CMLS, Cell. Mol. Life Sci. 60 (2003) 288–297
1420-682X/03/020288-10

CMLS Cellular and Molecular Life Sciences

Glycofection: facilitated gene transfer by cationic glycopolymers

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Abstract. Mammalian cells express several types of lectins involved in intracellular trafficking, including endocytosis, interorganelle routing and putatively nuclear import. In order to enhance the gene transfer efficiency, glycosylated cationic polymers have been used as nonviral vectors. We developed a simple method to convert reducing saccharides into glycosynthons. Glycosynthons are used to synthesize cationic glycopolymers, called Glycofectins. Glycofectins interact with a plasmid to give a glycoplex, a compacted form of a polymer/DNA complex. The high glycoplex efficiency depends on the sugar involved in the uptake and in the intracellular trafficking of glycoplexes. The present paper deals with glycoplexes, with gene transfer into cystic fibrosis airway epithelial and gland serous cells, and with some of the problems that have to be solved before clinical trials.

Key words. Cystic fibrosis; gene therapy; glycosylated polymers; intracellular traffic; endogenous lectins; nuclear import; polyethyleneimine; polylysine.

Introduction

Thanks to advances that occurred during the last 2 decades in molecular genetics and in molecular cell biology, new therapeutic approaches based on gene transfer have been set up [1]. Such therapies have already been successfully applied to correct severe combined immunodeficiency (SCID)-X1 disease, a mutation in the gene encoding the γ chain of the interleukin 2, 4, 7, 9 and 15 receptors [2, 3]. Several independent approaches are being developed involving viruses, including retroviruses [2], and nonviral vectors [4]. The latter, also called synthetic delivery systems [5], utilize lipo-cations and cationic polymers that interact with DNA to form lipoplexes and polyplexes, respectively. Using them to transfer genes corresponds to lipofection and polyfection [6]. Our work utilizes glycosylated cationic polymers, called Glycofectins, and gene transfer with glycofectins, Glycofection [7]. This approach is based on the presence of cell surface lectins on mammalian cells and their role in the specific cellular uptake of glycoconjugates [8, 9].

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Endogenous lectins

Animal lectins mediate biological processes ranging from protein folding and trafficking to the modulation of cell-cell and cell-matrix interactions as well as endocytosis. Membrane lectins expressed on the cell surface are good candidates to selectively target genes by making use of their specific ligands but intracellular (membrane and soluble) lectins are also important candidates for delivery to the right compartment, such as the cytosol and/or the nucleus.

Lectins are usually made of several domains, amongst them a single protein module responsible for sugar-binding activity, a carbohydrate-recognition domain or CRD. Different evolutionary patterns of sugar-binding activities emerge from the comparative analysis of lectins [10]. Lectins that contain CRDs fall broadly into two categories: those which are mostly located intracellularly (calnexin, L-type lectins: MR60/ERGIC-53, and VIP36, P-type lectin: Man6P receptors), in luminal compartments and involved in the trafficking, sorting and targeting of glycoproteins in the secretory and other pathways; and those which are either secreted or localized on the

plasma membrane and are involved in either cell interactions or in endocytosis of their ligands (C-type lectins such as Gal receptors, Man receptors, selectins, MBP or mannan-binding protein, galectins and I-type lectins, including Siglecs).

Endocytosis and trafficking from the plasma membrane to the cytosol

A wide variety of molecules enters cells by receptor-mediated endocytosis; an early step is the transfer of ligands and receptors from the cell surface into intracellular vesicles. Various membrane lectins mediate endocytosis [9]. Amongst them, hepatic ASGP-R and the ManR macrophage lectins are well known; others were described more recently. Glycoproteins with terminal mannose or fucose residues follow a well-known endocytic pathway leading to lysosomes. The receptor-ligand complex is taken up via clathrin-coated pits and directed to endosomes, where the complex dissociates – the affinity of ligands for the lectin is pH dependent, with less binding activity in acidic medium. Kinetic studies have shown that the ManR [11] and the ASGP-R [12] recycle to the cell surface with a vacant binding site. The mannose-6-phosphate/insulin-like growth factor II receptor (Man6P/IGF-II receptor) is a transmembrane glycoprotein that cycles through the Golgi, endosomes and the plasma membrane to carry out its role in the biogenesis of lysosomes and in the clearance of the IGF-II [13].

