

## Evidence for the role of proteoglycans in cation-mediated gene transfer

KIMBERLY A. MISLICK AND JOHN D. BALDESCHWIELER\*

Division of Chemistry and Chemical Engineering, 127–72, California Institute of Technology, Pasadena, CA 91125

Contributed by John D. Baldeschwieler, August 9, 1996

**ABSTRACT** We report evidence that gene complexes, consisting of polycations and plasmid DNA enter cells via binding to membrane-associated proteoglycans. Treatment of HeLa cells with sodium chlorate, a potent inhibitor of proteoglycan sulfation, reduced luciferase expression by 69%. Cellular treatment with heparinase and chondroitinase ABC inhibited expression by 78% and 20% with respect to control cells. Transfection was dramatically inhibited by heparin and heparan sulfate and to a smaller extent by chondroitin sulfate B. Transfection of mutant, proteoglycan deficient Chinese hamster ovary cells was 53× lower than of wild-type cells. For each of these assays, the intracellular uptake of DNA at 37°C and the binding of DNA to the cell membrane at 4°C was impaired. Preliminary transfection experiments conducted in mutant and wild-type Chinese hamster ovary cells suggest that transfection by some cationic lipids is also proteoglycan dependent. The variable distribution of proteoglycans among tissues may explain why some cell types are more susceptible to transfection than others.

Because of the current interest in gene therapy, a number of nonviral transfection agents have been developed. Among them are polylysine- (PLL) and lipid-based amphiphiles, which belong to a diverse class of macromolecules that form stable complexes with DNA. At optimum formulations, these agents surround DNA with a net positive charge, that in turn, enable the DNA complex to bind to anionic residues on the cell surface. Following entry into the cell by endocytosis, a fraction of the DNA enters the nucleus and the gene of interest is transcribed (1).

Despite the advances in this field, few studies have focused on the molecular mechanisms of gene delivery. In particular, the membrane molecules that are responsible for the transfection of cation–DNA complexes have never been investigated. Here, we report evidence that sulfated, membrane-associated proteoglycans serve as a receptor for transfection by PLL and possibly by some cationic lipids.

Sulfated proteoglycans are among the most negatively charged components of the cell. They consist of a core protein covalently linked to one or more sulfated glycosaminoglycans: heparin, heparan sulfate, dermatan sulfate, chondroitin sulfate, and keratan sulfate (2, 3). Proteoglycan synthesis begins in the endoplasmic reticulum with the formation of the protein core (4, 5). As the protein core is transported from the rough endoplasmic reticulum to the Golgi, serine residues are linked to D-xylose by xylosyltransferase (6). The protein core is further modified with two galactosyl residues and a glucuronosyl residue. Starting from this sugar, the glycosaminoglycan backbone is synthesized. Sulfates are transferred from adenosine 3'-phosphate 5'-phosphosulfate by sulfotransferase onto the glycosaminoglycans (7). Finally, the glycosaminoglycan residues are epimerized before the proteoglycans are exported to their final destination (8, 9).

Proteoglycans are a fundamental component of basement membranes (10) and the extracellular matrix, and play a pivotal role in cellular proliferation, migration, and differentiation (11, 12). As integral and glycosylphosphatidyl inositol-linked membrane proteins, heparan sulfate and chondroitin sulfate proteoglycans sequester protease inhibitors, growth factors, and proteases and serve as a receptor for infection by the herpes simplex virus (for reviews, see refs. 13 and 14).

Because glycosaminoglycans are highly anionic, the interactions between proteoglycans and their ligands occur largely through ionic forces. Based on this information, we proposed that membrane-associated proteoglycans mediate the binding and delivery of cation–DNA complexes into cells. To test this hypothesis, a number of assays were used. Since proteoglycan charge is derived mostly from the sulfation of the glycosaminoglycan chains (2), transfection in HeLa cells cultured in the presence of sodium chlorate, an inhibitor of glycosaminoglycan sulfation (15). Transfection was also tested in cells pretreated with glycosaminoglycan lyases to remove extracellular glycosaminoglycans (16) and in the presence of purified glycosaminoglycans. In addition, the transfection efficiency of PLL-and cationic lipid–DNA was tested in wild-type Chinese hamster ovary (CHO) cells and in mutant CHO cells unable to synthesize proteoglycans (17).

### MATERIALS AND METHODS

**Cell Culture.** HeLa cells were obtained from the American Type Culture Collection and grown in DMEM (GIBCO) containing basal Eagle's medium amino acids (GIBCO), non-essential amino acids (GIBCO), 10% fetal bovine serum (HyClone), and 40 mg/ml gentamicin (GIBCO).

