Specific gene transfer mediated by lactosylated poly-L-lysine into hepatoma cells

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Received November 23, 1992; Revised and Accepted January 19, 1993

ABSTRACT

Plasmid DNA/glycosylated polylysine complexes were used to transfer *in vitro* a luciferase reporter gene into human hepatoma cells by a receptor-mediated endocytosis process. HepG2 cells which express a galactose specific membrane lectin were efficiently and selectively transfected with pSV2Luc/lactosylated polylysine complexes in a sugar dependent manner: i) HepG2 cells which do not express membrane lectin specific for mannose were quite poorly transfected with pSV2Luc/mannosylated polylysine complexes, ii) HeLa cells which do not express membrane lectin specific for galactose were not transfected with pSV2Luc/lactosylated polylysine complexes. The transfection efficiency of HepG2 cells with pSV2Luc/lactosylated polylysine complexes was greatly enhanced either in the presence of chloroquine or in the presence of a fusogenic peptide. A 22-residue peptide derived from the influenza virus hemagglutinin HA2 N-terminal polypeptide that mimics the fusogenic activity of the virus, was selected. In the presence of the fusogenic peptide, the luciferase activity in HepG2 cells was 10 fold larger than that of cells transfected with pSV2Luc/lactosylated polylysine complexes in the presence of chloroquine.

INTRODUCTION

The introduction of a foreign gene in a cell is of great interest both for academic purposes and for gene therapy. While in *in vitro* experiments, general methods using calcium phosphate precipitation, DEAE dextran, or cationic lipids are suitable, more selective methods are required to specifically transfer a gene in a given cell population with the aim of developing gene therapy. Amongst these selective methods, gene transfer may be achieved by making use either of modified virus material starting with vaccinia virus or retrovirus, or of targeted liposomes or of targeted macromolecule gene complexes. DNA/carrier complexes such as asialoorosomucoid, insulin or transferrin substituted with polylysine have been proposed as targeted carriers of plasmid allowing cell transfection upon an endocytotic process induced by the corresponding receptors: the galactose specific receptor with asialoorosomucoid (1–6), the insulin receptor (7) and the

transferrin receptor (8–16). Membrane lectins (sugar specific receptors) have been evidenced at the surface of many normal cells (including hepatocytes, monocytes, macrophages, endothelial cells, lymphocytes, etc...) and tumor cells (for reviews see: 17–19); the specificity of these lectins depends on the cell type, and therefore membrane lectins are good candidates for gene transfer by DNA/glycoconjugate complexes as specific carriers. Soluble glycoconjugates bearing defined sugar moieties have been used to efficiently target drugs including cytotoxic drugs, toxins, immunomodulators, antiviral drugs (for reviews see: 18,20) and oligonucleotides (21,22); such soluble glycoconjugates could be efficient also in targeting genes.

In this report, we show that a plasmid/glycosylated polylysine complex allows a very efficient cell selective transfection. Poly-L-lysine partially substituted with lactose (Gal β Glc) residues, as recognition signal specific for cell surface lectin of hepatoma cells, gave a soluble DNA/polymer complex upon mixing with a plasmid. The plasmid (pSV2Luc) used contains the luciferase gene under the control of the early SV40T promoter. HepG2 cells were selectively transfected by pSV2Luc/lactosylated polylysine complex in a sugar dependent manner; however, the transfection efficiency was highly improved when the incubation was conducted either in the presence of chloroquine or of a fusogenic peptide. Indeed, the transmembrane passage of plasmid DNA is a critical process for its delivery into the cytosol and/or the nucleus where the gene will be expressed. Usually, endocytosed macromolecules are routed intracellularly *via* endocytotic vesicles derived from the plasma membrane to endosomes and to lysosomes, where they are degraded (23–25). To avoid hydrolytic degradation and then to increase the efficiency of the plasmid transfer to the cytosol, it is worthwhile to slow down the hydrolytic process and/or the translocation to the lysosomes either by using amphipathic amines such as chloroquine or by using membrane destabilizing agents such as amphipathic lipids or fusogenic peptides. Accordingly, the transfection efficiency obtained by using DNA/transferrin polylysine complexes (8–12) was much higher in the presence of chloroquine, a weak base which neutralizes the acidic pH of endocytotic vesicles, inhibiting hydrolases contained in endosomes and lysosomes and then decreasing the degradation of endocytosed macromolecules. Another approach should be used the efficient strategy developed by enveloped viruses.

