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RESEARCH ARTICLE

Intracellular rate-limiting steps of gene transfer using glycosylated polylysines in cystic fibrosis airway epithelial cells

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To identify the intracellular barriers to efficient gene transfer, we studied the intracellular trafficking of biotinylated plasmid DNA complexed with either fluorescein-conjugated lactosylated or mannosylated polylysine by confocal microscopy. Both are known to be taken up by cystic fibrosis airway epithelial cells (ΣCFTE29o− cells), but their gene transfer efficiencies differ markedly: lactosylated polylysine is the most efficient glycosylated polylysine while mannosylated polylysine is quite inefficient for gene transfer. Mannosylated complexes appeared to stay longer in endosomes labeled by anti-transferrin receptor antibody than lactosylated complexes (from 30 min to 3 h and from 10 min to 30 min, respectively). At 24 h, higher percentages of mannosylated than lactosylated complexes were localized inside lysosomes labeled by anti-LAMP-1 antibody (41.8 ± 6.6% versus 19.8 ± 5.2%, respectively, $P < 0.05$). Between 30 min and 8 h, complexes reached the nuclei labeled by anti-lamin A/C antibody and no difference was observed between the nuclear amounts of mannosylated and lactosylated complexes. However, as analyzed by a nuclease S1 transcription assay, initiation of transcription was prevented when plasmid DNA was complexed to mannosylated polylysine. Our results indicate that the major limiting steps for mannosylated versus lactosylated polylysine transfer of plasmid DNA are delayed exit from endosomes, high accumulation in lysosomes and limited transcription of the complexed plasmid DNA.
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Keywords: *glycosylated polylysine; intracellular trafficking; cystic fibrosis; endosomes; lysosomes; transcription*

Introduction

Gene therapy represents a potentially important advance in the treatment of many diseases, including cystic fibrosis (CF), which is the most common lethal autosomal recessive disorder in the Caucasian population. To allow routine clinical application of this therapeutic approach, safe, efficient and non-toxic gene delivery vectors are needed. Synthetic delivery systems, such as cationic lipids or polycations, provoke less host inflammatory and immune responses than viral vectors. They are also easier to prepare in large quantities. However, the efficiency of gene transfer by means of synthetic vectors is far from being sufficient to allow human clinical applications. Detailed knowledge of the mechanisms by which synthetic vectors transfer genes and identification of the intracellular barriers to efficient gene transfer should lead to improved efficiency. Two main rate-limiting steps of gene transfer using synthetic vectors are plasmid escape from the endosomal network and its translocation to the nucleus (for reviews see Refs 1 and 2).

Polyplexes are vehicles generated by condensing nucleic acids with a cationic polymer, such as polylysine or polyethylenimine, which can be chemically substituted with a ligand in order to target a specific cell. The notion of a ligand is usually associated to a polypeptide. However, polycations substituted with protein ligands are difficult to synthesize and are potentially immunogenic. We and others have previously shown that polylysine substituted with sugar residues can efficiently transfect various cell types.[3–8] Indeed, membrane lectins, ie cell-surface sugar-specific receptors, are thought to mediate the uptake of plasmid DNA complexed to glycosylated polylysine ('glycoplex') and to carry it into endosomes. The subsequent intracellular trafficking of glycoplexes is largely unknown, but appears to vary according to the sugar moiety substituting the polylysine. Indeed, although the major membrane lectin expressed at the surface of both normal and CF airway epithelial cells recognized α-D-mannopyranosides, and although the most efficient uptake into these cells was observed for glycoplexes bearing α-D-Man residues, glycoplexes made with mannosylated polylysine were quite inefficient for gene transfer. In contrast, although a membrane lectin recognizing β-D-lactopyranosides was poorly expressed at the surface of these airway epithelial cells, glycoplexes made with polylysine bearing lactosyl residues were the most efficient vectors in these cells.[7,8] To better understand the mechanisms by which glycosylated polylysines transfer a gene, we undertook this study to analyze the intracellular trafficking of complexes made either with lactosylated or

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mannosylated polylysine in CF airway epithelial cells. We were able to show that the main limiting steps for plasmid DNA transfer using mannosylated polylysine, as compared with lactosylated polylysine, were delayed exit from endosomes, high accumulation in lysosomes and limited transcription of the complexed plasmid DNA.

Results

Overall intracellular trafficking of plasmid DNA/lactosylated or mannosylated polylysine complexes

To examine the overall intracellular trafficking of glycosylated complexes, we used biotinylated plasmid DNA complexed to fluorescein-conjugated glycosylated polylysines. To ascertain that labeling did not interfere with complex trafficking, experiments were also performed with a Texas-red-stained plasmid complexed to glycosylated and fluorescein-free polylysine or to glycosylated and fluoresceinated polylysine; experiments were also performed with an unlabeled plasmid complexed to glycosylated and fluoresceinated polylysine. Similar results were obtained in all instances.