The presence of membrane lectins and their capacity to internalize their ligands can be demonstrated by using labeled glycoproteins or neoglycoproteins. By using fluorescein-labeled neoglycoproteins, we have shown that monocytes mediate the uptake of mannose-6-phosphate bearing neoglycoproteins [14]. Dendritic cells (DC), which are the most efficient antigen-presenting cells utilizing unique transport pathways from endosomes to the cytosol [15], express several cell surface membrane lectins. Monocyte-derived dendritic cells express mannose-specific lectins which efficiently mediate endocytosis of mannosylated neoglycoproteins [16, 17]. One of these, called DC-SIGN, for **d**endritic **c**ell-**s**pecific **i**CAM-3 **g**rabbing **n**on-integrin [18], binds the gp120 envelope glycoprotein of human immunodeficiency virus-1 [19]. The extracellular domain is a tetramer stabilized by an α-helical stalk [20], and the CRD binds $Man_9GlcNAc_2$ oligosaccharide. The internalization motif in the cytoplasmic tail suggested that DC-SIGN functions as an endocytic receptor, targeting ligand to late endosomes and lysosomes [21]. DC-SIGN has a tyrosine/dileucine internalization motif in its cytoplasmic tail, which mediates rapid internalization of intact human immunodeficiency virus (HIV) in a low pH non-lysosomal compartment, as evidenced by the failure to colocalize with Lamp-1 antibody. Complexes are targeted to late endosomes and lysosomes, where the ligands can dissociate, allowing recycling of DC-SIGN to the cell surface. So, in DC which express both ManR and DC-SIGN, the trafficking will be different if a glycosylated ligand binds one or another lectin.

Trafficking from the cytosol to the nucleus: nuclear and cytosolic lectins and glycoproteins

Nuclear lectins were first detected by Sève et al. [22] using neoglycoproteins, which were associated with nucleoli and nucleoplasmic ribonucleic elements. Galectin 3, also named CBP35, was shown to be predominantly localized in the nucleus of proliferating cells [23, 24]. Two C-type lectins were isolated from nuclei: CBP22 and CBP70, a Glc/GlcNAc-binding protein [25]. CBP70 coprecipitates the cellular prion protein (PrPC) in a sugar-dependent fashion [26].

Recently, HSP-70 was identified [27] as an *N*-acetyl glucosamine-specific lectin, putatively identical to CBP70. Some of the nuclear lectins may be involved in interactions with O-glycosylated proteins. Indeed, many cytosolic and nuclear proteins have some of their seryl or threonyl residues substituted with a β-GlcNAc moiety. This peculiar O-glycosylation modulates the function of nuclear and cytosolic proteins [28].

Proteins of molecular mass larger than 40,000 (the limit for a globular protein to freely diffuse across a nuclear pore complex) do not enter the nucleus unless they contain or are substituted with a peptide nuclear localization signal (NLS) [29]. Thus, bovine serum albumin (BSA), a nonnuclear protein of M_r 67,000 which normally does not go through the nuclear pores, migrates from the cytosol to the nucleus when it is substituted with either the SV40 large T antigen NLS [30] or the nucleoplasmin NLS [31]. Besides the NLS-mediated nuclear import pathway, NLS-free BSA substituted with specific sugars (neoglycoproteins), when introduced into the cytosol of HeLa cells upon electroporation or digitonin-permeabilization, can be transported into the nucleus in a sugar-dependent manner [32–34]. BSA bearing 25 ± 4 α-D-glucosyl (Glc-BSA), β-di-*N*-acetylchitobiosyl (GlcNAcβ4GlcNAc-BSA), α-D-mannosyl (Man-BSA) or α-L-fucosyl (Fuc-BSA) residues entered the nucleus of HeLa cells. Under the same conditions, sugar-free BSA or BSA bearing about 25 β-D-lactosyl (Galβ4Glc-BSA) or α-D-mannosyl-6-phosphate (M6P-BSA) moieties remained in the cytosol. The specificity of the import system was demonstrated by competition experiments using an excess of unlabeled neoglycoproteins. This discovery strongly suggested that sugar residues could act as nuclear targeting signal [35, 36]. This has been recently confirmed upon microinjection of various glycoconjugates into the cytosol [C. Rondanino et al., in preparation].