Paramyxoviruses and orthomyxoviruses possess, indeed envelope glycoproteins which direct a fusion between the viral and the host plasma membranes, thereby allowing the transfer of the viral genome to the cytosol (26–28). In the case of adenovirus upon endocytosis, the membrane of intracellular vesicles containing the virus are destabilized and their genome material is delivered into the cytosol (29). On these bases, defective adenovirus particules were conjugated to DNA/transferrin polylysine complexes with the aim of increasing the gene transfer into hematopoietic and hepatic cells (13–16). Fusogenic peptides contained in the viral fusogenic glycoprotein have been characterized and their interactions with lipid bilayers have been studied. In particular, the N-terminal fusogenic peptide of influenza virus hemagglutinin HA2 was found to induce fusion and leakage of lipid bilayers at pH between 6.0 and 5.0 which are the pH of the lumen of the endocytotic vesicles (30–31). We show that a peptide derived from that previously shown to mimic the fusogenic activity of the N-terminal peptide of the influenza hemagglutinin HA2 (32–35) increases dramatically the transfection efficiency of human hepatoma cells by a plasmid carried by a lactosylated polylysine conjugate and that this fusogenic peptide is even more efficient than chloroquine.

MATERIAL AND METHODS

Chemicals

Luciferin, chloroquine, bovine serum albumin fraction V, Triton X 100 and 1,4-diazobicyclo[2.2.2]octane (DABCO) were purchased from Sigma (St. Louis, MO); L-glutamine, dimethylsulfoxide (Me₂SO), ATP, glycerol and MgCl₂ from Merck (Darmstadt, Germany); dithiothreitol from Serva (Heidelberg, Germany); diisopropylethylamine, *p*-toluene sulfonic acid, EDTA from Aldrich (Strasbourg, France); Dowex 2×8, 20–50 mesh from Bio-Rad (Richmond, CA). 4-isothiocyanatophenyl-β-D-lactoside (Galβ4Glcβ-O-C₆H₄-NCS), 4-isothiocyanatophenyl-α-D-mannopyranoside (Manα-O-C₆H₄-NCS) and fluoresceinylated neoglycoproteins were prepared as previously described (36,37); Poly-L-lysine, HBr (Mr 30 000–50 000) containing about 190 lysine residues was from Bachem Feinchemikalien (Bubendorf, Switzerland).

Preparation of glycosylated poly-L-lysine

Poly-L-lysine, HBr (1 g in 200 ml H₂O) was passed through an anion exchange column (Dowex 2×8, OH[−] form, 20–50 mesh, 35×2.5 cm) in order to remove bromide (38) which is highly cytotoxic for cells (39). The effluent solution was neutralized with 10% *p*-toluene sulfonic acid in water (a non cytotoxic compound) and freeze-dried. 4-Isothiocyanatophenyl-β-D-lactoside (40 mg; 81 μmol) or 4-isothiocyanatophenyl-α-D-mannopyranoside (22 mg; 70 μmol) was added to *p*-toluene sulfonate poly-L-lysine (66 mg; 1.25 μmol) in 2 ml Me₂SO in the presence of diisopropylethylamine (36 μl; 0.25 mmol) and reacted for 24 h at 20°C. The polymer was precipitated by adding 10 volumes of isopropanol and spun down by centrifugation (1800 g for 15 min). The pellets were washed with isopropanol, collected by centrifugation (1800 g for 15 min), solubilized in distilled water and freeze dried to give 89 mg of lactosylated poly-L-lysine (LacpLK) and 61 mg of mannosylated poly-L-lysine (ManpLK). The average number of lactose and mannose residues bound per poly-L-lysine molecule, calculated from the sugar content determined by using the resorcinol sulfuric acid micromethod (40), was found to be 68 and 50, respectively.

Molecular weights of LactpLK, of ManpLK and of pLK were estimated to be 76 000, 61 000 and 52 800, respectively.

Preparation of fluoresceinylated lactosylated poly-L-lysine (FTC-LactpLK)

Lactosylated poly-L-lysine (10 mg; 0.17 μmol) was dissolved in 1 ml Me₂SO containing diisopropylethylamine (5 μl; 8.5 μmol) and substituted with FITC (isomer I, Molecular Probes, La Jolla, CA) (0.5 mg; 1.3 μmol) for 1 h at 20°C. The fluoresceinylated polymer was purified as described above.

Peptide synthesis

The peptide I (GLFEAIAEFIEGGWEGLEGCA) was synthesized by the Boc methodology using [[(Bocalanil-phenylacetamido)methyl]amino] polystyrene (Neosystem, Strasbourg, France) as matrix. Peptide coupling was carried out with benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate reagent (BOP) (41) and monitored with the ninhydrine test (42). Peptide I was cleaved from matrix and deprotected with the low-high HF method in the presence of *p*-cresol at 0°C for 3 h (43). The peptide was washed with diethylether. The peptide was purified by HPLC on a reverse phase C18 column (Vydac, Hesperia, CA) using water/13 mM ammonium acetate and -acetonitrile/13 mM ammonium acetate (75/25, v/v) as solvent. Amino acid composition was determined by amino acid analysis with a Waters 991 HPLC system using the phenylisothiocyanate pre derivatization method and was found to be: E = 5.2; G = 5.0; A = 2.8; I = 2.9; L = 2.4; F = 1.8.

Peptide II (nLKTEEYVQTSRESA) and peptide III (YnLGLVRGEKARKGSSSA) were synthesized as described above for peptide I.