After 1 h at 4°C, and as expected, plasmid DNA/lactosylated polylysine complexes were observed at the cell membrane (Figure 1A). After 10–15 min of incubation at 37°C, complexes began to be seen inside the cells (Figure 1B). Between 1 and 4 h, complexes were detected inside almost all cells, but some were still present at the plasma membrane at 4 h. After 24 h, most of the complexes had gathered in one area of the cell. After 48 h, few complexes remained inside the cell and most of them were located in the extracellular space. At all times assessed, no dissociation of the plasmid and its polycation could be detected. When the overall intracellular trafficking of the labeled plasmid/mannosylated polylysine complexes was examined, a similar pattern was observed (data not shown). However, at each time assessed (30 min, 8 h and 16 h) and in agreement with the predominant expression of mannose-specific lectin at the surface of ΣCFTE29o− cells,[7] significantly more mannosylated than lactosylated complexes were seen inside the cells (at 30 min, 7.2 ± 0.7 *versus* 4.2 ± 0.5 complexes/cell, respectively; $P < 0.01$).

Cellular uptake of glycosylated complexes

To determine the exact mechanism by which glycosylated complexes enter ΣCFTE29o− cells, we studied the effect of hypertonic medium, which is known to inhibit receptor-mediated endocytosis by rendering clathrin unavailable for assembly into coated pits.[9] We first verified that incubation of ΣCFTE29o− cells in hypertonic medium indeed modifies receptor-mediated (clathrin-dependent) endocytosis and we used fluorescein-coupled transferrin (Molecular Probes, Eugene, OR, USA) as a marker of receptor-mediated endocytosis. Incubation of cells in hypertonic medium containing 0.45 M sucrose resulted in a 90% decrease in fluoresceinated transferrin uptake, as compared with the uptake observed in control medium ($P < 0.01$) (Figure 2a). In hypertonic medium, incorporation of either mannosylated or lactosylated complexes was decreased by more than 95% ($P < 0.01$) (Figure 2a). In addition, gene transfer with lactosylated polylysine complexes was significantly poorer in hypertonic medium, than control medium ($P < 0.05$) (Figure 2b). Because gene transfer with mannosylated complexes was quite inefficient in control medium, its modification in hypertonic medium was not investigated. Uptake of complexes and gene expression level in isotonic sucrose were similar to those obtained in control medium, showing that sucrose did not compete with glycosylated complexes for binding to sugar receptors.

To investigate the possibility that phagocytosis or pinocytosis could also be involved in complex uptake, we studied the effect of cytochalasin B, which inhibits phagocytosis by disrupting the assembly of the F-actin microfilament network beneath the plasma membrane, and of colchicine which inhibits pinocytosis.[10] In the presence of cytochalasin B (10 μg/ml), incorporation of lactosylated or mannosylated complexes was unchanged. However, colchicine (50 μg/ml) decreased the uptake of

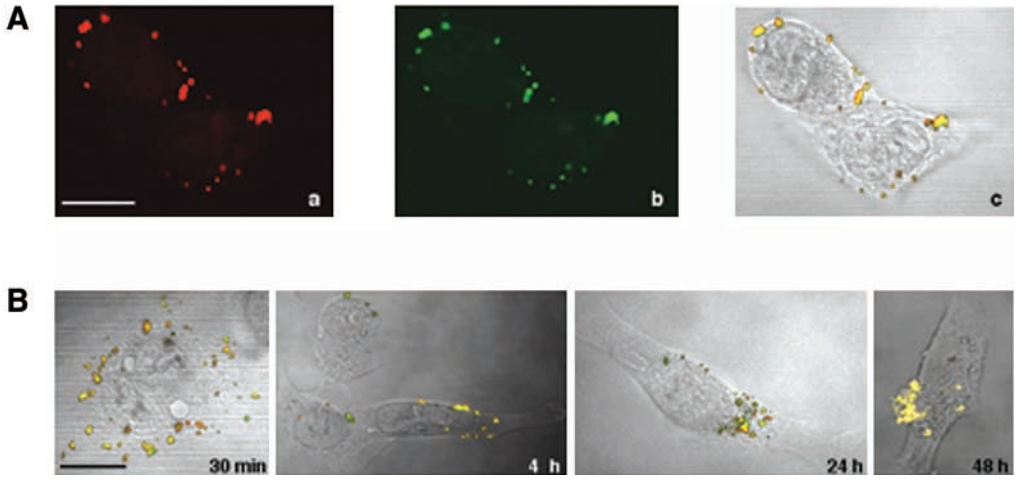

Figure 1 *Overall intracellular trafficking of plasmid DNA/ lactosylated polylysine complexes. (A) ΣCFTE29o− cells were incubated for 1 h at 4°C with biotinylated plasmid DNA/fluorescein-conjugated lactosylated polylysine complexes, then washed, fixed with 3% paraformaldehyde and biotinylated DNA was labeled with rhodamine-conjugated streptavidin. Cells were analyzed by confocal microscopy: (a) the rhodamine-labeled DNA is shown in red, (b) the fluorescein-conjugated lactosylated polylysine is shown in green, (c) merged phase-contrast image of cells with red and green fluorophore-labeling. (B) After a 1-h incubation at 4°C, cells were washed, incubated at 37°C for the indicated times, fixed and examined by confocal microscopy. Merged phase-contrast images of cells with fluorescein and rhodamine fluorophores-labeling (bars = 10 μm).*