Mutant (CHO-*pgs745*) and wild-type (CHO-K1) cells were generously donated by Jeffrey Esko (University of Alabama School of Medicine, Birmingham). The mutant cell line lacks xylosyltransferase, an initiator of glycosaminoglycan synthesis. Both cell lines were grown in Ham's F-12 medium, supplemented with 7.5% fetal bovine serum, and subcultured every 4 days.

Media for PLL transfections consisted of regular growth media (DMEM/10% fetal bovine serum or Ham's F-12/7.5% fetal bovine serum) supplemented with 100 μM chloroquine (Sigma). Lipid–DNA transfections were carried out in wild-type and mutant CHO cells in serum-free Ham's F-12 medium. Experiments were conducted using subconfluent cultures.

**Preparation of Complexes and Transfection.** An aliquot of poly-L-lysine (100 μg/ml, Sigma, molecular mass 56 kDa) was added to DNA (1.5 μg/100 μg/ml) diluted in 150 liters Hepes-buffered saline (50 mM Hepes/150 mM NaCl, pH 7.4). PLL–DNA samples were mixed gently, incubated for 30 min at room temperature, and added to cells in transfection media. Four hours later, cells were rinsed twice in 1 ml PBS (8 mM Na<sub>2</sub>HPO<sub>4</sub>/3 mM KCl/138 mM NaCl/2 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) and placed into fresh culture media at 37°C. Following an

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CHO, Chinese hamster ovary; PPL, polylysine.  
\*To whom reprint requests should be addressed.

additional 21 hr at 37°C, the medium was removed and cells were rinsed twice with 2 ml PBS. Luciferase expression in cellular lysates was determined using an Enhanced Luciferase Assay kit (Analytical Luminescence Laboratory, San Diego) according to the manufacturer's instructions. Expression was quantitated in terms of relative light units on an Analytical Luminescence Laboratory model 2010 Luminometer.

**Preparation of Fluorescent and Nick-Translated Plasmids.** The PGL2 plasmid (Promega) encoding the firefly luciferase reporter gene was amplified in competent JM109 *Escherichia coli* (Promega) and purified by chromatographic methods (Qiagen, Chatsworth, CA). Plasmid DNA was diluted to 100 µg/ml in sterile, distilled water.

DNA (1.5 µg) was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq) using a nick translation kit (Boehringer Mannheim) and purified by repeated chloroform/phenol extractions and ethanol precipitation (18). A stock solution of DNA was prepared by mixing nick translated plasmid with  $\approx$ 30 µg unlabeled plasmid. The concentration of DNA was determined by absorbance ( $\lambda_{\text{max}} = 260$  nm).

To prepare fluorescent DNA, plasmid (100 µg/ml) was incubated with a 50-fold molar excess of YOYO-1 (Molecular Probes) and a fluorescent DNA intercalator (19, 20) for 2 hr at 4°C. The solution was loaded onto a Centricon-30 desalting unit and spun for 3 hr at 6000 rpm at 4°C to remove unbound YOYO. The retentate was diluted to its original concentration in sterile water. The concentration of YOYO per mole plasmid was determined by spectrophotometric analysis. The fluorescence of YOYO-DNA (YYDNA) was measured ( $\lambda_{\text{ex}} = 488$  nm,  $\lambda_{\text{em}} = 512$ ) in a Hitachi model F-4500 fluorescence spectrophotometer.

**Desulfation of Cells.** HeLa cells ( $2 \times 10^4$  cells per ml) were seeded into 12-well plates (Falcon). Twelve hours later, media were supplemented with sodium chlorate (35 mM, Aldrich) or a combination of sodium sulfate (80 mM, Baker) and sodium chlorate (35 mM). Following an additional 48-hr incubation period, cells were rinsed twice with 1 ml PBS and placed into 1 ml transfection media. Transfection, uptake, or binding experiments immediately followed. The transfection was conducted in the absence of chlorate to minimize the possible effects that this compound might have on cellular processes unrelated to proteoglycans.

**Treatment of Cells with Glycosaminoglycan Lyases and Purified Glycosaminoglycans.** HeLa cells were seeded ( $5 \times 10^4$  cells per ml) into 12-well plates (Falcon). Eighteen hours later, cells were rinsed twice with 1 ml PBS and placed into a BSA/PBS solution containing either 10 units/ml chondroitinase ABC (Sigma) or 10 units/ml heparinase II (Sigma) (16). Cells were incubated with lyases for 1 hr at 22°C. Following the removal of surface glycosaminoglycans, cells were rinsed twice with PBS and transfected according to the usual protocol.