Cells and cell culture

Human hepatoma cell line HepG2 (44), kindly given by Dr François Levrat (Hopital Cochin, Paris, France) and human cervix epitheloid carcinoma HeLa 229 cells (ATCC CCL 2.1, ATCC, Rockville, MA) were cultured in complete DMEM medium: DMEM (GIBCO, Reufrewshire, U.K.) plus 5% heat inactivated fetal bovine serum (GIBCO) plus 2 mM L-glutamine (Merck) plus antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) (Eurobio, Paris, France).

Plasmid DNA

Gene transfer was monitored with pSV2Luc, a 5.0 kb plasmid in which the firefly luciferase reporter gene is under control of the SV40 early region promoter (45), kindly given by Dr A.B. Brasier (Massachusetts General Hospital, Boston).

Formation of plasmid/glycoconjugate complexes

In a typical experiment, the complex was prepared by adding, dropwise with constant mixing, 50 μg glycosylated or unglycosylated poly-L-lysine in 0.6 ml DMEM to 20 μg pSV2Luc in 20 μl of 1 mM EDTA, 10 mM Tris buffer, pH 7.4 diluted in 1.4 ml DMEM. The solution was kept for 30 min at 20°C.

Gene transfer

5×10⁵ cells per well were plated on day 0 into a 12-well tissue culture plates. On day 1, after removing the medium, the solution (2 ml) containing the plasmid/glycoconjugate complex supplemented with 1% heat inactivated fetal bovine serum, and made either 100 μM in chloroquine or 10 μM in fusogenic peptide

(determined by using $\epsilon_{280\text{ nm}}^{\text{Trp}} = 5500 \text{ M} \times 1^{-1} \times \text{cm}^{-1}$), was added into a well. After 4 h incubation at 37°C, the supernatant was removed and 2 ml of fresh complete DMEM medium was added and cells were further incubated for 48 h at 37°C. Transfections were also performed by the classical DEAE dextran method: 2.5 μg pSV2Luc plasmid was complexed with 250 μg DEAE dextran in 1 ml DMEM and added at 37°C into a well. One hour later, the cells were washed and further incubated for 48h at 37°C.

Luciferase assay

Luciferase gene expression was measured by luminescence according to (46). The culture medium was discarded and cells were harvested upon incubation at 37°C in PBS containing 0.2 mg/ml EDTA and 2.5 $\mu\text{g}/\text{ml}$ trypsin (GIBCO) and washed three times with PBS. The homogenization buffer (200 μl ; 8 mM MgCl_2 , 1 mM dithiothreitol, 1 mM EDTA, 1% Triton X 100, 10 mg/ml bovine serum albumin and 15% glycerol, 25 mM Tris-phosphate buffer, pH 7.8) was added onto the pellet. The suspension was shaken with a vortex and kept for 10 minutes at 20°C. The solution was spun down (5 min 800 g). ATP (95 μl of a 2 mM solution in the homogenization buffer without Triton X 100) was added to 60 μl supernatant and the luminescence was recorded for 4 seconds by using a luminometer (Lumat LB 9501, Berthold, Wildbach, Germany) upon automatical addition of 150 μl of a 167 μM luciferin in water; measurements were done in triplicate.

Flow cytometry analysis

The presence of membrane lectins at the surface of HepG2 and HeLa cells and their efficiency in internalizing their ligands were tested by flow cytometry. The adhered cells were incubated at 37°C for 2 h with 25 $\mu\text{g}/\text{ml}$ of fluoresceinylated neoglycoproteins in DMEM containing 10 mg/ml BSA, washed and suspended in sheath fluid (134 mM NaCl, 3.75 mM KCl, 15.24 mM NaF, 1.9 mM KH_2PO_4 , 16.53 mM Na_2HPO_4 , 0.2% 2-phenoxy-ethanol pH 7.4). The cell fluorescence intensity was measured using a FACS Analyzer (Becton Dickinson, Sunnyvale, CA, USA) equipped with the FACSlite unit (Becton Dickinson). A 488 nm excitation wavelength was produced by a 25 mW cold air argon laser. The cell fluorescence intensity was measured before and after a post incubation at 4°C with 50 μM monensin in order to estimate the quantity of fluorescent material that has been internalized as previously described (37,47).

Fluorescence microscopy

Confocal laser scanning microscopy was performed with a Bio-Rad instrument (MRC 600, Bio-Rad, Oxfordshire, UK) equipped with an air-cooled krypton/argon laser on a Nikon Optiphot epifluorescence microscope (Nikon, Tokyo, Japan). An objective with a 60 fold magnification and a 1.4 numerical aperture was used to detect the fluorescence emission from the fluoresceinyl-labeled polymers. Specimens were excited with the 488 nm laser beam.