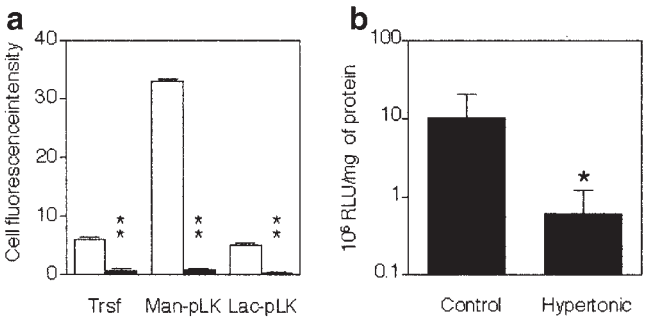

Figure 2 *Hypertonic pretreatment reduces both uptake of glycoplexes and gene transfer efficiency. (a) After a 45-min preincubation in 0.45 M sucrose (black), ΣCFTE29o− cells were incubated for 1 h in the presence of fluorescein-labeled transferrin (Trsf), plasmid DNA/fluorescein-conjugated mannosylated polylysine complexes (Man-pLK) or plasmid DNA/fluorescein-conjugated lactosylated polylysine complexes (Lac-pLK). Control experiments (white) were performed in growth medium. Cell fluorescence intensity was measured by flow cytometry (expressed as relative units). (b) Twenty-four hours after a similar transfection step using plasmid pCMVLuc/lactosylated polylysine complexes incubated in 0.45 M glucose (hypertonic) or growth medium (control), cells were lysed and the luciferase activity was measured by chemiluminescence in a luminometer. The relative light units (RLU) measured for 4 s are expressed as means ± s.e.m. per mg of protein. (∗: $P < 0.05$; ∗∗$P < 0.01$).*

lactosylated and mannosylated complexes by 51 and 45%, respectively. These results suggest that both receptor-mediated endocytosis and pinocytosis are probably involved in the uptake of glycosylated complexes.

Endosomal localization of glycosylated complexes

To determine the subcellular localization of complexes, we labeled the early endosomes using an antibody directed against the human transferrin receptor. At 10 min, 24.1 ± 5.5% of the lactosylated complexes appeared to be in early endosomes (Figure 3a). This localization

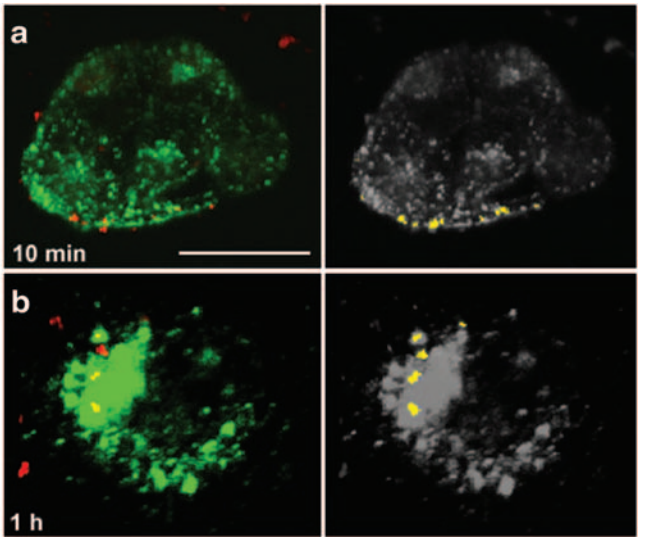

Figure 3 *Localization of glycoplexes in endosomes. ΣCFTE29o− cells were incubated with biotinylated DNA/lactosylated polylysine (a) or /mannosylated polylysine (b) complexes for 1 h at 4°C, then washed, incubated at 37°C for the indicated times, fixed and examined by confocal microscopy. Biotinylated DNA labeled with rhodamine-coupled streptavidin appears red and endosomes immunolabeled with anti-transferrin receptor antibodies and fluorescein-conjugated anti-mouse antibodies appear green in the left-hand panels. On the right, pixel analysis of colocalization of plasmid DNA and endosomes appears in yellow (bar = 10 μm).*

was transient since at 30 min, lactosylated complexes were no longer observed in early endosomes. In contrast, mannosylated complex localization in early endosomes began at 30 min with 14.9 ± 3.7% of complexes present in transferrin receptor-labeled compartments and was maximal at 1 and 3 h with 26.6 ± 4.6 and 23.2 ± 5.5% of the complexes present in early endosomes, respectively (Figure 3b). Similar results were obtained when an antibody directed against the early endosome antigen 1 was used (data not shown).

Lysosomal localization of glycosylated complexes

Since entrapment of complexes in lysosomes is thought to be associated with their degradation in these vesicles, we studied the lysosomal localization of glycosylated complexes. Lysosomes were labeled with an antibody recognizing the lysosomal membrane glycoprotein LAMP-1. At early times, neither glycosylated complex was found in vesicules expressing LAMP-1. However, they appeared in such vesicles at 4 h; at 8 h, 13.5 ± 4.2% of the lactosylated complexes and 19.9 ± 4.8% of the mannosylated complexes were seen in LAMP-1^+ organelles. Maximal accumulation in LAMP-1^+ vesicules was seen at 16 h and involved 34.7 ± 6.1% of the lactosylated complexes and 49.1 ± 6.5% of the mannosylated complexes (Figure 4a and c). However, at 24 h, the complex-localization patterns in LAMP-1^+ vesicles differed with 19.8 ± 5.2% of the lactosylated complexes and 41.8 ± 6.6% of the mannosylated complexes being found there ($P < 0.05$) (Figure 4b and d). Similar results were obtained when an anti-cathepsin D antibody was used (data not shown).

