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The reduction of the positive charges of polylysine by partial gluconoylation increases the transfection efficiency of polylysine/DNA complexes

Patrick Erbacher, Annie Claude Roche, Michel Monsigny, Patrick Midoux *

Glycobiologie, Centre de Biophysique Moléculaire, CNRS et Université d'Orléans, 1 rue Charles-Sadron, F-45071 Orléans Cedex 02, France

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Abstract

A polylysine partially substituted with polyhydroxyalkanoyl residues and specially with gluconoyl residues was developed in order to increase the transfection efficiency by decreasing the strength of the electrostatic interactions between the DNA and the cationic polymer. Partially gluconoylated polylysine/DNA complexes were more easily dissociated in solution and their transfection efficiency in the presence of chloroquine, evaluated with HepG2 cells, a human hepatocarcinoma line, was higher when $43 \pm 4\%$ of the ϵ-amino groups of polylysine were blocked with gluconoyl residues. Partially gluconoylated polylysine/plasmid complexes were efficient in transfecting different adherent as well as non-adherent cell lines. Partially gluconoylated polylysine formed highly soluble (above 100 μg/ml in DNA) complexes with DNA plasmids. In addition, partially gluconoylated polylysine bearing few lactosyl residues increased the transfection efficiency of HepG2 cells which express a galactose-specific membrane lectin.

Keywords: Gene therapy; Gene transfer; Polylysine; Chloroquine

1. Introduction

Polylysine conjugated to either proteins, peptides, carbohydrates or metabolites as recognition signals are suitable non-viral vectors when complexed with DNA plasmids to selectively deliver genes into eukaryotic cells (for reviews, see Refs. [1–3]). The DNA in such complexes is highly condensed with polylysine conjugates [4–6] due to strong electrostatic interactions between the negative charges of the DNA and the positive charges of the polylysine conjugates. As previously shown [7], the strength of the electrostatic interactions between DNA and lactosylated polylysine appears to be a limiting factor for a highly efficient gene delivery: the dissociation of the plasmid from lactosylated polylysine and the transfection efficiency of HepG2 cells by lactosylated polylysine/plasmid complexes were higher when one-third of the amino groups of polylysine (DP 190) were substituted with lactosyl residues than with an unsubstituted polylysine or a polylysine substituted with less than one-fourth of lactosyl residues. Thus, the decrease in the strength of the electrostatic interactions between a plasmid and polylysine could improve the transfection efficiency of DNA/polylysine

Abbreviations: DP, degree of polymerization; FBS, fetal bovine serum; GlcA, gluconoyl residue; Lact, lactosyl residue; PBS, phosphate-buffered saline, pH 7.4; pLK, poly-L-lysine.

* Corresponding author. Fax: +33 2 38 690094.

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complexes by increasing the dissociation of the complexes inside the cells in order to allow genes to become available to the nucleus machinery.

In this report, we describe the preparation of a polylysine partially acylated upon treatment with δ-gluconolactone in order to decrease its number of positive charges. We show that a plasmid complexed with a polylysine having 43 ± 4% of its ϵ-amino groups blocked with gluconoyl residues was more easily dissociated in solution and was much more efficient to transfect various cell lines than a plasmid complexed with an unsubstituted polylysine. Moreover, partially gluconoylated polylysine may be additionally substituted with a limited number of small recognition signals such as carbohydrates in order to transfect cells expressing membrane lectins, in a sugar-dependent manner.

2. Materials and methods

2.1. Preparation of gluconoylated polylysines (GlcApLK)

Poly-L-lysine, HBr (pLK, average Mr = 40 000; average degree of polymerization DP = 190) (Bachem Feinchemikalien, Bubendorf, Switzerland) (1 g in 200 ml H_2O) was passed through an anion exchange column (Dowex 2 × 8, $^{-}$OH form, 20–50 mesh) in order to remove bromide ions [8] which are highly cytotoxic [9]. The eluate was neutralized with a 10% *p*-toluene sulfonic acid solution in water and freezed-dried. Poly-L-lysine was partially substituted with gluconoyl residues (GlcA) as described [8]; briefly, δ-gluconolactone (11–35 mg; 61–194 μmol) (Aldrich, Strasbourg, France) was added to polylysine *p*-toluene sulfonate salt (50 mg; 0.86 μmol) in 3 ml dimethylsulfoxide (Aldrich) in the presence of diisopropylethylamine (12–37 μl; 68–205 μmol) (Aldrich) and 1% H_2O and the solution was stirred for 24 h at 20°C. GlcApLK was precipitated by adding 10 volumes of isopropanol and spun down by centrifugation (1800 × *g* for 15 min). The pellet was washed with isopropanol, collected by centrifugation (1800 × *g* for 15 min), solubilized in distilled water and freezed-dried. The average number of GlcA molecules bound per pLK molecule (x) was determined by ^{1}H-NMR spectroscopy at 300 MHz in D_2O