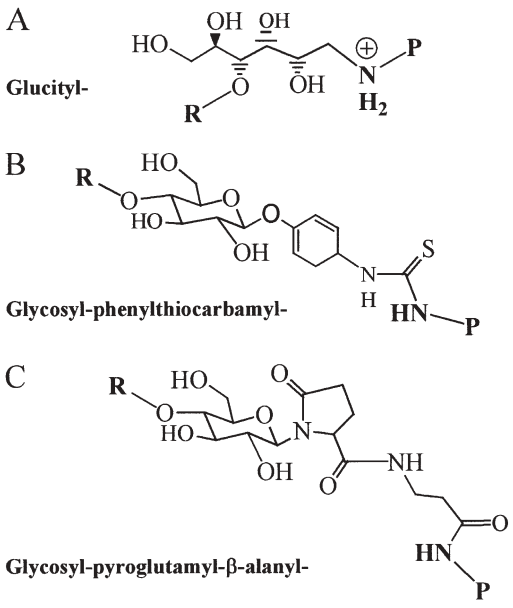

Figure 1. Glycosylated cationic polymers: three types of linkage between the sugar moieties and the cationic polymers. (*A*) A reducing sugar is linked to an amino group of the polymer through a glucitylamine linkage; the cationic charge is still present on the secondary amine. (*B*) A glycosyl-phenyl thiocarbamyl derivative of the cationic polymer is obtained upon reaction of a glycosyl-phenylisothiocyanate; the thiourea is devoid of charge. (*C*) A glycosynthon: glycosyl-pyroglutamyl-β-alanyl moiety is linked through an amide on the cationic polymer; this linkage is devoid of charge. Starting with lactose, R stands for a β-galactopyranosyl residue, NH-P stands for one of the amines of the polymer P. In A, the glucose has been reduced into a pentahydroxy-hexylamine, while in B and C the glucose residue is preserved.

Glycofectins and glycoplexes

Glycofectins, i.e. glycosylated cationic polymers [8, 37] fig. 1) have been developed in order to enhance the selective uptake of DNA by targeting cell surface lectins. In this way we hoped to increase the intracellular trafficking inside the cytoplasm, enhance nuclear import and favor DNA release from the complex. Polycations (such as polylysine, polyethyleneimine, chitosan and so on) interact with polyanions to form complexes. When the polyanion is a DNA, such as a plasmid, the complex is called a polyplex [6] in which the DNA is compacted. The size and structure of a polyplex depend on the size of both the plasmid and the polycation, but also on the absolute and relative concentrations of the plasmid and of the polycation, on the charge density of the polycation, the nature and the concentration of the buffer in which the plasmid and the polycations are dissolved and the presence of serum [38].

Glycosylated polylysines

Neoglycoproteins [9, 39] have been used to target antiviral drugs [40], cytotoxic drugs [41] or macrophage activators [42]. However, because such neoglycoproteins were glycosylated serum bovine or human serum albumin, it was not possible to test such devices in clinics. For this reason, polylysine has been used instead of serum albumin to obtain safe glycoconjugates usable as drug carriers [43, 44], e.g. to target plasmids to hepatocytes [45]. These carriers include asialo-orosomucoid/polylysine conjugate [46], polylysine substituted either with a tetraantennary galactose ligand or galactose or lactose [47], polylysine substituted with lactose [48], polylysine substituted with a tris-GalNAc-peptide [49] as well as polylysine substituted with lactosyl-PEG [50]. Similarly, mannosylated polylysine was used successfully to target genes into macrophages [51, 52]. Glycosylated polylysines were also shown to transfer antisense oligonucleotides into cells in culture (fucosylated polylysine for A549 cells [53] and galactosylated and mannosylated polylysine, respectively, into parenchymal and nonparenchymal liver cells upon intravenous injections of oligonucleotides and glycosylated polylysine complexes. In addition, polylysines substituted with asialo-orosomucoid [54] and galactosylated polylysines were shown to target DNA to the liver upon intravenous injections of mice and rats [55–57].