Nuclear localization of glycosylated complexes and transcription efficiency

To evaluate the nuclear localization of complexes, nuclei were stained with DAPI. Lactosylated or mannosylated polylysine complexes reached the nuclear membrane after 30 min of incubation at 37°C and were seen inside the nuclei after 2 h and up to 18 h, with maximal localization at 8 h (Figure 5a). After 24 h, almost no complexes remained inside the nuclei. This time course observed for nuclear localization of lactosylated complexes was consistent with the expression kinetics of a plasmid encoding the luciferase gene and complexed to lactosylated polylysine. Indeed, luciferase expression began to be detected after 2 h (Figure 5b). To confirm the results obtained at 8 h, the internal nuclear membrane was labeled using an anti-lamin A/C antibody: 8.2 ± 2.2% of the lactosylated complexes and 4.5 ± 1.7% of the mannosylated complexes were seen inside the nuclei (Figure 6a). In addition, lactosylated and mannosylated complexes were seen inside the nuclei of 42% and 27% of the cells, respectively, and it is noteworthy that nuclear complexes were always located at the periphery of the nucleus.

These high percentages of cells with nuclearly localized glycosylated complexes were not consistent with either the 5–10% of transfected ΣCFTE29o− cells usually obtained with lactosylated polylysine,[7] or with the even poorer gene transfer efficiency obtained with mannosylated complexes. Therefore, these results suggest that nuclear localization of complexes is not sufficient to allow efficient transcription of the plasmid DNA. Since we have never been able to observe the dissociation of the plasmid from its polycation (Figure 6b), we wondered whether the condensation of the plasmid with the polycation

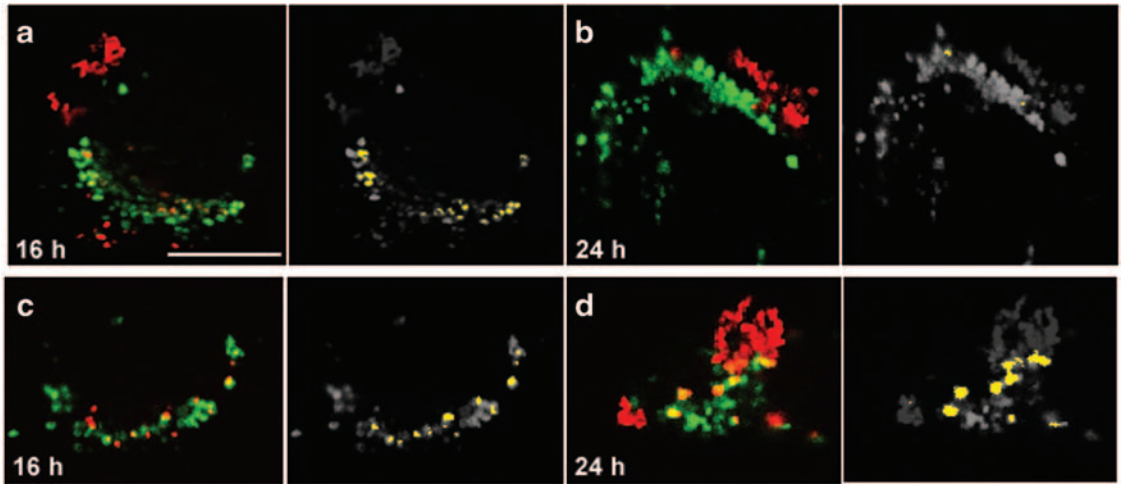

Figure 4 *Localization of glycoplexes in lysosomes. ΣCFTE29o− cells were incubated with biotinylated DNA/lactosylated polylysine (a and b) or /mannosylated polylysine (c and d) complexes for 1 h at 4°C, then washed, incubated at 37°C for the indicated times, fixed and examined by confocal microscopy. Biotinylated DNA labeled with rhodamine-coupled streptavidin appears red and lysosomes immunolabeled with anti-LAMP-1 antibodies, followed by fluorescein-labeled anti-mouse antibodies, appear green in the left-hand panels. On the right, pixel analysis of colocalization of plasmid DNA and lysosomes appears in yellow (bar = 10 μm).*