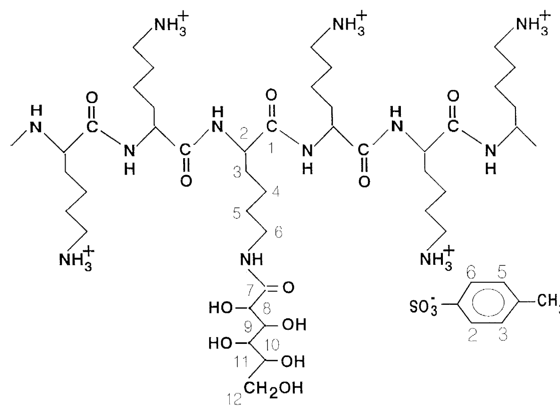

Fig. 1. Structure of partially gluconoylated polylysine.

according to: x = 3/2. (h_{GlcA}/h_{Lys}). DP, where h_{GlcA} was the value of the integration of the signal in the range from 3.6 to 3.9 ppm corresponding to the 4 protons (1H C_{10}, 1H C_{11} and 2H C_{12}) of GlcA (Fig. 1), h_{Lys} that in the range from 1.3 to 1.9 ppm corresponding to the 6 methylene protons (C_3, C_4 and C_5) of lysine residues (Fig. 1) and where DP was the degree of polymerization of pLK.

2.2. Preparation of lactosylated and gluconoylated polylysine (Lact-, GlcApLK)

Polylysine bearing an average number of 30 lactosyl residues per pLK molecule ($Lact_{30}$pLK, 50 mg; 0.75 μmol) prepared as described [7,10] dissolved in 3 ml of dimethylsulfoxide was stirred with δ-gluconolactone ranging from 1.3 to 6.6 mg (7.5–37.5 μmol) in the presence of diisopropylethylamine (22 μl; 150 μmol) and 1% H_2O for 24 h at 20°C. Lact-, GlcApLK was purified by precipitation as described above.

2.3. Cell culture

HepG2 (human hepatoma cells, ATCC HB 8065, ATCC, Rockville, MA) and Rb-1 (rabbit smooth muscle cells, M. Nachtigal, University of South Carolina, Columbia, SC) [11] cells were cultured in DMEM (Gibco, Renfrewshire, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco); HeLa cells (ATCC CCL 2.1) in DMEM with 5% FBS; B16 cells (murine melanoma cells, D. Klatzman, La Pitié Salepetrière, Paris, France) in

DMEM with 10% heat-inactivated newborn calf serum (Gibco); HOS cells (human osteosarcoma cells, ATCC CRL 1543) in MEM (Gibco) with 10% FBS; 3LL cells (Lewis lung carcinoma cells, F. Lavelle, Rhône-Poulenc Rorer, Paris, France) in MEM with Hanks' salts and 20% FBS; HEL (human erythroleukaemia cells, F. Smajda-Joffe, INSERM U268, Villejuif, France), K562 (human erythroleukaemia cells, ATCC CCL 243), HL60 (human promyelocytic cells, E. Le Floch, Rhône-Poulenc Rorer, Paris, France) and Jurkat (human T-cell leukaemia cells, ATCC TIB 152) cells in RPMI 1640 (Gibco) with 10% FBS; THP1 cells (human myelocytic cells, J. Balzarini, Leuven, Belgium) in RPMI 1640 with 10% FBS in the presence of 5×10^{-5} M 2-mercaptoethanol; RBE4 cells (rat brain endothelial cells, P.O. Couraud, Institut Cochin, Paris, France) in collagen-coated culture plates in α-MEM/Ham's F10 (1/1; v:v) (Gibco) with 10% FBS, bFGF (1 ng/ml) and geneticin G418 (Gibco). All culture media were supplemented with 2 mM L-glutamine (Merck, Darmstadt, Germany) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin, Eurobio, France). Cells were mycoplasma-free as evidenced by the bisbenzimidazole (Hoechst 33258) [12] and the BVC-kanamycin A staining methods [13].

2.4. Plasmids

pSV2*LUC* (5 kb) [14] and pCMV*LUC* (6.2 kb) (J.P. De Villartay, Hôpital Necker, Paris, France) were expression plasmids encoding the firefly luciferase gene under the control of the SV40 large T antigen and the human cytomegalovirus promoters, respectively. LPS content (measured with the BioWhittaker QCL-1000 chromogenic *Limulus* assay, BioWhittaker, Verviers, Belgium) [15] was 0.08 and 2 EU/μg DNA for pSV2*LUC* and pCMV*LUC* plasmids, respectively and 0.02 EU/μg for polymers. With this assay, 10 pg/ml LPS (from *Escherichia coli* 0.55: B5; BioWittaker batch 3L0120) gave 0.1 EU/ml.

2.5. Formation of polymer / plasmid complexes

Complexes were prepared by adding, dropwise and with constant mixing, various amounts of either pLK, GlcApLK, LactpLK or Lact-, GlcApLK in 0.3 ml serum-free DMEM to 5 μg (1.5 pmol) plasmid in 0.7 ml of serum-free DMEM. The mixed solution was kept for 30 min at 20°C before use. Complexes made with the lowest polymer to DNA molar ratio leading to a complete retardation of all the DNA in 0.6% agarose gel electrophoresis were used for transfection [7].