RESULTS

Uptake of fluorescein-labeled neoglycoproteins by HepG2 cells

In order to demonstrate that the gene targeting by glycosylated poly-L-lysine is sugar specific, we looked for the presence of various membrane lectins on the cell lines used (HepG2 and HeLa cells) in these experiments. Indeed, while liver parenchymal cells

express a galactose specific lectin on their surface (48), many hepatoma cells do not. Furthermore, upon passaging, cell lines may stop expressing a given protein. Schwartz *et al* (49) showed that one hepatoma cell line, called HepG2 expresses a galactose-specific lectin. The presence of membrane lectins on HepG2 and on HeLa cells was checked by flow cytometry with a series of fluorescein-labeled bovine serum albumin substituted with different sugars (so called neoglycoproteins) (37). Serum albumin bearing galactosyl or lactosyl residues were recognized and internalized by HepG2 cells but not by HeLa cells (data not shown). The internalization of the galactosylated and lactosylated serum albumin was ascertained by showing that the cell fluorescence intensity increased upon a post incubation at 4°C in the presence of monensin (37,47) which releases the fluorescence quenching of fluorescein by neutralizing all cellular acidic compartments (50). Neoglycoproteins bearing other sugars, including mannose residues, are neither associated with nor internalized by these cells. On these bases, sugar-selective targeting is expected to be efficient with lactosylated polymer in the case of HepG2 cells. Negative controls will include mannosylated polymer with HepG2 cells and lactosylated polymer with HeLa cells, and sugar free polylysine with both cells.

Specificity of pSV2Luc gene transfer in HepG2 cells by using lactosylated poly-L-lysine as a carrier

An expression plasmid pSV2Luc encoding the firefly luciferase gene was used as a reporter gene to monitor the efficiency of gene transfer and expression when using poly-L-lysine and its glycosylated derivatives as carriers. The complexes were prepared by mixing 20 μg (3nM) pSV2Luc plasmid and 50 μg (330 nM) LactpLK, ManpLK or unglycosylated pLK in 2 ml DMEM (polymer to DNA molar ratio close to 110 : 1). Cells

Figure 1. Carrier dependent gene transfer. HepG2 cells (5×10^5 cells per well) were incubated at 37°C in the presence of 100 μM chloroquine and of 10 $\mu\text{g}/\text{ml}$ (3 nM) pSV2Luc plasmid or complexes formed with 10 $\mu\text{g}/\text{ml}$ (3 nM) pSV2Luc plasmid and 25 $\mu\text{g}/\text{ml}$ (330 nM) of either LactpLK or ManpLK or unglycosylated pLK. After a 4 h incubation, the medium was removed and the cells were further incubated in complete culture medium in the absence of chloroquine for 48 h. Gene expression was determined by assaying the luciferase activity of three aliquots of cell lysates. The values of relative light units (RLU) shown represent the activity of 10^6 cells.

Figure 2. Concentration dependent gene transfer by using various concentration of the two components. A) Complexes with 25 µg/ml (330 nM) LactpLK and with either 0.1, 0.5, 1, 5 or 10 µg/ml pSV2Luc plasmid were formed in DMEM. B) Complexes with 10 µg/ml (3 nM) pSV2Luc plasmid and 6.25, 12.5, 25 or 50 µg/ml LactpLK were formed in DMEM. HepG2 cells (5×10^5 cells per well) were incubated at 37°C for 4 h in the presence of the indicated concentration of plasmid and of LactpLK and in the presence of 100 µM chloroquine for 4 h. The medium was removed and the cells were further incubated in complete culture medium in the absence of chloroquine for 48 h. The gene expression was determined by assaying the luciferase activity of three aliquots of cell lysate. The values of relative light units (RLU) shown represent the activity of 10^6 cells.

were incubated at 37°C with the plasmid/polymer complex in the presence of 100 µM chloroquine. Chloroquine, a cell permeant base, was used to partially neutralize acidic cell compartments and prevent the fusion of endosomes with lysosomes. After 4 h incubation, cells were washed and further incubated at 37°C in chloroquine-free and plasmid-free culture medium. The efficiency of the gene transfer into HepG2 cells was evaluated 48 h later by measuring the luciferase activity in the cell lysate (Figure 1). In the presence of chloroquine, the luciferase gene expression is much higher ($RLU = 6 \times 10^5$) than that obtained in the absence of chloroquine (Figure 1) ($RLU = 2.2 \times 10^3$). A maximum luciferase activity was obtained with cells incubated for 4 hours in the presence of pSV2Luc/LactpLK complexes, when chloroquine was added either at the same time or 1 h later; when chloroquine was added later the luciferase activity was much lower showing weaker transfection efficiency. When chloroquine was left over a longer incubation period, no further increase in luciferase activity was detected. Plasmid/polymer complexes present during the first 4 h incubation without or with 100 µM chloroquine present for up to 4 h, did not induce any cytotoxic effect nor cell detachment, the number of cells present in a well was identical with that of mock treated cells.