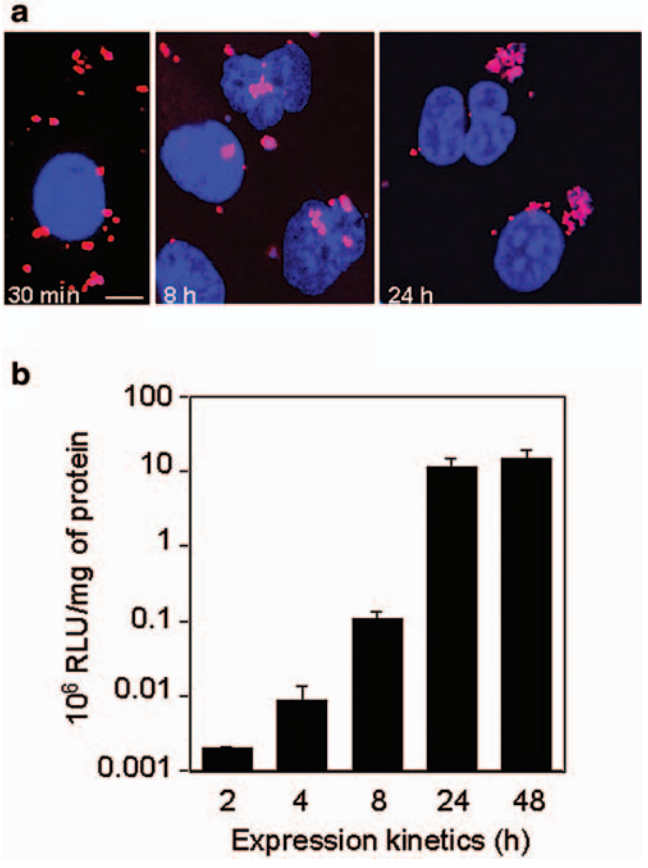

Figure 5 *Kinetics of nuclear localization (a) and gene expression (b) using lactosylated polylysine as the vector. ΣCFTE29o− cells were incubated with plasmid pCMVLuc/lactosylated polylysine complexes for 1 h at 4°C, then washed and incubated at 37°C in culture medium. (a) At the indicated times, cells were fixed and examined by confocal microscopy. Biotinylated plasmid DNA labeled with rhodamine-coupled streptavidin appears red; DAPI-stained nuclei appear blue (bar = 5 μm). (b) At the indicated times, cells were lysed and luciferase activity was measured by chemiluminescence in a luminometer. The relative light units (RLU) measured for 4 s are expressed as means ± s.e.m. per mg of protein.*

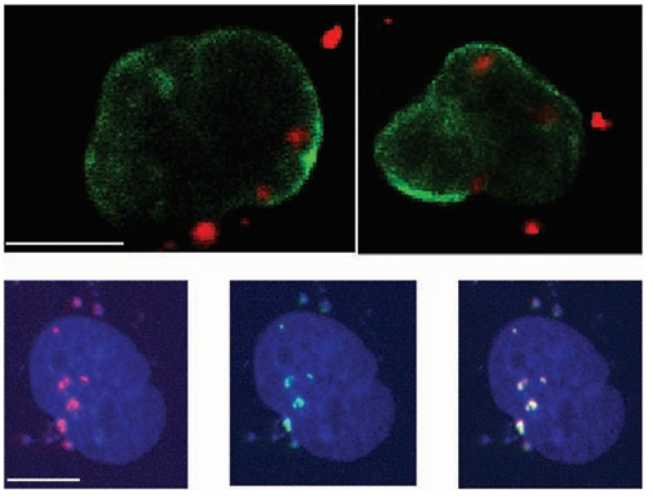

Figure 6 *Nuclear localization of glycoplexes (a) and lack of evidence for the dissociation of the complexes (b). (a) ΣCFTE29o− cells were incubated with biotinylated DNA/lactosylated polylysine (left panel) or /mannosylated polylysine (right panel) complexes for 1 h at 4°C, then washed, incubated at 37°C in culture medium for 8 h and fixed in methanol–acetone. Biotinylated DNA labeled with rhodamine-coupled streptavidin appears red. The nuclear membrane was immunolabeled with anti-lamin A/C antibodies, followed by fluorescein-conjugated anti-goat antibodies, and appears green. Cells were analyzed under a confocal microscope. (b) ΣCFTE29o− cells were incubated with biotinylated plasmid DNA/fluorescein-conjugated lactosylated polylysine complexes for 1 h at 4°C, then washed, incubated at 37°C in culture medium for 8 h and fixed in 3% paraformaldehyde. Biotinylated plasmid DNA was labeled with rhodamine-coupled streptavidin and nuclei were stained with DAPI. Cells were analyzed by confocal microscopy: the nucleus appears blue. Left panel: biotinylated DNA appears red; middle panel: fluorescein-labeled lactosylated polylysine appears green; right panel: merged images (bars = 10 μm).*

could limit transcription efficiency. Using an S1 nuclease transcription assay and HeLa cell nuclear extract (10 μg/μl, either 5 μl: Figure 7, lanes 1, 3, 5, 7 or 10 μl: lanes 2, 4, 6, 8), we compared the transcription efficiencies of free plasmid DNA and plasmid DNA complexed to glycosylated polylysines. Initiation of free plasmid DNA