Glycosaminoglycans (chondroitin sulfate A, bovine trachea; chondroitin sulfate B, bovine mucosa; chondroitin sulfate C, shark cartilage; heparan sulfate, bovine trachea; heparin, bovine trachea; hyaluronic acid, bovine mucosa) were purchased from Sigma. To evaluate the effect of free glycosaminoglycans on transfection, DNA complexes and glycosaminoglycans (40 g/ml heparan sulfate, heparin, chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C, or hyaluronic acid) were added together at the time of transfection. Transfected cells were treated according to the usual protocol.

**Fluorescence Uptake Experiments and Confocal Microscopy.** HeLa, CHO-pgs745, and CHO-K1 cells were incubated with PLL-YYDNA for 4 hr and then rinsed three times in PBS. To remove surface bound DNA, cells were treated with DNase (1 mg/ml, Sigma) for 15 min and then detached with trypsin-EDTA. Cell suspensions were pelleted, washed twice in PBS, and fixed in 4% paraformaldehyde/PBS for 10 min at room temperature. After a final rinse in PBS, cell pellets were resuspended in Biomed/PBS solution (90:10) and mounted

between a slide and coverslip. Fluorescence images were obtained using a Bio-Rad confocal microscope.

**Binding Assays.** Complexes were prepared as described using nick-translated plasmid and then added to cells that were prechilled to 4°C. After a 2–4 hr incubation at this temperature, cells were rinsed four times in ice-cold PBS and detached with trypsin-EDTA. Aliquots were dissolved in Safety Solve scintillation cocktail, radioactivity (counts per min) was quantitated in a Beckman model LS 5000 TD scintillation counter. Cellular protein was determined by the fluoroldehyde assay (Pierce).

## RESULTS

**PLL-Mediated Transfection Requires Positively Charged Complexes.** The net charge on a DNA complex can significantly influence transfection efficiency. Optimum formulations with cationic lipids consist of a molar excess of positive charge (1). To determine the optimum charge ratio for PLL, a series of complexes prepared with 1.5 µg DNA and varying amounts of PLL were transfected into HeLa cells.

Complexes composed of amine/nucleotide charge ratios <1 yielded very little expression (Fig. 1). Expression increased nonlinearly with PLL concentration and reached a peak ( $5 \times 10^5$  relative light unit/mg cell protein) at a charge ratio (amine/nucleotide) equal to 1.5. Agarose gel electrophoresis confirmed that complexes composed of this ratio were positively charged (data not shown). Cellular uptake assays revealed that this ratio was optimal for cellular uptake of nick-translated and fluorescently labeled DNA. Complexes with ratios larger than 1.5 yielded lower expression values according to previous observations (21). These results are consistent with the putative ionic model to describe the interactions between the cell membrane and cation-DNA complexes.

**Sulfates Mediate PLL-DNA Transfection.** The role of proteoglycans in cellular processes has been demonstrated by inhibiting glycosaminoglycan sulfation with sodium chlorate (22, 23). Chlorate is a nontoxic inhibitor of ATP-sulfurylase, an essential enzyme in the synthesis of 3'-phosphoadenosyl-5'-phosphosulfate, a glycosaminoglycan sulfate donor (15, 24).

To test the role of sulfated proteoglycans in transfection, cells were grown in the presence of sodium chlorate for 48 hr

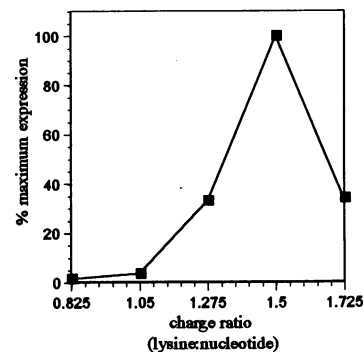


FIG. 1. Determination of the optimum charge ratio (lysine/nucleotide) for the transfection of PLL-DNA. Varying amounts of PLL (100 µg/ml) were added to 1.5 µg plasmid DNA diluted in 150 µl Hepes-buffered saline. Samples were gently agitated, equilibrated for 30 min, and added to HeLa cells in transfection media. Following a 4-hr incubation at 37°C, transfection medium was removed, and cells were washed twice in 2 ml PBS. Cells were grown for an additional 21 hr in fresh growth media and then washed in PBS. Luciferase expression in cellular lysates was determined using an Enhanced Luciferase Assay kit and a Monolight Luminometer (Analytical Luminescence Laboratory) according to the manufacturer's instructions. Results obtained from different charge ratios are presented as a percent of the maximum observed expression.