However, glycosylated polylysines appeared to have a very low efficiency in the absence of a helper agent such as chloroquine or a fusogenic peptide such as E5CA to induce the exit of the complex or plasmid from endosomes [48]. In the presence of such a helper agent, lactosylated polylysine allowed an efficient gene transfer into HepG2 hepatoma cells, which express the Ashwell receptor, but not into HeLa cells, which do not express such a lectin. Unfortunately, chloroquine as well as other small molecular weight helper molecules are not suitable for in vivo applications because it is not possible to reach the required concentration.

The size of polylysines and the number of sugar moieties linked to polylysines were shown to modulate the gene transfer efficiency: best results were obtained by using a relatively small polylysine (about 200 lysine units per molecule: $M_r = 24{,}000$) with about one-third of the amino groups substituted with a sugar unit (33% with lactosyl residues, 40% with galactosyl moieties) [58]. The substitution of the amino groups by either sugar moieties or gluconoyl moieties [59, 60] decreased the strength of the DNA/polylysine interactions. Indeed, by substituting a part of the polylysine amines, the number of cationic charges decreases because the amine is transformed into either a thiourea (lactosyl-phenylthiocarbamyl substituent) or an amide (gluconoyl substituent). The most efficient gluconoylated and glycosylated polylysines were those containing about 50% of the amines substituted. The higher efficiency in gene transfer with partially neutralized polycations is linked to an easier release of the plasmid from the complexes. It was shown that

chloroquine which spontaneously concentrates into acidic endosomal vesicles facilitates plasmid/polycation dissociation [61].

The roles of the sugar moieties borne by glycosylated polylysines include decreasing the strength of plasmid/polycation interactions, as mentioned above; cell surface lectin-mediated uptake of the glycoplexes by cells such as hepatocytes with a galactose-specific lectin [48], or macrophages which express various lectins [51] and sugar-dependent intracellular fates, as discussed below.

Glycosylated polyethyleneimines

PEI, a synthetic polycation, was shown to compact plasmids and to efficiently transfer them to cells both in vitro and in vivo [62]. PEI/DNA complexes have been shown to deliver transgenes to mouse lung upon intravenous injection [63] or aerosol delivery [64]. Glycosylated PEI led to glycofectins with gene transfer efficiencies which depended on the nature of the carbohydrates used and on the linkages between the carbohydrate moieties and PEI. For instance, PEI substituted with triglucosyl α1,4 glucityl units was shown to be up to 1000 times less efficient than sugar-free PEI in transfecting murine hepatocyte cell line BNL-CL2 at an N/P (number of amino groups/number of phosphate) ratio of 5. Similarly, glycoplexes containing PEI substituted with galactosyl-β-dithio-PEG were found to be much less efficient than polyplexes containing PEI itself [65]. Conversely, PEI substituted with 5 galactoses per 100 amines [66] was shown to efficiently transfect hepatocyte-derived cell lines but not fibroblast lines; furthermore, this enhanced efficiency was galactose specific and could be inhibited by an asialo-glycoprotein. Similarly, mannosylated PEI was more efficient than sugar-free PEI in transferring genes into cells expressing a mannose-specific receptor such as dendritic cells [67]; this trend also applied in the case of human carcinoma cells transfected with glycoplexes prepared with glucosylated or galactosylated PEI. In addition, these glycosylated PEI were less cytotoxic [68]. PEI (branched polymer, M_r 25,000) substituted with oligosaccharides: Galα1, 3Galβ1, 4Galα1, 3galactityl or Glcα1, 4Glcα1, 4Glcα1, 6glucityl moieties were found to compact plasmid into small 100-nm particles at an N/P ratio of 5. These particles were shown to efficiently transfect murine hepatoma cells and human HeLa cells: the PEI substituted with oligogalactose moiety being about 10 times more efficient than sugar-free PEI or PEI substituted with oligoglucose in transfecting the murine hepatoma cells. Interestingly, the PEI substituted with either the oligogalactose or the oligoglucose were about 10 times more efficient [69] than PEI itself in transfecting HeLa cells, although these cells seemed not to express cell surface membrane lectins (see the above comments on intracellular lectins).