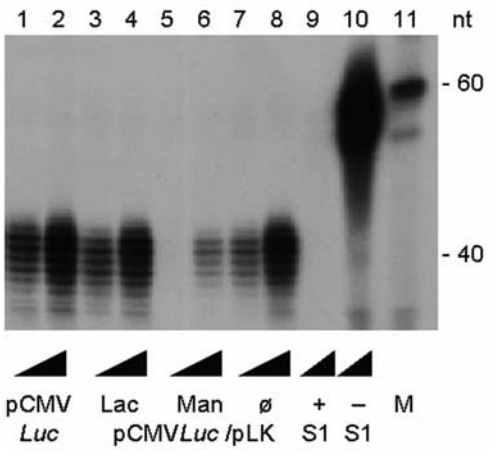

Figure 7 *Glycosylated polylysine-dependent transcription. Free pCMVLuc (pUT650) (lanes 1, 2) or pCMVLuc complexed to either lactosylated (Lac) polylysine (pLK) (lanes 3, 4), mannosylated (Man) polylysine (lanes 5, 6) or sugar-free (ø) polylysine (lanes 7, 8) were tested for transcription activity in a nuclease S1 assay: transcription was initiated in the presence of either 5 μl (lanes 1, 3, 5 and 7) or 10 μl (lanes 2, 4, 6 and 8) of a 10 μg/μl solution of an HeLa cell nuclear extract and NTPs. Transcripts were then hybridized with ^{32}P-labeled 60-mer oligonucleotide corresponding to the CMV sequence and the single-stranded DNA was digested with 100 U of nuclease S1. Control lanes 9 and 10 represent the nuclease S1 (100 U)-digested radiolabeled probe, and undigested, respectively; (lane 11, M, molecular mass markers; nt, nucleotides).*

transcription was efficient (lanes 1, 2), as were those of plasmid DNA complexed to lactosylated polylysine (lanes 3, 4) or to sugar-free polylysine (lanes 7, 8). In contrast, initiation of transcription of plasmid DNA complexed to mannosylated polylysine was impeded (lane 5). Indeed, it could only be detected in the presence of 10 μl of HeLa cell nuclear extract (lane 6).

Discussion

Substitution of polycations, like polylysine, with a ligand has gained increasing attention because it supposedly assures plasmid DNA entry into the cell through a receptor-mediated pathway and, therefore, enables the targeting of a specific cell type.[3,11–13] Indeed, such a mechanism is likely at work for glycosylated polylysines. Our previous findings[7] and those reported herein suggest such a mechanism: a mannose-specific membrane lectin is strongly expressed at the surface of ΣCFTE29o− cells and mannosylated complexes are the glycosylated complexes most efficiently incorporated into the cells, as shown by flow cytometry[7] and confocal microscopy (this study). Moreover, we showed here that mannosylated complexes entered the cells via receptor-mediated, clathrin-dependent endocytosis. However, the mechanism of uptake of large aggregates, eg as complexes, may not be exactly the same as that described for small molecules, like transferrin, and our results suggest that pinocytosis is also involved. Nevertheless, while mannosylated complexes entered ΣCFTE29o− cells more efficiently than lactosylated complexes, the level of gene expression obtained with lactosylated complexes was significantly higher.[7] Because of these differences, further investigations were required to identify the precise mechanism of intracellular trafficking of glycoplexes.

Localization of glycosylated complexes in transferrin receptor-labeled vesicles is consistent with the postulated uptake mechanism. However, this endosomal localization is thought to be a barrier preventing efficient gene transfer because the DNA retained in endosomes will eventually reach lysosomes where it will be inactivated and degraded.[14,15] To partly overcome this barrier, we used chloroquine, a endosomolytic agent which is thought to protect the internalized plasmid from intracellular degradation by neutralizing acidic compartments and delaying the transfer of material from endosomes to lysosomes.[3,16,17] It is pertinent to note that, although both mannosylated and lactosylated complexes were tested in the presence of chloroquine, their transfer kinetics from endosomes to lysosomes differed. Indeed, mannosylated complexes remained in endosomes longer and more were present in lysosomes, as compared with lactosylated complexes. These observations suggest that the sugar moieties bound to polylysine may interact with intracellular membrane lectins and, to some extent, drive the trafficking of glycoplexes.

Entry of DNA into the nucleus is usually considered to be a major limiting step for gene transfer with synthetic vectors. When complexed to cationic lipids, plasmid DNA has rarely been detected in the nucleus[14,18] and plasmid dissociation from the cationic lipid is considered to be necessary for efficient gene transfer.[19] In contrast, here, we observed that both the plasmid DNA and the glycosylated polylysines were present in the nucleus after 2 h and up to 18 h. In addition, at 8 h and depending on the sugar moiety linked to the polylysine, complexes were seen in the nucleus of 27–42% of the cells. These results are in agreement with previous studies showing that plasmid DNA complexed to cationic polymers, such as polyethylenimine[20] or polylysine[13,21,22] were present in the nucleus. The kinetics of entry into the nucleus were quite similar to those reported previously:[20,22] plasmid DNA was detected in the nucleus between 1 and 6 h and its nuclear accumulation was time-dependent. In addition, when basic fibroblast growth factor-substituted polylysine or lactosylated polylysine were used, both the plasmid DNA and the vector were detected in the nuclei of 30% of cells at 4 and 6 h, respectively.[21,22] However, the mechanism by which plasmid DNA complexed to cationic polymer enters the nucleus remains largely unknown. Polylysine might function as a nuclear localization signal,[23] but this possibility remains controversial.[24] For plasmid complexed to glycosylated poylysines, it has been suggested that the sugar moiety may form a complex with lectins acting as a shuttle between cytosol and nucleus.[25] However, even if the sugar moiety does facilitate nuclear entry, it remains a highly inefficient process since 90–95% of the complexes present inside the cells never enter the nucleus.