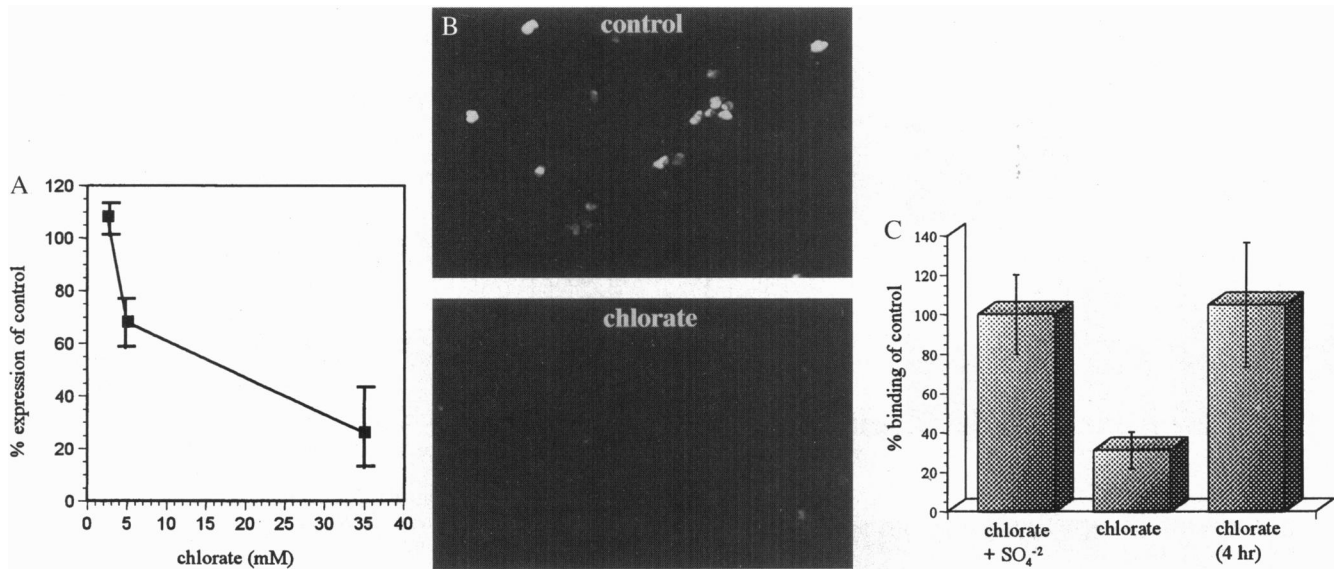


FIG. 2. Effect of sodium chlorate concentration on transfection. HeLa cells were grown for 48 hr in the presence of varying amounts of sodium chlorate or were left untreated as a control. (A) Cells were transfected as described (Fig. 1) using PLL-DNA complexes made with the optimum charge ratio. (B) Complexes composed of YOYO-labeled DNA (YYDNA) and PLL were prepared and incubated with chlorate treated (35 mM) and untreated HeLa cells at 37°C. After 4 hr, cells were rinsed extensively and incubated in 1 ml PBS/deoxyribonuclease (Sigma, 50  $\mu$ g/ml) for 15 min. Cells were trypsinized, fixed in 4% paraformaldehyde, and suspended in Biomeda/PBS (90:10) solution. Cells were mounted onto a glass slide and coverslip and imaged 12 hr later using a Bio-Rad Confocal microscope. Each panel contains approximately the same number of cells. (C) HeLa cells were treated with sodium chlorate (35 mM) or with sodium chlorate (35 mM) and sodium sulfate (80 mM) for 48 hr or with sodium chlorate (35 mM) for 4 hr. Then, PLL-<sup>32</sup>P-DNA complexes were prepared and incubated with cells for 4 hr at 4°C to inhibit endocytosis. Cells were rinsed extensively in ice cold PBS and dissolved in 1.0 M NaOH. Aliquots of dissolved cells were added to 10 ml Scintillation fluid and counted for one minute in a Beckman model 2010 scintillation counter. Samples were assayed in duplicate and normalized per mg cell protein. Error bars represent the actual data points of duplicate samples.

and transfected with PLL-DNA. As demonstrated by Fig. 2A, expression was inhibited by chlorate in a concentration dependent manner. At 35 mM chlorate, luciferase expression was 30% relative to untreated cells. The inhibition of gene expression was probably not caused by chlorate toxicity, because cellular morphology and proliferation remained normal at all chlorate concentrations.

However, these results could possibly be due to intracellular artifacts unrelated to the delivery mechanism of PLL-DNA. To elucidate the effects of chlorate more clearly, we tested whether chlorate treatment inhibited the uptake of DNA into the cell. DNA was labeled with YOYO, a fluorescent intercalator, complexed with PLL at the optimum ratio, and incubated with chlorate treated (35 mM) and untreated HeLa cells. After 4 hr, cells were washed, fixed, and imaged by confocal microscopy to localize intracellular fluorescence. As shown in Fig. 2B uptake of YYDNA into chlorate-treated cells was severely impaired suggesting that the chlorate induced decrease in expression was due to an inhibition of YYDNA entering the cell.