The luciferase expression was at a similar level when cells were transfected with the pSV2Luc complexed with ManpLK ($RLU: 1.85 \times 10^4$) and with pLK ($RLU: 1 \times 10^4$). Therefore, with pSV2Luc carried by LactpLK, the luciferase expression based on emitted light, was 60 fold and 33 fold larger than in the cases of DNA complexed with pLK and with ManpLK, respectively, showing that the galactose residues present on LactpLK specifically enhance the gene transfer into HepG2 cells. When the same plasmid carrier preparations were used with HeLa cells (that do not express the galactose specific lectin) the luciferase activity measured ($RLU = 1.1 \times 10^4$) was as low (insert Figure 1) as by using pLK plasmid complex with HepG2 cells,

Figure 3. Concentration dependent gene transfer by using various concentration pSV2Luc/LactpLK complex. HepG2 cells (5×10^5 cells per well) were incubated at 37°C for 4 h with various dilutions of a pSV2Luc/LactpLK complex (3 nM DNA and 330 nM polymer) in the presence of 100 µM chloroquine. Then, the medium was removed and the cells were further incubated in complete culture medium in the absence of chloroquine for 48 h. The gene expression was determined by assaying the luciferase activity of three aliquots of cell extracts. The values of relative light units (RLU) shown represent the activity of 10^6 cells.

confirming that the high activity obtained with HepG2 cells was related to the presence of lactose on the carrier. Comparatively, the luciferase activity measured upon transfection of pSV2Luc by the classical DEAE dextran method was quite low ($RLU = 960$) even in the presence of chloroquine ($RLU = 3260$).

Optimal conditions for transfection of HepG2 cells by pSV2Luc/LactpLK complexes

The luciferase gene expression measured 24 h, 48 h and 72 h after transfection with pSV2Luc/LactpLK complex (polymer to DNA molar ratio, 110:1) for 4 h in the presence of chloroquine, was found to be maximal after 48 h (data not shown).

Various ratios of LactpLK and pSV2Luc plasmid were tested in order to determine the optimal conditions for DNA/polymer complex formation and for gene delivery. Different complexes were made either with a constant amount of LactpLK (25 µg/ml) and an increasing amount of pSV2Luc plasmid (0.1–10 µg/ml) (Figure 2A) or with a constant amount of pSV2Luc plasmid (10 µg/ml) and an increasing amount of LactpLK (6.25–50 µg/ml) (Figure 2B). The luciferase activity was similar when HepG2 cells were transfected with complexes formed with 25 µg/ml LactpLK and either 5 or 10 µg/ml DNA i.e. polymer to DNA molar ratios of 220:1 and 110:1, respectively (Figure 2A), suggesting that above a concentration of 5 µg/ml plasmid and 25 µg/ml LactpLK, the system is apparently saturated. The luciferase activity drastically decreased when a smaller quantity of plasmid was used, probably due to a competition between endocytosis of free LactpLK and LactpLK complexed with the plasmid (Figure 2A). When 10 µg/ml DNA was complexed with either 12.5 or 6.25 µg/ml LactpLK i.e. polymer to DNA molar ratio of 55:1 and 22.5:1, respectively, the luciferase activities were 20 and 170 fold, respectively, smaller than that obtained with the complex formed with 25 µg/ml LactpLK (Figure 2B). When 10 µg/ml DNA was complexed with either 25 or 50 µg/ml of LactpLK i.e. polymer to DNA molar ratio of 110:1 and 220:1, respectively, the luciferase activities were in the same range indicating that these complexes had similar transfection efficiency; however, the complex formed with 50 µg/ml LactpLK was not

Figure 4. Confocal fluorescence microscopy of HepG2 incubated with pSV2Luc/FTC-LactpLK complex. HepG2 cells were incubated for 4 h at 37°C in the presence of 100 μ M chloroquine and of a pSV2Luc/FTC-LactpLK complex (0.5 μ g/ml, 0.15 nM DNA and 1.25 μ g/ml, 16.5 nM polymer). Cells were washed three times with PBS, fixed for 5 minutes with a cold (-20°C) ethanol/acetic acid solution (95: 5; v/v), washed twice with PBS and mounted in PBS/glycerol (v/v) containing 1% DABCO as antifading agent. Fluorescence images from serial sections of the same field were recorded. 6 sections distant from 3 μ m were presented (scale bar, 20 μ m).

as soluble as the complex formed with 25 μ g/ml LactpLK (Figure 2B). By using diluted solution of this complex (polymer to DNA molar ratio of 110:1), the gene transfer efficiency was dependent upon the overall concentration of the complex (Figure 3). It is however striking that a 20 fold dilution (less than 16.5 nM i.e. 1.25 μ g/ml LactpLK with 0.15 nM i.e. 0.5 μ g/ml luciferase encoding plasmid) still led to a high signal (RLU = 1×10^5).