2.6. Gene transfer with polymer / plasmid complexes

Adherent cell lines ($2–4 \times 10^5$ cells) were plated (day 0) into 4 cm^2 culture dishes (12-well culture plates). On day 1, the medium was removed and 1 ml of a solution containing a polymer/plasmid complex supplemented with 1% FBS and made 100 μM in chloroquine (Sigma), was added into each well. Non-adherent cell lines in exponential growth phase, were collected by centrifugation ($600 \times g$ for 5 min) and suspended (1×10^6 cells) in 1 ml of a solution containing a polymer/plasmid complex supplemented with 1% FBS and made 100 μM in chloroquine. After 4 h incubation at 37°C in a humidified atmosphere (95% air, 5% CO_2), the medium was removed and cells were further incubated at 37°C in 2 ml of the relevant complete culture medium in a humidified atmosphere (95% air, 5% CO_2). HepG2 cells were also transfected in the presence 10 μM of the fusogenic peptide (E5CA: GLFEAIAEFIEGGWEGLIEGCA) [10] instead of 100 μM chloroquine.

2.7. Transfection with DEAE-dextran

Transfections were performed as described by Rupprecht and Coleman [16]. HepG2 cells (4×10^5) were plated on day 0 into 4 cm^2 culture dishes; the medium was removed on day 1 and cells were incubated for 4 h at 37°C in a humidified atmosphere (95% air, 5% CO_2), in 1 ml of DMEM containing 2% FBS, 250 μg/ml of DEAE-dextran (50 mg/ml stock solution sterilized through a 0.22-μm filter) and 5 μg (1.5 pmol) pSV2*LUC*. Then the medium was removed, cells were washed twice with DMEM and further incubated for 48 h at 37°C in 2 ml of fresh complete DMEM supplemented with 10% FBS in the absence of any other additive in a humidified atmosphere (95% air, 5% CO_2).

2.8. Lipofection

pSV2*LUC* was complexed with lipofectin reagent [17,18] according to the manufacturer's recipe.

pSV2*LUC* (10 μg in 50 μl H_2O) and lipofectin reagent (10, 20 and 30 μg in 50 μl H_2O; DNA to lipofectin weight ratio 1:1, 1:2 and 1:3, respectively) were gently mixed in a polystyrene tube and incubated at 20°C for 15 min. The lipofectin/pSV2*LUC* complexes (0.1 ml) were then added to 2 ml of serum-free DMEM. HepG2 cells (4×10^5) were plated on day 0 into 4-cm^2 culture dishes; the medium was removed on day 1 and cells were incubated for 4 h at 37°C in a humidified atmosphere (95% air, 5% CO_2), with 1 ml of lipofectin/pSV2*LUC* complexes (1.5 pmol DNA) in serum-free DMEM. The medium was then removed and cells were incubated for 48 h at 37°C in 2 ml fresh complete DMEM supplemented with 10% FBS in the absence of any other additive in a humidified atmosphere (95% air, 5% CO_2).

2.9. Luciferase and protein assays

Gene expression was measured by assaying the luciferase-induced luminescence [19] as previously described [7,10]. The luminescence was recorded for 4 s with a luminometer (Lumat LB 9501, Berthold, Wildbach, Germany); RLU/mg protein expressed the light emitted by one mg protein. RLU of 5 pg/ml luciferase was 10 000. Data shown are the mean values from three independent experiments. Protein content was determined for each sample by using the bicinchoninic acid (BCA, Sigma) colorimetric method [20] modified according to Ref. [21] as described [7].

2.10. Analysis of the strength of the polymer/DNA interaction

pSV2*LUC* (5 μg; 1.5 pmol in 0.7 ml of PBS) was complexed by adding, dropwise with constant mixing, either pLK (2.5 μg; 43 pmol), $GlcA_{74}$pLK (7.5 μg; 108 pmol) or $GlcA_{110}$pLK (10 μg; 170 pmol) in 0.3 ml of PBS. The solution was kept for 30 min at 20°C. The effect of the ionic strength on the stability of the complexes was studied by increasing the concentration of NaCl upon adding aliquots of a 4-M stock solution. After 15 min at 20°C, solutions were passed through nitrocellulose filters (0.45 μm), presoaked and rinsed with a solution containing the same concentration of salt. The amount of free DNA was determined from the fluorescence intensities ($\lambda_{exc} = 360$ nm; $\lambda_{em} = 450$ nm) of the filtrates made 2.8 μM in 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) [22]. The percentage of polycation/DNA complex was calculated according to: $[(I_T\text{-}I)/(I_T\text{-}I_0)] \times 100$, where '$I_T$' was the fluorescence intensity of DAPI in the presence of the total amount of DNA, 'I' that of DAPI in the filtrate containing free DNA and 'I_0' that of DAPI in the absence of DNA in solution containing the same concentration of salt as in the sample, in order to take into account the effect of salt on the fluorescence intensity of DAPI in the absence of DNA.