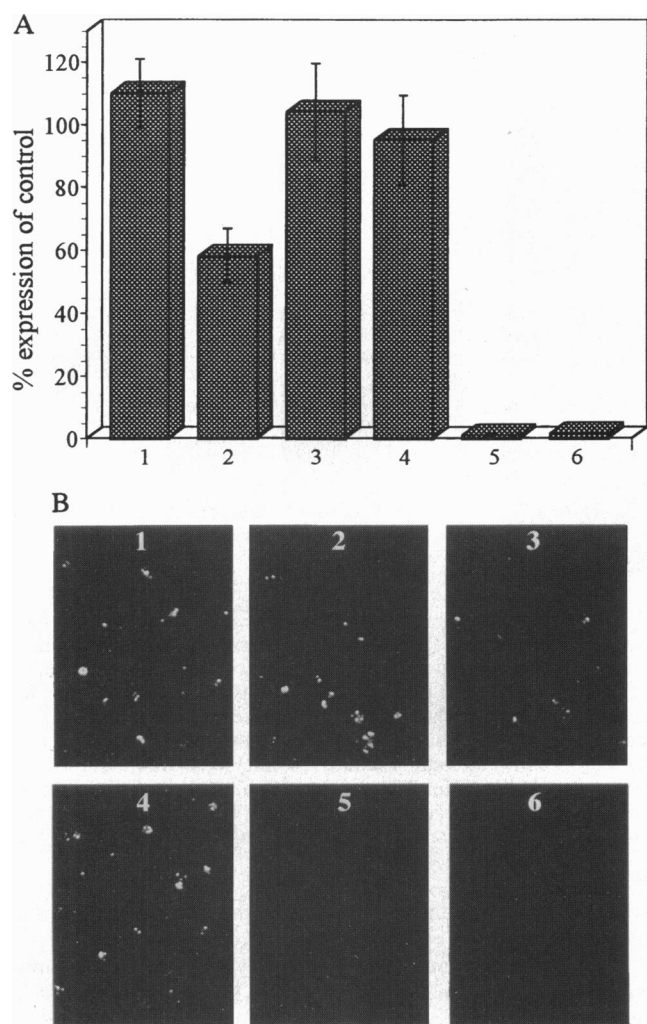
To demonstrate a direct correlation between the uptake of PLL-DNA and sulfated cell membrane residues, we tested whether chlorate treatment inhibited binding of DNA to the cell surface. Complexes of PLL and nick translated <sup>32</sup>P-labeled plasmid were prepared and incubated with HeLa cells, previously treated with chlorate, for 4 hr at 4°C to inhibit endocytosis. Cells were washed extensively with ice-cold PBS, dissolved, and assayed for cell-associated radioactivity. As shown in Fig. 2C, binding of PLL-<sup>32</sup>P-DNA to chlorate treated cells was inhibited 69% with respect to untreated cells. In HeLa cells cotreated with chlorate and excess sulfate, the inhibitory effect of chlorate was reversed. Furthermore, HeLa cells incubated with chlorate for only 4 hr bound as much PLL-<sup>32</sup>P-DNA as untreated cells. We conclude that chlorate inhibits luciferase expression by inhibiting the sulfated receptors,

possibly proteoglycans, that mediate transfection. Additional evidence was sought to implicate proteoglycans directly.

**Effect of Exogenous Glycosaminoglycans and Glycosaminoglycan Lyases on Transfection of PLL-DNA.** To test if glycosaminoglycans could competitively inhibit gene delivery, HeLa cells were cotransfected in the presence of exogenous glycosaminoglycans (40  $\mu$ g/ml). Following exposure to DNA complexes and glycosaminoglycans, cells were washed, incubated in fresh culture medium, and assayed for luciferase expression 22 hr later (Fig. 3A). The most anionic glycosaminoglycans, heparin and heparan sulfate, nearly eliminated luciferase expression, whereas chondroitins A and C and hyaluronic acid had no effect. Chondroitin sulfate B, the third most anionic glycosaminoglycan, reduced expression by  $\approx$ 40%.