Gene therapy for cystic fibrosis

When the CFTR gene was cloned [70], gene therapy treatment for cystic fibrosis was thought to be an easy way to care this frequent disease. Several teams started to investigate the feasibility of gene transfer to airway epithelial cells both in vitro and in vivo, including in clinical trials. Reporter genes and the CFTR gene were included into viral vectors as well as nonviral vectors, including lipocations and cationic polymers [4].

Several viral vectors [71] have been developed and used in vitro and in vivo. Viral vectors are so far more efficient than synthetic nonviral vectors, but they are not ready to be used in clinics, because they induce inflammatory responses [72]. Amongst nonviral vectors, liposomes and cationic lipids have been used [73]. Used instead of the more classical lipocations containing long alkyl moieties, cationic cholesterol has been shown to transfer a reporter gene into primary human airway epithelial cells and the mouse airway epithelium in vivo [74]. However, clinically, aerosolized lipoplexes were shown to induce an inflammatory syndrome [75].

Glycofection of the airway epithelial cells

Cystic fibrosis epithelial cells were shown to be efficiently transfected in the presence of 50 or 100 µM chloroquine using a series of glycosylated polylysines, the more efficient one being lactosylated polylysine [76]. Subsequently, Kollen et al. [77] showed that luciferase gene transfer into cystic fibrosis (CF) epithelial cells with glycoplexes containing lactosylated polylysine was even more efficient when 5% glycerol was used, the best conditions being the use of glycoplexes containing lactosylated polylysine in the presence of both glycerol and chloroquine. They also showed that the transfer of the CFTR gene into CF epithelial cells with glycoplexes containing a lactosylated polylysine was efficient in the presence of 100 µM chloroquine and 100 µg/ml E5CA peptide.

Efficient gene expression is not always related to a higher uptake

Glycoplexes prepared with a series of different glycosylated polylysines led to a high luciferase gene expression in rabbit vascular smooth muscle cells with polylysine bearing β-GalNAc or α-Gal residues but not with polylysine substituted with lactose residues [7]. The uptake of glycoplexes by the same cells was maximal with lactosylated polylysine and very poor with polylysine substituted with β-GalNAc or α-Gal residues. Such an apparent discrepancy was also observed in the case of ∑CFTE29o–,

an immortalized human CF tracheal epithelial cell line, and of 16 HBE14o–, an immortalized human non-CF bronchial epithelial cell line [78]. Both cells express on their surface one or more lectins which bind and internalize neoglycoproteins containing α-mannose residues and, to a lower extent, neoglycoproteins containing lactose. Similarly, these cells take up very efficiently glycoplexes prepared with mannosylated polylysine and moderately those prepared with lactosylated polylysine. With both cell lines, glycoplexes containing mannosylated polylysine are dramatically inefficient, while glycoplexes containing lactosylated polylysine were very efficient. Furthermore, glycoplexes containing β-GlcNAc residues were hardly taken up, but they were almost as efficient as glycoplexes made with lactosylated polylysine. Even more surprising, when glycoplexes were prepared with partially glycosylated and gluconoylated polylysine, those prepared with gluconoylated polylysine containing β-GlcNAc were much more efficient than those prepared with lactosylated and gluconoylated polylysine. Glycoplexes prepared with gluconoylated polylysine substituted with β-GalNAc residues were at least as efficient as polyplexes prepared with PEI, Lipofectin or Lipofectamine. Similar results were also observed while using gland serous MM39, a non-CF immortalized human tracheal gland serous cell line, and CF-KM4, a CF cell line [79]. The CF-KM4 line was very efficiently transfected with glycoplexes prepared with glycosylated polylysine containing either α-Glc, α-GalNAc, α-Gal, α-Fuc, β-Lac and β-GlcNAc, but not with polylysine containing α-Man. Similarly, gluconoylated polylysines bearing either β-Lac, β-GlcNAc or α-Fuc led to efficient gene transfer, but not those bearing α-Man [80].