Fluorescence microscope analysis of HepG2 cells incubated with pSV2Luc/FTC-LactpLK

The complex (1.25 μ g/ml FTC-LactpLK, 0.5 μ g/ml DNA) was incubated for 2h at 37°C in the presence of chloroquine with HepG2 cells. Cells were analyzed by fluorescence microscopy using a confocal microscope. Numerous small fluorescent vesicles were detected in more than 95% of the cells. The fluorescence microscopy patterns of successive slices through the cells showed that the complex entered the cells and that fluorescence was mainly localized inside intracellular vesicles (Figure 4). Using such a transfection method, all the cells (more than 95%) were fluorescent, showing that the transfer was very efficient. It appears therefore that transfection using LactpLK polymer complex occurs by a specific recognition process involving the galactose residues borne by the glycosylated polymer and the galactose specific membrane lectin of HepG2 cells which mediate the endocytosis.

Effect of the fusogenic peptide I on the transfection efficiency of HepG2 cells by pSV2Luc/LactpLK

The peptide I, a peptide with sequence close to that of the N-terminal moiety of influenza hemagglutinin HA2 which induces membrane fusion and leakage of lipid bilayers, was synthesized

Figure 5. Effect of the fusogenic peptide I (GLFEAIAEFIEGGWEGLIEGCA) on the gene expression into HepG2 cells. HepG2 cells (5×10^5 cells per well) were incubated at 37°C with 1.5 nM pSV2Luc free or 1.5 nM pSV2Luc complexed with 165 nM LactpLK in the absence or in the presence of either 10 μ M fusogenic peptide I or 100 μ M chloroquine. After 4h incubation, the medium was removed and the cells were further incubated at 37°C for 48h in plasmid-free, chloroquine-free and fusogenic peptide-free complete medium. Gene expression was determined by assaying the luciferase activity of cell lysate in triplicate. The values of relative light units (RLU) shown represent the activity of 10^6 cells.

Figure 6. Effect of the fusogenic peptide I concentration on the transfer efficiency. HepG2 cells were incubated at 37°C in the presence of 1.5 nM pSV2Luc complexed with 165 nM LactpLK and various concentrations of either peptide I (●) or peptide II (■) or peptide III (▲). After 4h incubation, the medium was removed and the cells were further incubated in complete culture medium in the absence of both plasmid and peptides for 48 h. The gene expression was determined by assaying the luciferase activity of cell lysate in triplicate. The values of relative light units (RLU) shown represent the activity of 10^6 cells.

and used as a cofactor for transfection of HepG2 cells by pSV2Luc/lactpLK complexes. Cells were incubated with either pSV2Luc plasmid free or complexed with LactpLK (glycosylated polymer to DNA molar ratio close to 110) for 4h at 37°C in the absence or in the presence of either 10 μ M peptide I or 100 μ M chloroquine (Figure 5). The efficiency of the gene transfer in HepG2 cells was assessed 48 h later by measuring the activity of expressed luciferase. The luciferase activity in cells incubated for 4h in the presence of 10 μ M peptide I was about 500 fold larger than cells incubated in the absence of peptide I (Figure 5). The luciferase activity of cells incubated in the presence of 10 μ M peptide I was 10 fold larger than that of cells incubated in the presence of 100 μ M chloroquine. The luciferase activity in

cells incubated with pSV2Luc alone was very low and not dependent on the presence of 10 μ M peptide I. These data show that the incubation in the presence of peptide I does not increase the internalization of the plasmid but increases the expression of the gene internalized thanks to the glycosylated carrier. The effect of the peptide on the expression of the enzyme upon transfection with pSV2Luc/LactpLK complexes was optimal for concentrations between 5 to 10 μ M (Figure 6). In control experiments with 10 μ M peptide II or with 10 μ M peptide III, the luciferase activity was as low as in experiments conducted in the absence of any peptide. In control experiments, it was shown that peptides (I, II or III) at any concentration up to 50 μ M did not impair the cell growth of HepG2 cells over a period 3 days (data not shown).