Since excess glycosaminoglycans in media have been shown to affect DNA transcription (25), we tested whether the intracellular uptake of YYDNA was affected by exogenous glycosaminoglycans. PLL-YYDNA complexes and glycosaminoglycans were incubated with HeLa cells for 4 hr at 37°C. Cells were rinsed, fixed, and mounted onto glass slides. Confocal microscopy images of intracellular fluorescence (Fig. 3B) revealed a correlation between expression and YYDNA uptake in the presence of competing glycosaminoglycans. Heparin and heparan sulfate eliminated the uptake of YYDNA into the cells, but chondroitin sulfates A and C did not have any obvious effect. Chondroitin sulfate B moderately reduced the uptake of YYDNA into the cell. Binding of <sup>32</sup>P-DNA to the cell surface was tested in the presence of glycosaminoglycans and a similar pattern of inhibition was observed (data not shown).

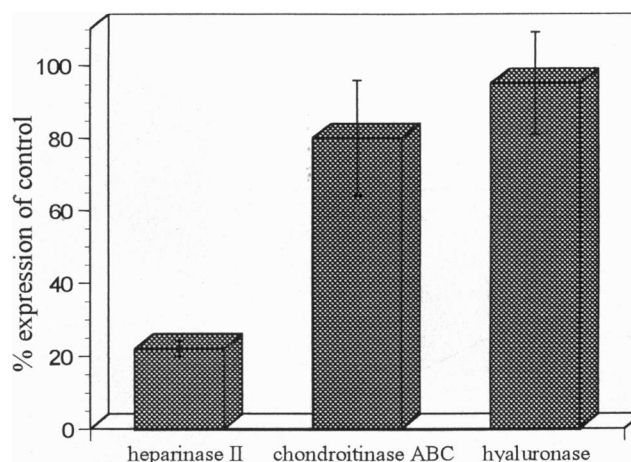
It could be argued that any highly sulfated, anionic polymer in the media could inhibit transfection by destabilizing the interactions between PLL and DNA. Indeed, when dextran sulfate, a synthetic glycosaminoglycan analog, was added to the media, reductions in uptake and expression comparable to



**FIG. 3.** Effect of exogenous glycosaminoglycans on transfection. (A) HeLa cells were transfected with PLL-DNA in the presence of purified glycosaminoglycans (40 μg/ml). Results are presented as a percentage of luciferase expression relative to cells transfected in the absence of glycosaminoglycans. Error bars represent the actual data points of duplicate samples. Lanes: 1, chondroitin sulfate A; 2, chondroitin sulfate B; 3, chondroitin sulfate C; 4, hyaluronic acid; 5, heparin; 6, heparan sulfate. (B) HeLa cells were transfected with PLL-YYDNA in the presence of purified glycosaminoglycans (40 μg/ml). Cells were prepared and imaged as described (Fig. 2C). Lanes: 1, control; 2, chondroitin sulfate A; 3, chondroitin sulfate B; 4, chondroitin sulfate C; 5, heparin; 6, heparan sulfate. Each panel contains approximately the same number of cells.

those induced by heparin were observed (data not shown). As an additional control, HeLa cells were transfected following treatment with glycosaminoglycan lyases. By removing heparin/heparan sulfate and chondroitin sulfates with heparinase II and chondroitinase ABC, expression was reduced by 78% and 20%, respectively (Fig. 4). Moreover, treatment of cells with hyaluronase that removes nonsulfated hyaluronic acid glycosaminoglycans found in the extracellular matrix did not inhibit luciferase expression. These results suggest that heparin/heparan sulfate proteoglycans and, to a smaller extent, chondroitin sulfate B proteoglycans are mediators of transfection by PLL-DNA.

**Cells Lacking Proteoglycans Are Transfection Deficient.** To demonstrate that proteoglycans play a role in gene delivery into other cell lines, transfection efficiency was tested in wild-type CHO-K1 cells and in mutant CHO-*pgs745* cells deficient in xylosyltransferase, an essential enzyme in proteoglycan synthesis. Despite their inability to produce proteogly-



**FIG. 4.** Transfection of cells treated with glycosaminoglycan lyases. To test the effect of lyases on transfection, cells were incubated in the presence of either chondroitinase ABC or heparinase for 1.5 hr and transfected according to methods described in Fig. 1. Error bars represent the actual data points over duplicate samples.

cans, CHO-*pgs745* cells are highly proliferative and are morphologically indistinct from their wild-type counterparts (17).

Both CHO-K1 and CHO-*pgs745* cells were transfected with the same amount of DNA and with the same charge ratio, but luciferase expression in proteoglycan-deficient cells was 53× lower than in wild-type cultures (Fig. 5A). Consistent with previous observations, the uptake of PLL-YYDNA into CHO-*pgs745* cells was dramatically lower than into CHO-K1 cells (Fig. 5B), and the binding of PLL-<sup>32</sup>P-DNA to CHO-*pgs745* cells was inhibited 80% with respect to wild-type CHO cells (data not shown).