The uptake of glycoplexes containing either lactosylated or mannosylated polylysine by CF-KM4 cells is a receptor-mediated clathrin-dependent endocytosis, as suggested by the very efficient inhibition induced by pretreatment of the cells in the presence of an hypertonic medium (0.45 M sucrose) [81]. The gene expression level depends on the differentiation state of the cells. When CF-KM4 cells were cultured up to 70% confluence, the expression of luciferase gene upon transfection with glycoplexes containing lactosylated polylysine was quite high. Conversely, using the same glycoplexes, gene expression was several orders lower when the transfected cells were fully confluent. The low expression in confluent cells was not related to an impaired uptake, since both neoglycoproteins and glycoplexes were taken up to a similar extent in 70% and fully confluent cells [82]. The differential intracellular traffic of glycoplexes may explain the apparent discrepancy between the uptake efficiency and the gene expression extent. It was found [83] (fig. 2) that glycoplexes containing mannosylated polylysine remained for a larger time in endosomes than glycoplexes containing lactosylated polylysine. Furthermore, a large part of glycoplexes with mannose (42%) was inside lysosomes 24 h upon transfection, while a smaller part of glycoplexes with lactose (20%) was present in lysosomes stained with LAMP-1 antibodies. These results show that the most efficient uptake may not induce the highest gene expression [81, 84]. Knowing that after binding to different membrane lectins, ligands are conveyed in different vesicular compartments, we hypothesized that the intracellular trafficking of glycoplexes may be different according to the sugar signal recognition.

In addition, it was shown that similar amounts of glycoplexes with lactose and with mannose were imported into the nucleus. Nevertheless, the gene carried by glycoplexes with mannose was much less efficiently expressed than that carried by glycoplexes with lactose. This discrepancy could be related to a limited transcription of genes associated with mannosylated polylysine as shown by an in vitro transcription assay [83]. Indeed, the glycoplex obtained by compaction of a plasmid DNA with mannosylated polylysine prevented the initiation of transcription, while the glycoplex made with lactosylated polylysine did not.

Using the glycosylated polylysine synthesized according to Midoux et al. [48], Klink et al. [85] showed that polylysine substituted with both 4-isothiocyanatophenyl β-lactoside and fluorescein isothiocyanate efficiently reached the nucleus. As complexed with a plasmid, the lactosylated polylysine was internalized by CF airway epithelial cells, and reached the perinuclear region but did not enter the nucleus. However, when such lactosylated polylysine/DNA complexes were used in the presence of agents known to enhance transfection efficiency such as chloroquine, fusogenic peptides and glycerol, some complexes reached the nucleus. This behavior was restricted to complexes made of polylysine substituted with lactose. Complexes made of sugar-free polylysine or of polylysine substituted with mannose were not transported into the nucleus. In contrast, the nuclear import of glycoconjugates from the cytosol to the nucleus was shown to be quite efficient when the macromolecules were substituted with glucose or mannose [32, 33], but not at all with lactose.

However, the success of any gene therapy for pulmonary diseases has so far been impaired by several barriers associated with gene delivery in the airways. Those barriers include host inflammatory responses and physical barriers. In addition, various other barriers at the cell level need to be overcome, specially when synthetic nonviral vectors are used. Those include the low uptake of complexes, the low efficiencies in DNA exit from endosomes, DNA release from the complexes, DNA diffusion in the cytoplasm, DNA nuclear import and finally DNA migration in the nucleoplasm as well as promoter downregulation.