3. Results

3.1. GlcApLK/plasmid complexes

pLK (DP 190) was partially substituted with GlcA (Fig. 1), in order to decrease the number of positive charges born by the polypeptide and consequently to lower the strength of the electrostatic interactions between the polycation and a plasmid DNA. GlcA residue is a polyhydroxyalkanoyl moiety which does not contain any charge but five hydroxyl groups, does not contain any aldehyde or ketone group but a carboxyl group and therefore has no relation with a carbohydrate molecule. In addition, there are no GlcA receptors known on the surface of cells so far.

pSV2*LUC* was complexed with various amounts of GlcApLK containing up to 130 GlcA. The complexes were made with the lowest polymer to DNA molar ratio leading to a complete retardation of all the DNA in electrophoresis through a 0.6% agarose gel. As previously described [7], under these conditions more than 95% of GlcApLK was complexed with the plasmid and this clear solution was used to transfect cells without any further purification.

3.2. Analysis of the strength of the GlcApLK/plasmid interaction

The strength of the interaction between pSV2*LUC* and GlcApLK was investigated in an acellular system in the presence of salts as previously described [7] (Fig. 2). Free DNA passed through a nitrocellulose filter (0.45 μm in diameter) while DNA complexed with GlcApLK as well as GlcApLK were retained on the filter. The dissociation between DNA and

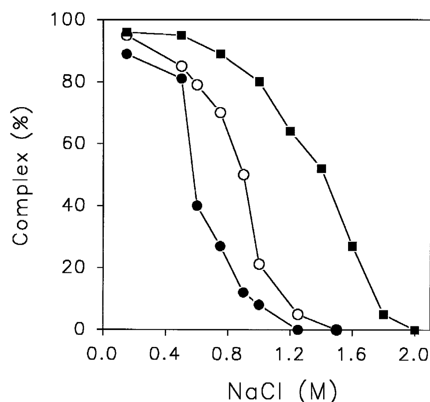

Fig. 2. Analysis of the strength of the polymer/DNA interaction. pSV2*LUC* (1.5 pmol) was complexed with either (■) pLK (2.5 μg; 43 pmol), (○) GlcA$_{74}$pLK (6 μg; 108 pmol) or (●) GlcA$_{110}$pLK (10 μg; 170 pmol) in 1 ml of PBS for 30 min at 20°C. Then, the NaCl concentration was increased up to 2 M by addition of aliquots of a 4 M NaCl solution in PBS. After 15 min at 20°C, each solution was passed through a nitrocellulose filter and the amount of free DNA in filtrates was determined upon adding DAPI by measuring the fluorescence intensity with a spectrofluorometer (λ_{exc} = 360 nm; λ_{em} = 450 nm). The percentage of GlcApLK/DNA complex was calculated as described in Section 2.

GlcApLK depended on the number of GlcA molecules bound per pLK molecule. The NaCl concentration required to dissociate 50% of the complex was 1.5 M, 1.0 M and 0.6 M when pLK was substituted with 0, 74 and 110 GlcA, respectively, showing that the strength of the complexes decreased when the number of positive charges of pLK decreased (Fig. 2).

3.3. Gene transfer into HepG2 cells with GlcApLK/plasmid complexes

The transfection of HepG2 cells by GlcApLK/pSV2*LUC* complexes in the presence of chloroquine was quite efficient and with a maximum when 43 ± 4% of the amino groups of pLK (82 ± 8 GlcA in the case of a pLK with a DP = 190) were substituted with GlcA (Fig. 3A). The luciferase activity was very low when cells were incubated in the presence of chloroquine by using complexes made with pLK substituted with a small number of GlcA (40 or less) or with a large number of GlcA (100 or more) (Fig. 3A). The transfection efficiency by GlcA$_{74}$pLK/pSV2*LUC* complexes depended on the GlcApLK/DNA molar ratio (Fig. 3B). It was more efficient when this molar ratio was between 70 and 170 than below 70 or above 170. These results were expected on the basis of the ratio required to make complexes leading to a complete retardation of all the plasmid in electrophoresis. Indeed, when complexes were made with a GlcApLK/DNA molar ratio: (1) lower than 70, all the plasmid was not completely retarded, the medium contained GlcApLK-free DNA molecules and the transfection was not efficient; (2) between 70 and 170, all the plasmid was completely retarded and the medium contained less than 5%