A rather puzzling finding of the present study was that the nuclear localization of the plasmid DNA did not imply an efficient gene transfer. Indeed, although we detected lactosylated complexes in the nucleus of 40% of ΣCFTE29o− cells in this study, we previously showed with the same vector and the same cells, an efficient gene transfer in only 5–10% of the cells.[7] Poor gene expression once the plasmid has entered the nucleus suggests inefficient transcription and/or translation processes. It should be recalled that the complexes inside the nucleus always had a peripheral localization, which might facilitate a transcription-silencing process.[26] In addition, it

seems that the plasmid and the polymer remained associated even inside the nucleus. Consequently, poor accessibility of the plasmid DNA to the transcription machinery may also be involved. If efficient gene expression requires that the plasmid DNA be free, the observation that very little DNA became dissociated from the glycosylated polylysine could explain our inability to detect free DNA with the techniques used and may account for the low level of gene transfer. However, microinjection of complexes into the nucleus showed that gene expression was not prevented by the condensation of the DNA with cationic polymers.[19] Focusing on transcription by using an *in vitro* assay, we have shown that the dissociation of DNA from sugar-free polylysine or lactosylated polylysine was not a prerequisite, at least for initiation of transcription. In contrast, the condensation of the DNA complexed with mannosylated polylysine clearly prevented the initiation of transcription. However, because lactosylated and mannosylated polylysine vectors have very similar physico-chemical characteristics, it is unlikely that their condensations of plasmid DNA differ. Therefore, we hypothesize that the mannosyl residues themselves inhibit the initiation of transcription by an as yet unknown mechanism. However, further studies using other synthetic vectors and other cell types are needed to establish if, like exit from endosomes and entry into the nucleus, transcription may represent another major limiting step for efficient gene transfer using synthetic vectors.

In an attempt to identify some cellular barriers to efficient gene transfer, we have taken advantage of two vectors, lactosylated and mannosylated polylysines, which are taken up by the cells, but display very different gene transfer efficiencies. We demonstrated rate-limiting steps similar to those previously described for other synthetic vectors, ie poor exit from endosomes and likely degradation in lysosomes where complexes accumulate. Moreover, we showed that the presence of plasmid DNA in the nucleus does not guarantee efficient gene expression and that transcription of the plasmid may be prevented by minor modifications of the vector. It would appear that to obtain efficient gene transfer with synthetic vectors, all cellular steps including the nuclear ones, must be optimized and, therefore, need to be fully understood in order to design highly efficient vectors.

Materials and methods

Cell culture

The immortalized, human tracheal epithelial ΣCFTE29o− cells, kindly given by DC Gruenert (University of Vermont, Colchester, VT, USA), are from a CF patient homozygous for the △F508 *cftr* mutation and show no cAMP-dependent chloride transport.[27] They were cultured as previously described.[27]

Glycosylated poly-L-lysine conjugates

Poly-L-lysine (40 kDa; degree of polymerization: 190) (Bachem Feinchemikalien, Budendorf, Switzerland) was partially substituted with sugar residues as previously described.[28] The average number of lactosyl or mannosyl residues bound per poly-L-lysine molecule was calculated from the sugar content determined using the resorcinol sulfuric acid micromethod[29] and was found to be 66 ± 5 and 76 ± 4, respectively. Fluorescein-conjugated glycosylated polylysines were prepared as previously described[3] and contained 4 ± 2 fluorescein residues per poly-L-lysine molecule.

Gene transfer procedure

One day 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 plasmid DNA, respectively.

The glycoplexes (glycosylated polylysine/plasmid DNA complexes) were prepared as previously described.[30] Briefly, glycosylated poly-L-lysine (10 μg of mannosylated polylysine or 15 μg of lactosylated polylysine in 0.3 ml of serum-free mimimal essential medium (MEM) was mixed with the reporter gene plasmid pCMV*Luc* (pUT 650, 5.15 kb) (Cayla, Toulouse, France) which contains the gene encoding the firefly luciferase under the control of the human cytomegalovirus (CMV) promoter (5 μg in 0.7 ml of serum-free MEM). This transfection medium was kept for 30 min at 37°C, supplemented with 100 μM chloroquine and added to each culture well after removal of the growth medium. To investigate the uptake of complexes by the cells, cells were preincubated in hypertonic media (0.45 M sucrose), isotonic sucrose (0.25 M), MEM containing cytochalasin B (10 μg/ml) or colchicine (50 μg/ml) for 45 min at 37°C and transfection was performed in the same medium for 1 h. After transfection, cells were rinsed twice with sodium citrate buffer, pH 4.6, to remove cell surface-bound complexes and cells were either incubated in growth medium until analysis of gene expression 24 h later or trypsinized to determine cell fluorescence intensity when the gene transfer procedure was performed with fluorescein-conjugated glycosylated polylysine complexes. 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. To render the specific fluorescence intensity of each type of complex comparable, the cell fluorescence intensities were corrected with the $I^{comp}/I^{glycoplex}$ ratio, where I^{comp} and $I^{glycoplex}$ are respectively, the fluorescence intensities of 1 μg/ml of fluorescein-conjugated polylysine/DNA complex and a given fluorescein-coupled glycosylated polylysine/DNA complex.

