actin plays a minor role in the subsequent complex intracellular trafficking from endosomes to lysosomes. In the presence of nocodazole and for both types of complexes, the percentages of complexes localized in endosomes were similar to that observed in control medium. However, nocodazole caused a 50% decrease in lysosomal localization of complexes, as compared with that observed in control medium (p<0.05) (Table 1).

These results confirm the major role of microtubules in the intracellular trafficking of complexes made with PEI derivatives since complex delivery to lysosomes was impaired when microtubules were disrupted. We [12] and others [29] have reported that, when complexed with a PEI derivative, a plasmid enters the nucleus mostly as a complex. However, some degree of plasmid DNA/PEI derivative dissociation might occur and it is still unclear if microtubules interact with the plasmid alone, the plasmid complexed with the PEI derivative or vesicles containing the complexes. It has been suggested that plasmid DNA, like most viruses, is able to associate with motor proteins to traffic to the nucleus by using microtubules [5]. Our data do not exclude that uncomplexed plasmid or polyplexes may be physically associated with the motor protein themselves. However, our results suggest that nocodazole, by depolymerizing microtubules, disrupts the targeting of complexes from endosomes to lysosomes. Therefore, the involvement of microtubules in the trafficking of polyplexes is probably mainly due to complex transport in endosomes undergoing motor protein-driven movement guided by microtubules. It was suggested that microtubule-depolymerizing agents inhibit lipoplex transport from endosomes to lysosomes and, by hence limiting plasmid degradation, increase gene transfer efficiency [7,9]. In contrast, with polyplexes, this slower complex transport to lysosomes does not favor an efficient gene transfer, maybe because most of the polyplexes exit early from the endosomal vesicles. Indeed, the inhibition of perinuclear polyplex accumulation by microtubule-depolymerizing agents [10] is likely to decrease the amount of plasmid able to cross the nuclear envelope.

In conclusion, our study shows a major cytoskeletal involvement in the cellular trafficking of complexes made with PEI derivatives: actin filaments in complex uptake and early steps of intracellular trafficking; and microtubules in complex trafficking towards the nucleus, probably through guided transport of complex-containing endosomal vesicles. This new insight into PEI complex behavior in relation with cytoskeleton might guide rational improvements in vector design, such as enhancing complex interaction with microtubules by promoting association with motor proteins.

| Table 1 | Cytoskeletal involvement in the intracellular trafficking of complexes made with PEI or Lac-PEI | | | | |
|---------------------------------|--------------------------------|-----------------|-----------------|-----------------|
| | Endosomes | | Lysosomes | | | |
| | Number of dots per cell | % of colocalization | Number of dots per cell | % of colocalization | | |
| PEI DMSO | 10±1 | 24±4 | 11±2 | 22±4 | | |
| Nocodazole | 9±2 | 22±5 | 10±3 | 6±1 | | |
| Cytochalasin D | 5±2* | 19±7 | 6±1* | 10±3 | | |
| Lac-PEI DMSO | 13±1 | 31±6 | 17±2 | 30±3 | | |
| Nocodazole | 13±2 | 32±3 | 13±2 | 15±3** | | |
| Cytochalasin D | 5±1** | 25±5 | 6±1*** | 20±4 | | |

ΣCFTE29o- cells were incubated for 1 h in the presence of biotinylated DNA/PEI or /Lac-PEI complexes in culture medium containing DMSO, nocodazole or cytochalasin D. Cells were then washed and incubated in the same medium up to the pre-determined times of maximal endosomal or lysosomal localizations [12]. Cells were fixed, biotinylated DNA, endosomes and lysosomes were immunolabeled and complex localization inside or outside a given organelle was analyzed by confocal microscopy: *p<0.05, **p<0.005, ***p<0.0005 when the numbers of complexes (dots) per cell or the percentages of colocalization obtained after incubation in the presence of DMSO or inhibitors were compared (Mann–Whitney U-test).
References