**Role of Proteoglycans in Cationic Lipid-Mediated Transfection.** The experiments described support the hypothesis that membrane-associated proteoglycans mediate the delivery of PLL-DNA. In practice, unmodified PLL is not a widely used agent for transfection, because the transfection efficiency is low compared with cationic lipids. To evaluate the role of proteoglycans in a more common transfection system, the transfection efficiency of a series of cationic lipids (PerFect Transfection kit, Invitrogen) was tested in wild-type and mutant CHO cells. The formulations tested were either a mixture of two cationic lipids (pfx-1), a 1:1 mixture of dioleoylphosphatidylethanolamine (DOPE)/cationic lipid (pfx-6 and pfx-7), or a single cationic lipid (pfx-8).

Each formulation was transfected into CHO-K1 and CHO-*pgs745* cells ( $\approx 2 \times 10^5$  cells per well) using 4 μg DNA and the optimum amount of lipid (as determined by the manufacturer). Cells were exposed to lipid-DNA complexes for 4 hr in serum-free Ham's F-12 media, rinsed twice with PBS, and incubated in regular growth media. Twenty hours later, a significant number of dead cells and cellular debris was observed especially in cultures transfected with pfx-7 and pfx-8. Cationic liposome induced toxicity has been observed previously, although the reasons for this toxicity are unclear.

To eliminate the possible artifacts that might arise from toxicity, cells were transfected again with a 20-fold lower concentration of lipid-DNA. At these concentrations, very few dead cells were observed in any of the cultures. Following the transfection, all cultures were rinsed and assayed for luciferase activity.

A correlation quotient,  $X$ , defined as (relative light units in wild-type cells/relative light units in mutant cells) was plotted for each type of lipid (Fig. 6). For pfx-1 formulations,  $X$  was equal to 81, indicating that wild-type cells expressed 81× more luciferase than proteoglycan deficient cells. The correlation quotient determined for the other lipid formulations was 10.7,

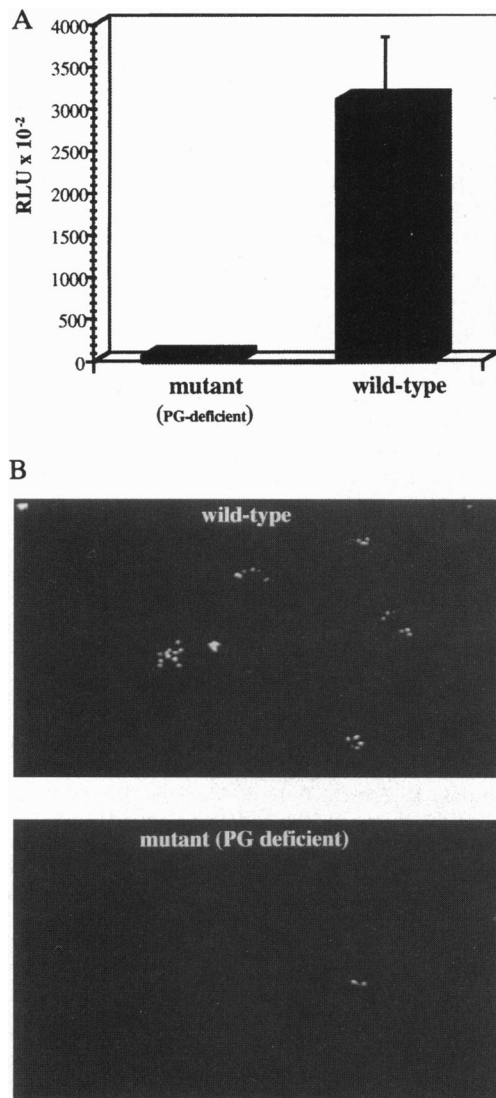


FIG. 5. Transfection of wild-type and mutant CHO cells by PLL. (A) PLL-DNA complexes were transfected into wild-type and mutant CHO cells according to the methods described in Fig. 1. (B) Cells were prepared and imaged as described (Fig. 2B). Each panel contains approximately the same number of cells.

1.7, 4.8, and 1.7 for pfx-4, pfx-6, pfx-7, and pfx-8, respectively. The highest levels of raw luciferase expression in both CHO cell lines were obtained with pfx-1-DNA and pfx-4-DNA and the lowest levels from pfx-6, pfx-7, and pfx-8-DNA.