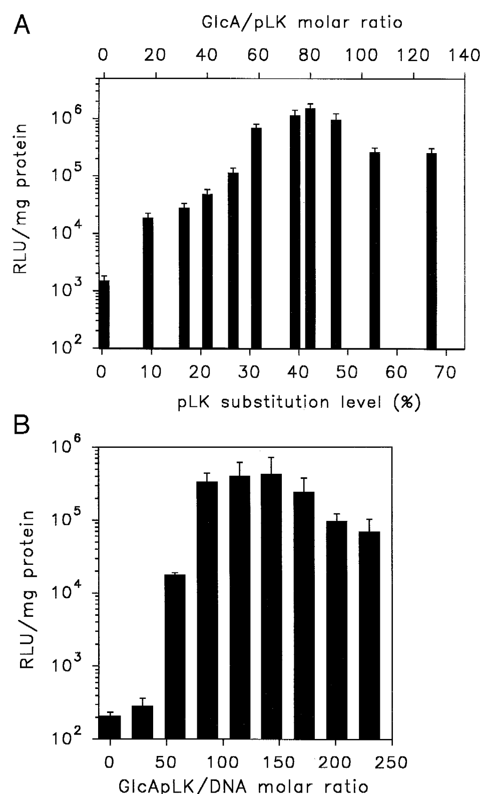

Fig. 3. Gene transfer into HepG2 cells with GlcApLK/pSV2*LUC* complexes. (A) Influence of the number of GlcA molecules bound per pLK molecule (GlcA/pLK). Complexes were formed between pSV2*LUC* (1.5 pmol) in 0.7 ml of DMEM and pLK bearing either 0, 17, 31, 40, 50, 59, 74, 90, 105 or 127 GlcA in 0.3 ml of DMEM. HepG2 cells (4×10^5) were transfected at 37°C for 4 h in 1 ml of DMEM containing a complex, 1% FBS and 100 μM chloroquine. (B) Influence of the polymer to plasmid molar ratio (GlcApLK/DNA). HepG2 cells (4×10^5) were transfected at 37°C for 4 h in 1 ml of DMEM containing 1% FBS, 100 μM chloroquine and pSV2*LUC* (1.5 pmol) complexed with various amounts of GlcA$_{74}$pLK. Gene expression in (A) and (B) was determined 48 h later by assaying the luciferase activity in cell lysates.

DNA-free GlcApLK molecule; and (3) above 170, all the plasmid was completely retarded but the medium contained DNA-free GlcApLK (for instance, 30% at a ratio of 300) which may interact with the cell surface, impairing the uptake of GlcApLK/pSV2*LUC* complexes and inducing toxicity (for instance, with 0.25 μM $GlcA_{74}pLK$, a concentration present in a complex made with a GlcApLK/DNA molar ratio of 300, 10% of HepG2 cells were killed). Upon transfection in the absence of chloroquine, the luciferase activity was very low (RLU/mg $< 10^3$). Alternatively, the luciferase-induced luminescence of HepG2 cells transfected with a $GlcA_{74}pLK$/plasmid complex in the presence of 10 μM E5CA fusogenic peptide [10] was as high as that obtained in the presence of 100 μM chloroquine (data not shown). The transfection efficiency of HepG2 cells with $GlcA_{74}pLK$/pSV2*LUC* complexes was compared with that obtained with two other non-viral methods. The transfection with DEAE-dextran (even after a glycerol shock) was 17-fold lower than that obtained with $GlcA_{74}pLK$/pSV2*LUC* complexes in the presence of chloroquine and the transfection efficiency of $GlcA_{74}pLK$/pSV2*LUC* complexes in the presence of chloroquine was as great as that obtained with the same amount of plasmid complexed with 5 μg lipofectin (data not shown).

3.4. Gene transfer into different cell lines

Plasmids encoding the firefly luciferase gene under the control of either the SV40 large T antigen (pSV2*LUC*) or the human cytomegalovirus (pCMV*LUC*) promoters were used to monitor the transfection efficiency of various cell lines with $GlcA_{74}pLK$/plasmid complexes in the presence of chloroquine (Table 1). Adherent cell lines including those of a rabbit smooth muscle cells (Rb-1), rat brain endothelial cells (RBE4), mouse melanoma cells (B16), human osteosarcoma cells (HOS) or human hepatoma cells (HepG2), were efficiently transfected. Lewis Lung carcinoma cells (3LL) and HeLa cells were transfected with a relatively low efficiency. Non-adherent cell lines including human erythroleukaemia cells (HEL and K562) were also transfected. On the opposite, some non-adherent cell lines such as human myelocytic cells (THP1), human promyelocytic cells (HL60) and human lymphoid cells (Jurkat) were transfected with a very low efficiency. The luciferase activity into each cell line transfected with gluconoyl-free pLK/DNA complexes was always very low. The luciferase gene expression depended both on the promoter used and on the cell line: the luciferase activity in HEL and RBE4 cells was rather low after transfection with pSV2*LUC* but quite high with pCMV*LUC*; HepG2 cells were transfected with a very high activity in both cases.