DISCUSSION

The membrane lectin present at the surface of HepG2 cells which binds and induces the uptake of β -galactose terminated glycoproteins was targeted by lactosylated poly-L-lysine in order to induce the internalization of a luciferase encoding plasmid (pSV2Luc). Thirty five per cent of the 190 lysine residues of the poly-L-lysine used was substituted with lactose residues as the recognition signals by using the 4-isothiocyanatophenyl derivative of β -D-lactopyranoside; the remaining cationic charges of lysine residues serve as counterions to form stable complexes with plasmid DNA. pSV2Luc/LactpLK complexes were shown to be efficient to specifically transfer the plasmid into HepG2 cells upon binding and internalization of the complexes, this behaviour is similar to that of β -D-galactose terminated glycoproteins which are recognized and internalized by an endocytotic process mediated by the galactose specific membrane lectin (48,49): i) HepG2 cells which do not possess membrane lectin specific for mannose terminated glycoproteins were not efficiently transfected by a pSV2Luc/ManpLK complex; ii) HeLa cells which do not possess membrane lectin for galactose terminated glycoproteins were not efficiently transfected by a pSV2Luc/LactpLK complex; iii) HepG2 and HeLa cells were not efficiently transfected by a pSV2Luc/pLK complex in which pLK is sugar free. Taking into account that the lactosylated poly-L-lysine possess 122 remaining cationic charges and that pSV2Luc is a plasmid of 5.0 kb, the electroneutrality of a complex between LactpLK and pSV2Luc is obtained when a pSV2Luc plasmid molecule is covered by 82 LactpLK molecules. It appears that the optimized complex is formed with a LactpLK to DNA molar ratio of 110:1 (10 μ g/ml DNA and 25 μ g/ml LactpLK) which is close to the electroneutrality. That is in agreement with the optimal conditions found with transferrin-polylysine conjugates (transferrin-pLK to DNA molar ratio was 120:1) (11). The efficiency of transfection by diluted solution of a pSV2Luc/LactpLK complex (polymer to DNA molar ratio of 110:1) decreased because the amount of internalized complexes *via* the galactose membrane lectin of HepG2 cells is supposed to decrease as expected in the case of a receptor mediated endocytosis process. Other carrier systems have already been developed to target and to introduce DNA into cells by receptor-mediated endocytosis: asialoorosomucoid-polylysine conjugates have been used to transfect HepG2 cells *in vitro* (1,2) and hepatocytes *in vivo* (3–6); insulin-polylysine conjugates to transfect hepatocytes *in vitro* (7); transferrin-polylysine conjugates to transfect hematopoietic and leukemic cells *in vitro* (8–15). The use of lactosylated polylysine instead of asialoorosomucoid-

polylysine offers several advantages: it is a synthetic compound, rapidly prepared, easily purified and highly soluble at physiological ionic strength and pH. In contrast, asialoorosomucoid which is obtained by careful removal of sialic acid from orosomucoid purified from serum, it is weakly water-soluble. Moreover, asialoorosomucoid as well as transferrin and insulin are proteins; the preparation of polylysine-protein conjugates requires high ionic strength conditions (i.e 2 M guanidium chloride) in order to prevent the precipitation of the scarcely soluble protein-polylysine complexes. The polylysine-protein conjugates in a further step have to be made free of the excess of salt to allow them to form complexes with a plasmid; unfortunately, in a low ionic strength buffer, the polylysine-protein conjugates are weakly soluble. In addition, the transferrin receptor is present at the surface of numerous cells and as such has a limited value to target genes to specific cells *in vivo*.

After specific binding by HepG2 cells, pSV2Luc/LactpLK complexes are internalized in acidic vesicles by a receptor-mediated endocytosis process. The key steps of this mechanism involve the routing of the endocytosed material *via* intracellular vesicles derived from the plasma membrane. These vesicles are progressively acidified: 2 to 5 minutes after internalization, endocytosed macromolecules are contained in early endosomes (intraluminal pH: 6.1 ± 0.1); 15 minutes later they are contained in late endosomes ($5.5 < \text{pH} < 6.0$) and from 30 minutes to few hours later they are contained in lysosomes ($\text{pH} < 5.0$) (see 23, for a review). To avoid the degradation which occurs mainly in lysosomes, the endocytosed plasmid should leave the endocytic vesicles and enter the cytosol promptly. The presence of chloroquine during the incubation of cells with DNA/LactpLK complexes increased 275 times the transfection efficiency. In the absence of chloroquine, the transfection efficiency of HepG2 cells by LactpLK was as low as that obtained by the classical DEAE dextran method. This result is in agreement with those obtained by Zenke *et al.* (9) and Cotten *et al.* (10) using a similar plasmid and transferrin-polylysine conjugate as vector: the luciferase activity expressed by the human hematopoietic HD3 cells was 40 fold that measured in the absence of chloroquine and the enhancement was greater in the case of the human leukemic K-562 cells in which no luciferase activity was obtained in the absence of chloroquine. Chloroquine is a weak base used in endocytosis studies in order to neutralize the acidic pH of endocytotic vesicles, to inhibit hydrolases contained in lysosomes, to inhibit the fusion of endocytotic vesicles and lysosomes and thereby to decrease the degradation of endocytosed macromolecules. In the presence of chloroquine, the DNA carried by LactpLK or transferrin-pLK conjugates could escape degradation by endosome and lysosome hydrolases, allowing a correlative larger number of DNA molecules to reach the cytosol and/or the nucleus. The membrane crossing of DNA molecules from the lumen of endocytic vesicles to the cytosol and/or to the nucleus could take place either in late endosomes, in trans Golgi network, or in the Golgi apparatus, however the mechanism is not yet known. Recently, in order to release DNA molecules into cytosol, defective adenovirus particles, able to disrupt endosome membrane, were associated with DNA/transferrin-polylysine complexes and were found to drastically increase the transfection efficacy compared to that previously obtained in the presence of chloroquine (13–15). The mechanism by which paramyxoviruses deliver their nucleic acid material from the endosomes to the cytosol is well established. Thus, the influenza virus particles enter the cell by receptor-mediated endocytosis;