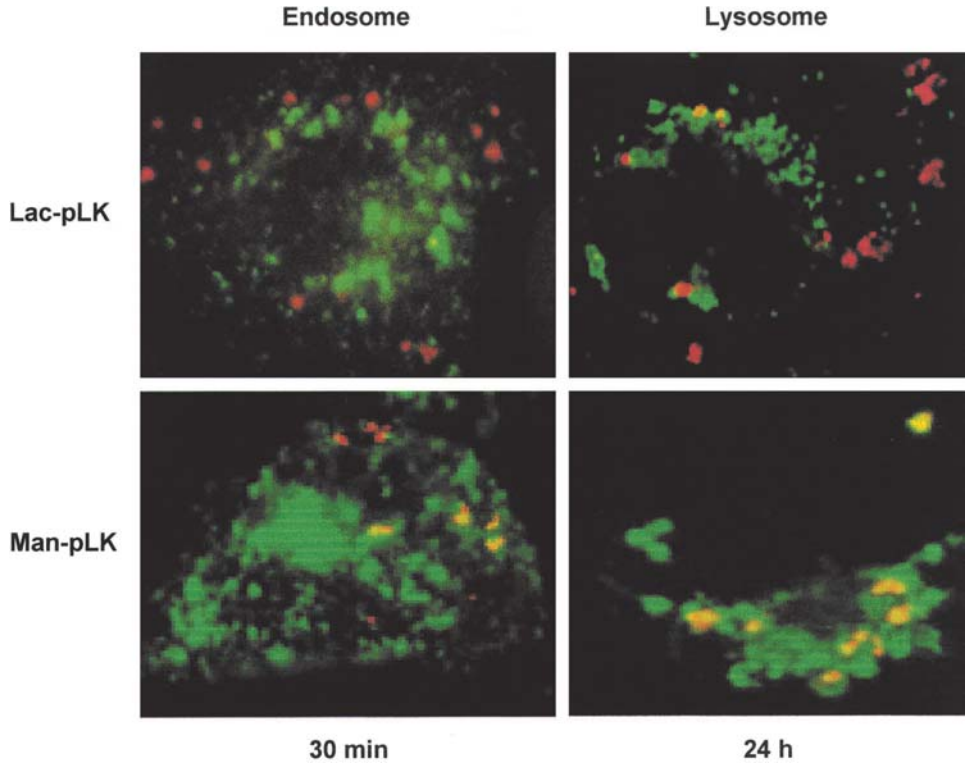

Figure 2. Intracellular trafficking of glycoplexes in immortalized CF airway epithelial cells (∑CFTE29o- cells). Cells were incubated with biotinylated DNA/lactosylated polylysine (Lac-pLK) or mannosylated polylysine (Man-pLK) complexes for 1 h at 4 °C, then washed, incubated at 37 °C for 30 min or 24 h and fixed. Endosomes were immunolabeled with anti-transferrin receptor antibodies and fluorescein-conjugated anti-mouse antibodies. Lysosomes were immuno-labeled with anti-LAMP-1 antibodies, followed by fluorescein-labeled anti-mouse antibodies. Cells were examined by confocal microscopy. Biotinylated DNA, which binds rhodamine-labeled streptavidin, appears red, while cellular organelles appear green. Plasmid DNA/glycosylated polylysine complexes which colocalized with cellular organelles appear in yellow. Bar, 5 µm.

Overcoming physical and chemical barriers

At the level of the lung, the low efficiency of gene therapy is related to poor access of the vectors to epithelial cells through the mucosal surface. This barrier can be partially overcome by using surface active agents such as nonionic surfactants. For example, polidocanol was shown to enhance the in vivo adenoviral gene transfer of LacZ as well as CFTR [86]. Alternatively, the use of Ca^{2+} chelators in order to increase the epithelial junction permeability could help glycoplexes to reach the surface of airway epithelia of the lung as in the case of retrovirus and adenovirus-mediated gene transfers [87]. Biocompatible organic solvents could be used in conjunction with glycoplexes to open the way to the cell surface, as suggested by Schughart et al. [88], who showed that a solvent such as di-*n*-propylsulfoxide itself could be used as a vehicle for intrapulmonary delivery. Extracellular glycosaminoglycans (GAGs), which are linear, negatively charged polysaccharides, interact with the positively charged polyplexes and decrease the gene expression because they change the intracellular distribution of polyplexes without lowering the cellular uptake [89]. In addition, mucolytic agents including recombinant human DNase improved liposome and adenovirus-mediated gene transfer in vitro [90]. A partial degradation of mucus and/or GAG by relevant hydrolases before the introduction of glycoplexes could overcome this low accessibility.