Table 1
Efficiency of gene transfer with $GlcA_{74}pLK$/plasmid complexes into various cell lines

Cells		RLU/ μg protein			
		pSV2*LUC*		pCMV*LUC*	
		$GlcA_{74}pLK$	pLK	$GlcA_{74}pLK$	pLK
HepG2 [‡]	+ [†]	1540	10	4000	20
HOS [‡]	+	100	1	820	10
HEL [§]	−	20	1	790	10
K562 [§]	−	210	nd	nd	nd
THP1 [§]	−	1	1	1	1
HeLa [‡]	+	50	0.5	nd	nd
HL60 [§]	−	1	nd	nd	nd
Jurkat [§]	−	1	1	nd	nd
3LL [‡]	+	100	5	nd	nd
B16 [‡]	+	910	10	nd	nd
Rb-1 [‡]	+	390	5	nd	nd
RBE4 [§]	+	5	1	260	10

All transfections were performed with 5 μg plasmid complexed with the minimal amount of polymer leading to a complete retardation of the plasmid DNA in agarose gel electrophoresis, i.e., either 6 μg $GlcA_{74}pLK$ or 2.5 μg pLK per assay. [†] +, adherent cells; −, non-adherent cells; the luciferase activity was measured [§] 24 h and [‡] 48 h after the transfection conducted for 4 h in the presence of 100 μM chloroquine. nd, not determined. RLU/ μg protein in non-transfected cells was 0.1.

3.5. Solubility of polymer / plasmid complexes

The solubility of complexes containing pSV2*LUC* and either GlcApLK, pLK, LactpLK or Lact-, GlcApLK was compared by dispersing them in PBS and estimating the turbidity by measuring the absorbance at 610 nm (Fig. 4). pLK and $GlcA_{74}pLK$ formed highly soluble complexes with pSV2*LUC* from 5 μg/ml up to 100 μg/ml (30 nM) DNA. On the opposite, the solubility of the plasmid complexed with LactpLK was low and depended on the number

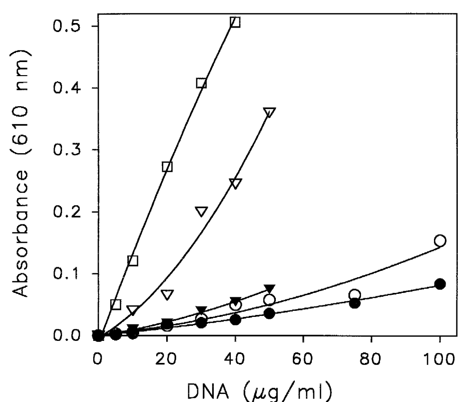

Fig. 4. Concentration-dependent solubility of the polymer/plasmid complexes. Complexes were formed by mixing pSV2*LUC* (1.5–30 pmol; 5–100 μg/ml) with the minimal amount of polymer leading to a complete retardation of all the plasmid in agarose gel electrophoresis, i.e., either (●) pLK (polymer/DNA molar ratio = 28), (○) GlcA$_{74}$-pLK (polymer/DNA molar ratio = 74), (▽) Lact$_{30}$-pLK (polymer/DNA molar ratio = 52), (□) Lact$_{60}$-pLK (polymer/DNA molar ratio = 114) or (▼) Lact$_{30}$, -GlcA$_{50}$-pLK (polymer/DNA molar ratio = 78) in 1 ml of PBS. The solutions were kept for 30 min at 20°C and then their turbidity assessed by measuring the absorbance at 610 nm.

of Lact molecules bound per pLK molecule: the absorbance of the solutions containing a plasmid complexed with lactosylated polylysines (Lact$_{60}$pLK and Lact$_{30}$pLK) increased drastically above 10 μg/ml and 30 μg/ml, respectively. In contrast, the addition of 50 GlcA on a slightly lactosylated polylysine (Lact$_{30}$-, GlcA$_{50}$pLK) increased drastically the solubility of the DNA complex which became as soluble as that formed with GlcA$_{74}$pLK or with pLK.

3.6. Gene transfer with plasmid complexed with a lactosylated and gluconoylated polylysine

HepG2 cells bind and internalize glycoproteins containing β-D-galactosyl residues in a non-reducing terminal position due to the presence of a galactose-specific membrane lectin [23]. We previously showed that HepG2 cells were easily transfected by Lact-pLK/plasmid complexes in a sugar-dependent manner [10] when the polypeptide was substituted with 59 ± 4 Lact residues (when 31 ± 4% of the ϵ-amino groups of the pLK was substituted) [7]. The luciferase activity was high when pLK contained 60 Lact, whereas it was 40-fold lower when pLK contained 30 Lact (Fig. 5). In order to decrease both the number of Lact and the number of positive charges of the lactosylated polylysine, pLK substituted with 30 Lact molecules was partially gluconoylated. The luciferase activity into HepG2 cells transfected with the Lact$_{30}$-, GlcA$_{50}$pLK/pSV2*LUC* complex was 40-fold higher than that obtained with the Lact$_{30}$pLK/pSV2*LUC* complex and was as great as that obtained with the Lact$_{60}$pLK/pSV2*LUC* complex (Fig. 5). A lower substitution of Lact$_{30}$pLK with GlcA residues did not enhance the transfection efficiency: the luciferase activity after a transfection with the Lact$_{30}$-, GlcA$_{30}$pLK/pSV2*LUC* complex was as low as that obtained with the Lact$_{30}$pLK/pSV2*LUC* complex (Fig. 5). Similarly, a higher substitution of Lact$_{30}$pLK with GlcA residues decreased the transfection efficiency: after transfection with the Lact$_{30}$-, GlcA$_{80}$pLK/pSV2*LUC* complex, it was 25-fold less than with the Lact$_{30}$-, GlcA$_{50}$pLK/pSV2*LUC* complex (Fig. 5).