in the endosomes, at a pH close to 6.0, the conformation of the envelope glycoprotein called hemagglutinin HA containing two subunits HA1 and HA2 is modified; the N-terminal peptide of HA-2 subunit leaves the protein core and interacts with the endosomal membrane, inducing the fusion of both the endosomal and virus membranes with the consequent release of the nucleocapsid into the cytosol (26–29). Murata *et al.* (30) and Lear and DeGrado (31) demonstrated that dodecapeptides with sequences similar to that of the N-terminal segment of influenza hemagglutinin HA2 from strains A/PR/8/34 or X31F/68 mimic the fusogenic activity of the influenza virus HA2 hemagglutinin. Several water-soluble anionic peptides derived from the HA2 subunit have been synthesized and were found to induce membrane fusion of small unilamellar vesicles as well as leakage of the luminal content at pH lower than 6.0–6.5 (32, 33, 35). In particular, the anionic peptide E5, derived from the N-terminal peptide (GLFGAIAGFIEGGWGTGMIDG) of A/PR/8/34 influenza virus strain in which G-4, G-8, T-15 and D-19 were replaced by glutamic acid, has a random coil structure at neutral pH and forms an α -helix structure at pH lower than 6.0; the protonation of the 5 glutamic residues increases the hydrophobicity, allowing interactions with lipid bilayers and induces the leakage of egg phosphatidylcholine small and large unilamellar vesicles between pH 6.0 and 5.0 up to a very large extent (32, 33, 35). The 22 residue peptide used in the present work has the sequence of the peptide E5 of Takahashi *et al.* (32) and contains an additional cysteine and one alanine residues in position 21 and 22, respectively; the thiol function was added in order to link the peptide I on a carrier able to increase its specific cellular uptake by a receptor-mediated endocytosis in further studies. Upon incubation of HepG2 cells for 4h in the presence of the pSV2Luc plasmid/lactosylated carrier complex, the luciferase activity is relatively low, but when the transfection is conducted in the presence of 10 μ M peptide I, the luciferase activity increases 500 fold. The fusogenic peptide is supposedly internalized by pinocytosis whereas the plasmid/lactosylated polylysine complex is internalized by receptor dependent endocytosis. As expected from the physicochemical studies (32, 33, 35), the fusogenic peptide is protonated in the acidic endosomal compartments endowing a α -helix structure which induces the destabilization of the endosomal membrane and increases the transmembrane passage of the plasmid DNA from the endosomes into the cytosol. The fusogenic peptide is 10 times more efficient than chloroquine in increasing the luciferase activity upon transfection with the plasmid DNA/lactosylated polylysine complex. The fusogenic peptide is supposed to disrupt the endosomal membrane allowing the release of endocytosed plasmid molecules into the cytosol. Very recently, Wagner *et al.* (51) showed that a tricosapeptide having the sequence of the N-terminal segment of influenza virus X31F/68 hemagglutinin HA2 (GLFGAIAGFIENGWEGMIDG) coupled to polylysine increases the transfer efficiency of a plasmid DNA/transferrin-polylysine complex to human hematopoietic cells, to HeLa cells and to murine hepatocytes. Therefore, the expression of a transfected enzyme gene is greatly enhanced by using a fusogenic peptide alone as defined by Takahashi (32) and Murata *et al.* (33, 35) or the X31F/68 influenza hemagglutinin N-terminal peptide linked to polylysine (51). Because fusogenic peptides are more active than chloroquine, the association of targeted plasmid DNA/glycosylated polylysine complex together with targeted fusogenic peptides could open the way to very efficient and specific cell transfections *in vivo*.

Knowing that membrane lectins are present at the surface of many normal cells (human, murine, bovine, etc) as well as on various tumor cells, and that this sugar specificity is depending on the cell types: mannose-6-phosphate is recognized by monocytes, mannose by macrophages and some B cells, Lewis ^x and sialyl Lewis ^x by endothelial cells, *N*-acetyllactosamine by T cells, galactose by melanoma cells, glucose by colon carcinoma cells, etc (for review see 18, 52), polylysine substituted with specific sugar moieties are good candidates to selectively target genes to define cell populations *in vivo*. Polylysine substituted with the corresponding sugar moieties are being prepared and will be used to target genes to selected cells in a membrane lectin dependent manner.

ACKNOWLEDGMENTS

We thank Gilbert Aschwell (Bethesda) and Pascale Briand (Cochin, Paris) for their kind advices. We thank Suzanne Nuques, Martine Dubois Marie-Thérèse Boussier, Emmanuelle Martin and Philippe Bouchard for their skillful technical help. This work was partly supported by grants from 'Agence Nationale de Recherche sur le Sida' (ANRS) and Association Recherche sur le cancer (ARC 6132). PM is 'Chargé de recherche INSERM'. ACR is 'Directeur de recherche INSERM'.

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