Gene expression

Luciferase gene expression was measured by luminescence according to de Wet *et al*.[31] The luminescence generated upon automatic addition of 150 μl of 167 μM luciferin dissolved in water was recorded in duplicate samples for 4 s by a luminometer (Lumat LB 9501; Berthold, Wildbach, Germany). The luminescence is reported as relative light units (RLU). Protein concentrations in each sample were determined using the bicinchoninic acid (BCA) colorimetric method,[32] modified according to Hill and Straka.[33] Results are expressed as RLU per mg of protein.

Intracellular localization of plasmid DNA/glycosylated polylysine complexes

The plasmid was biotinylated or Texas red-labeled using the FastTag nucleic acid-labeling system (Vector Labora-

tories, Burlingame, CA, USA) and complexed to lactosylated or mannosylated polylysine. In some experiments, fluorescein-labeled glycosylated polylysines were used. ΣCFTE29o− cells on coverslips were incubated with glycoplexes for 1 h at 4°C to allow glycoplex binding to the cell membrane, but not their incorporation. Then, the complexes remaining free in the supernatant were withdrawn and cells were incubated in growth medium at 37°C for 10 min up to 48 h. At the indicated times, cells were washed twice with phosphate buffer saline (PBS), fixed in 3% paraformaldehyde for 15 min at room temperature, incubated for 10 min with 0.1 M glycine in PBS and then for 15 min with 0.2% bovine serum albumin (BSA) and 0.05% saponin in PBS. Finally, the biotinylated plasmid DNA was labeled with rhodamine-coupled streptavidin (Molecular Probes; dilution: 1/200). Then, coverslips were either mounted in Vectashield-DAPI solution (Vector Laboratories) and examined with an MRC-1024 BioRad confocal system (Hercules, CA, USA) mounted on a Diaphot 300 inverted microscope (see below) to study the overall trafficking of the complexes or were further processed for organelle immunolabeling by incubation for 1 h in the presence of primary antibody diluted in 0.2% BSA and 0.05% saponin in PBS. Cells were washed four times with PBS and incubated for 45 min with fluorescein-labeled secondary antibody diluted in PBS. Coverslips were then washed with PBS and distilled water and mounted in Vectashield-DAPI solution and examined by confocal microscopy. The krypton/argon laser was tuned to produce 488-nm and 568-nm excitation wavelengths and the ion/argon laser was tuned to generate a 363-nm excitation wavelength. Serial sections collected at increments of 0.5-μm thick for endosomal and lysosomal localizations, and 0.1-μm thick for nuclear localization, were used to define the intracellular localization of plasmid DNA/glycosylated polylysine complexes. In quantification experiments, 25 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. 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, 6.0 software.

Antibodies for organelle immunolabeling

The following primary antibodies were used: the mouse monoclonal antibody (mAb) directed against the human transferrin receptor CD71, clone DF 1513 (Sigma, St Louis, MO, USA; diluted 1/10), the mouse mAb directed against the early endosome antigen 1 (EEA1), clone 14 (Transduction Laboratories, Lexington, KY, USA; diluted 1/200) as markers of early endosomes; the mouse mAb directed against the lysosomal-associated membrane protein 1 (LAMP-1), clone H4A3 (PharMingen, San Diego, CA, USA; diluted 1/500) and the rabbit polyclonal Ab to cathepsin D (Upstate Biotechnology, Lake Placid, NY, USA; diluted 1/200) as markers of lysosomes. The goat polyclonal Ab directed against a peptide mapping at the amino terminus of lamin A/C (Santa Cruz Biotechnology, Santa Cruz, CA, USA; fixation: methanol-acetone and diluted 1/100) as a marker of the internal nuclear membrane. The following secondary antibodies were used: fluorescein-conjugated goat anti-rabbit or anti-mouse Ab (Molecular Probes; diluted 1/200) and fluorescein-coupled donkey anti-goat Ab (Jackson ImmunoResearch, West Grove, PA, USA; diluted 1/100).

Transcription assay

To investigate the putative inhibition of transcription efficiency attributed to the condensation of DNA with the vector, a nuclease S1 transcription assay adapted from Sambrook *et al*[34] was used: free plasmid DNA pCMV*Luc* (75 ng) or plasmid DNA/glycosylated polylysine complexes were incubated with a HeLa cell nuclear extract (final volume: 45 μl) for 20 min at 25°C to allow the formation of the preinitiation complex. Transcription was then initiated by adding NTPs and allowed to proceed for 30 min at 30°C. After protein digestion with proteinase K and phenol–chloroform extraction, the transcripts were hybridized overnight at 42°C with ^{32}P-labeled (10 000 c.p.m./assay) 60-mer oligonucleotide corresponding to the CMV sequence (from –20 to +40) in buffer containing 200 mM PIPES (pH 6.5), 5 mM EDTA, 2 M NaCl, and 50% formamide. The single-stranded DNA was then digested with 100 U of nuclease S1 in buffer containing 30 mM sodium acetate, 30 mM $ZnCl_2$, and 4 M NaCl for 2 h at 25°C. The reaction was stopped by adding 10 μg of tRNA and loaded on to an 8% polyacrylamide gel.

Statistical analysis

Data are expressed as means ± s.e.m. of three independent experiments or of the analysis of 25 cells for confocal studies. Comparisons were made using the non-parametric Mann–Whitney *U* test. Values of $P \leq 0.05$ were considered to be statistically significant.

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