3.7. Charge ratio in polymer / plasmid complexes

Cationic compounds interact strongly and non-specifically with the cell surface and in addition activate the complement system [24]. The global

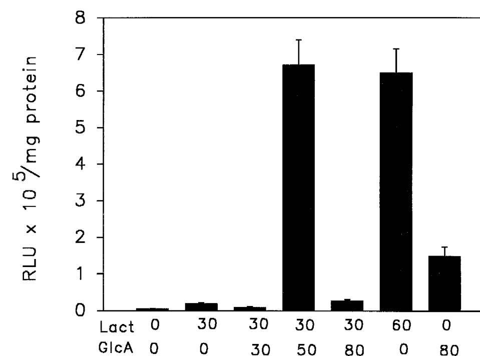

Fig. 5. Gene transfer with lactosylated and gluconoylated polylysine/plasmid complexes. HepG2 cells were transfected at 37°C for 4 h in 1 ml of DMEM containing 1% FBS, 100 μM chloroquine and pSV2*LUC* (1.5 pmol) complexed with the minimal amount of polymer leading to a complete retardation of all the plasmid in agarose gel electrophoresis, i.e., either pLK (2.5 μg; 43 pmol), Lact$_{30}$pLK (5 μg; 78 pmol), Lact$_{30}$-, GlcA$_{30}$pLK (6 μg; 98 pmol), Lact$_{30}$-, GlcA$_{50}$pLK (7.5 μg; 117 pmol), Lact$_{30}$-, GlcA$_{80}$pLK (10 μg; 156 pmol), Lact$_{60}$pLK (12.5 μg; 170 pmol) or GlcA$_{74}$pLK (6 μg; 108 pmol). Gene expression was determined 48 h later by assaying the luciferase activity in cell lysates. Lact and GlcA were the number of lactosyl and gluconoyl residues per polylysine molecule, respectively.

charge of polymer/plasmid complexes was calculated from the ratio between the average number of positive charges (free ϵ-amino groups of polymer) per negative charges (phosphate groups of a nucleotide residue) in complexes made with the lowest polymer to DNA molar ratio leading to the complete retardation of all the DNA in electrophoresis (Fig. 6). For this calculation, it was assumed that 100% of the free ϵ-amino groups were protonated and that 100% of the phosphodiester groups were charged. GlcApLK/pSV2*LUC* complexes had an excess of negative charges that weakly depended on the number of gluconoyl residues bound per pLK molecule: the NH_3^+/nucleotide ratio was 0.54 with pLK and 0.8 with $GlcA_{74}$pLK. In contrast, LactpLK/pSV2*LUC* complexes had an excess of positive charges that increased when the number of lactosyl residues bound per pLK molecule increased: the NH_3^+/nucleotide ratio was 0.75, 1.6 and 2.8 with $Lact_{30}$pLK, $Lact_{60}$pLK and $Lact_{80}$pLK, respectively. The addition of 50 GlcA residues on a slightly lactosylated polylysine ($Lact_{30}$-, $GlcA_{50}$pLK) allowed the formation of lactosylated polymer/DNA complexes containing an average number of positive charges close to that of negative charges.

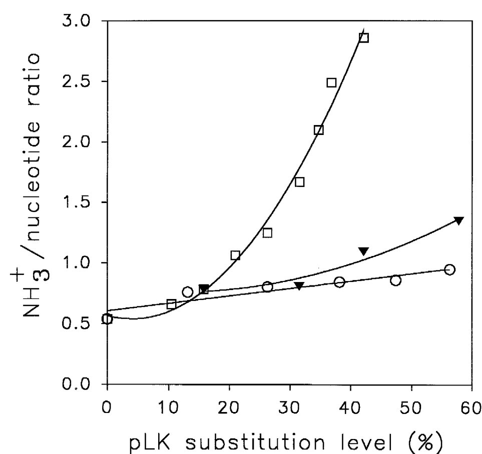

Fig. 6. Charge ratio in polymer/plasmid complexes. Influence of the polylysine substitution level on the NH_3^+/nucleotide ratio in complexes between pSV2*LUC* and either (○) $GlcA_x$pLK ($0 < x < 105$), (□) $Lact_x$pLK ($0 < x < 76$) or (▼) $Lact_{30}GlcA_x$pLK ($0 < x < 80$). The NH_3^+/nucleotide ratio is the ratio of the average number of free ϵ-amino groups (assuming that 100% of them are protonated) and of phosphate groups (assuming that 100% of them are charged) in polymer/DNA complexes made with the lowest polymer to DNA molar ratio leading to the complete retardation of all the DNA in electrophoresis.