Overcoming cellular barriers

Glycoplexes containing glycosylated polylysine require the presence of an endosomolytic agent such as chloroquine, fusogenic peptide or glycerol, or a combination of those. The presence of such agents is not required when histidylated polylysine is used instead of polylysine to prepare the polymer/DNA complexes [91]. Such polyplexes have been used successfully to transfer genes into immortalized cystic fibrosis airway surface epithelial cells (ΣCFTE29o– cells) and airway gland serous cells (CF-KM4 cells) [92]. Glycosylated PEIs behave as sugar-free PEIs, with similar endosomolytic properties and the ability to efficiently transfer genes into differentiated airway epithelial cells [82]. New glycosylated PEIs obtained

by substitution of PEI with glyco-clusters are under development and could be of interest in applying glycoplexes in vivo.

When a glycoplex enters a cell, it must reach the nucleus to allow expression of the transgene. This is a difficult task, especially in quiescent cells. However, several ways may be used to improve the efficiency of this step. PEI (branched, 25,000 M_r) by itself to some extent facilitates DNA importation into the nucleus [93, 94]. PEI is even more efficient when a linear PEI (22,000 M_r) is used [95]. Recently, Brunner et al. [96] showed that linear PEI was almost 10 times less efficient when HeLa cells were transfected in G1 phase than when they were transfected in the late S phase. The choice of the cationic polymer is therefore a way to improve the nuclear import of DNA.

The use of a nuclear localization signal peptide directly linked to a short (3.3 kbp) DNA also improved nuclear import [97]. A neutral nuclear localization signal (NLS), containing the M9 sequence of heterogeneous nuclear ribonucleoprotein hnRNP-A1, was linked to a cationic peptide scaffold to allow binding to DNA. The conjugate/DNA complex localized into the nucleus of permeabilized cells and enhanced marker gene expression upon lipofection of confluent endothelial cells [98]. Another approach, in development, uses peptide nucleic acids as a DNA-binding device on which NLS can be linked to improve the nuclear import of DNA [99]. GAL4 is inefficient in targeting DNA to the nucleus because its DNA-binding and nuclear targeting activities are mutually exclusive. However, by switching the GAL4 nuclear import pathway to a more conventional one by adding a modified polylysine to which an optimized NLS peptide was linked, Chan and Jans [100] showed that the modified GAL4 significantly enhanced gene transfer thanks to interactions with desired components of the cellular nuclear import machinery. Dean et al. [101] showed that the intracellular nuclear import machinery could directly interact with selected DNA sequences and improve the nuclear translocation of the plasmid. More precisely, the sequence involved in facilitating optimally the transport is the 72-bp repeats of the SV40 enhancer [102]. Other cellular barriers include a limited diffusion of the glycoplexes and of the free DNA in the cytosol as well as in the nucleoplasm. Therefore, molecular devices favoring selective interactions with intracellular proteins involved in energy-dependent motions of macromolecules could lead to a large improvement in the nonviral approaches. Finally, the limited expression of a gene which is successfully imported into the nucleus is linked to several regulatory systems, including downregulation of the imported gene [103]. Improvements will come from engineering the genes and especially by including in the construction genomic regulation sequences and introns.

Conclusions

In conclusion, glycoplexes are improved vectors for gene transfer. They increase selectivity thanks to targeting to cells which express a membrane lectin with a corresponding specificity. They are also involved in intracellular trafficking in the cytoplasm and putatively from the cytosol to the nucleus, and facilitate the release of DNA from the complex, though they are not yet efficient enough in comparison with viral vectors. However, improvements using more efficient ligands, such as the newly developed glycoclusters, and combining other approaches briefly reported above may in the future lead to useful reagents for gene therapy of diseases such as cystic fibrosis.

Acknowledgements. We are grateful to Philippe Bouchard and Philippe Marceau for preparing glycoconjugates, and to Marie-Thérèse Bousser, Yolande Aron and Guiti Thévenot for their skillful help. This research was supported by Vaincre la Mucoviscidose grants and fellowships, the Chancellerie des Universités de Paris and INSERM (CReS 003). A.C.R. is research director at INSERM, N.F. received a fellowship from the Région Centre Council and C.R. received a fellowship from the MENESR.

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