4. Discussion

The transfection efficiency of mammalian cells with polylysine/plasmid DNA complexes which is generally low, became more efficient when about one-third of the ϵ-amino groups of the polypeptide was blocked by acylation with δ-gluconolactone. Polylysines (DP 190) partially substituted with gluconoyl residues form complexes with plasmids in which the electrostatic interactions are reduced. In solution, the dissociation of the plasmid from pLK substituted with 74 or 110 GlcA molecules was easier than the dissociation of the plasmid from pLK, i.e., required a lower ionic strength (Fig. 2). The GlcApLK/DNA complexes were highly efficient in transfecting HepG2 cells when pLK was substituted with 81 ± 7 GlcA residues (when 43 ± 4% of the ϵ-amino groups were substituted) (Fig. 3A); the transfection efficiently reached a plateau when GlcApLK/plasmid complexes were formed with the lowest polymer to plasmid molar ratio giving a complete retardation of all the DNA in electrophoresis (Fig. 3B). Under these conditions (1) GlcApLK was totally associated with the plasmid and no further purification was required to remove free GlcApLK since the concentration of GlcApLK remaining free was negligible; (2) GlcApLK/DNA complexes were anionic (Fig. 6) and (3) the luciferase activity was as great as that obtained with cationic complexes containing an excess of GlcApLK (Fig. 3B). Because GlcApLK/DNA complexes enter the cells through an endocytic pathway, transfections must be performed in the presence of either chloroquine or a fusogenic peptide to help the DNA to cross intracellular membranes and to reach the cytosol. Chloroquine prevents the delivery of the endosomal content to lysosomes [25], induces the formation of large vacuoles [26] and in addition allows the dissociation of polylysine/plasmid complexes as shown previously [27]. A fusogenic peptide, able to disrupt the membrane of acid vesicles, is known to help the DNA to reach the cytosol [10,28,29]. When compared to other transfection methods, transfection of HepG2 cells with the same amount of plasmid complexed with DEAE-dextran was very low; transfection with lipofectin reagent was as efficient as with $GlcA_{74}$pLK/plasmid complex in the presence of chloroquine.

We showed that a $Lact_{30}$pLK/pSV2*LUC* complex, which was much less efficient than a $Lact_{60}$pLK/pSV2*LUC* complex in transfecting HepG2 cells [7], became highly efficient after addition of 50 GlcA residues in order to reduce the number of remaining positive charges on the polycation (Fig. 5). Therefore, partially gluconoylated polylysine containing reduced number of positive charges can serve as a polycation to link a small number of molecules acting as recognition signals such as carbohydrates in order to target gene into cells expressing specific membrane lectins. The advantages of using a partially gluconoylated polylysine bearing a small number of sugar residues over polylysine bearing a great number of sugar residues, are that gluconoylated and glycosylated polylysine/plasmid complexes (1) are highly soluble (above 100 μg/ml in DNA) (Fig. 4) which is of great interest for in vivo applications; (2) are not cationic (Fig. 6) and (3) form small particles (25–50 nm in diameter), the size of which are close to those obtained with sugar-free gluconoylated polylysine (Delain et al., in preparation). In addition, the reduction of the number of sugar residues required on gluconoylated polylysine for efficient and specific delivery will be much less expensive, especially when complex oligosaccharides are used.

Partially gluconoylated polylysine and those bearing a small number of sugar moieties acting as recognition signals are synthetic compounds, rapidly prepared and easily purified; it was shown that gluconoylated and glycosylated polylysine is not immunogenic [1] and is totally biodegradable [8]. Recently, synthetic cationic molecules, including polylysine, involved in non-viral gene delivery systems and their complexes with DNA were shown to induce a strong activation of the complement system which decreased when the charge ratio of complexes decreased [24]. Therefore, DNA complexes made with gluconoylated polylysine and gluconoylated polylysine bearing a small number of sugar moieties which are not cationic (Fig. 6) should be inefficient in activating the complement.

$GlcA_{74}$pLK/plasmid complexes were suitable to transfect different cell lines including adherent and non-adherent cells (Table 1). The transfection efficiency depends on the cell lines and is also related to (1) the nature of the promoter which controls the gene expression; (2) the efficiency of the uptake of the complexes by a receptor-independent endocytosis – the gluconoyl molecule is not known to be recognized by cell surface receptors; (3) the intracellular traffic of the complexes after internalization and (4) the efficiency of the transmembrane passage of the plasmid into the cytosol in relation to the intracellular concentration of chloroquine or to the membrane-disrupting efficiency of a fusogenic peptide. In conclusion, partially gluconoylated polylysine, and those bearing a small number of sugar moieties acting as recognition signals specific for membrane lectins, are good non-viral vehicles for gene transfer into several eukaryotic cells in a receptor-independent and in a receptor-dependent manner, respectively.

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