## DISCUSSION

In this paper, we investigated the identity of the anionic membrane sites that mediate transfection by PLL. Because proteoglycans bind and internalize a wide range of cationic substrates in the body, we hypothesized that gene delivery is mediated by proteoglycans bound to the cell surface. Transfection was tested in cells treated with chlorate to evaluate the role of sulfated proteoglycans in the transfection mechanism. Chlorate diminished binding of PLL-DNA to the cell surface at 4°C, which suggested that the first step in the transfection process involved binding to sulfated proteoglycans. Chlorate treatment also diminished the uptake of DNA as well as the expression of the luciferase reporter gene. Taken together, these results indicate that DNA complexes are effectively bound and internalized by membrane-associated proteoglycans.

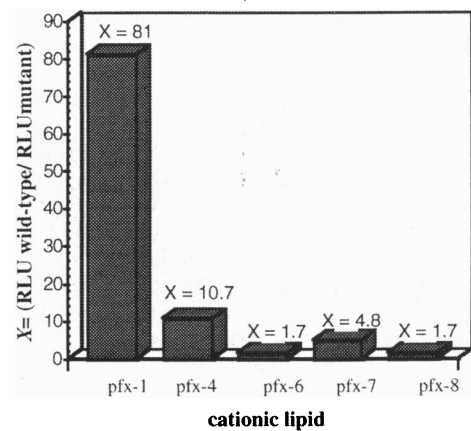


FIG. 6. Transfection of wild-type and mutant CHO cells by cationic lipids. An optimum amount of each type of lipid was used to transfect CHO-K1 and CHO-pgs745 cells ( $\approx 2 \times 10^5$  cells per well) with 0.2  $\mu$ g DNA. Cells were exposed to lipid-DNA complexes for 4 hr in serum-free Ham's F-12 media, rinsed twice with PBS, and incubated in regular growth media for an additional 20 hr. Luciferase expression in cellular lysates was determined as described (Fig. 1).

We also demonstrated that exogenous heparin and heparan sulfate preferentially inhibited transfection, probably by binding and destabilizing complexes in solution and preventing intact complexes from entering the cell. These results do not directly support a role for membrane-associated proteoglycans in transfection but explain why heparin is a serious inhibitor of transfection and also support the results of recently published work in this area (26). Since the least anionic glycosaminoglycans, the chondroitin sulfates, had very little effect on transfection and dextran sulfate, an anionic glycosaminoglycan analog, dramatically inhibited transfection, the inhibition by heparin and heparan sulfate proteoglycans is probably a non-specific effect, dependent on charge effects rather than on structural features.

However, direct evidence was found to implicate membrane-associated heparin/heparan sulfate proteoglycans in transfection. Treatment of cells with heparinase was an effective transfection inhibitor. Thus, PLL-DNA complexes may preferentially transfect cells via heparin/heparan sulfate proteoglycans. By showing that binding, uptake, and expression of PLL-DNA was reduced in proteoglycan deficient cells relative to wild-type cells, we confirmed that proteoglycans participate in PLL-mediated gene delivery into other cell lines as well.

It is unclear whether the transfection inefficiency of CHO-pgs745 cells is due solely to inefficient binding and transport of PLL-DNA. In CHO-pgs745 cells, cellular binding of PLL-<sup>32</sup>PDNA at 4°C was 5-fold lower than binding to wild-type cells, but expression was inhibited 50-fold. To account for this difference, it is possible that CHO-pgs745 cells possess other intracellular deficiencies that can affect the expression of the reporter gene. Alternatively, in the absence of proteoglycans PLL-DNA may still bind to other anionic sites but may not enter the cell efficiently. The lack of DNA uptake into CHO-pgs745 cells is consistent with this hypothesis. Regardless of whether proteoglycans are the sole membrane binding sites for transfection, we conclude that a correlation exists between membrane-associated proteoglycans and the efficiency of PLL-mediated transfection.

Since PLL is not a widely used transfection agent, preliminary experiments were conducted to evaluate the possible role of proteoglycans in the transfection by cationic lipids. Luciferase expression in wild-type cultures relative to proteoglycan deficient cells varied depending on the lipid. The strongest correlation between transfection and proteoglycans, as well as the highest expression levels, were obtained with pfx-1 and pfx-4 lipids that consist of a mixture of two cationic lipids. The



weakest correlation and the lowest expression levels were obtained with lipid formulations consisting of a 1:1 mixture of a cationic lipid and DOPE or a single cationic lipid. Experiments were not conducted to verify that the inhibition of expression was directly caused by decreased binding and uptake of lipid-DNA, but they are currently in progress. These preliminary results are consistent with the hypothesis that proteoglycans participate in the transfection of certain cationic lipids, but we cannot conclude yet that other mechanisms do not play an important role. Future studies will be conducted to test the role of proteoglycans in the transfection by cationic lipids of known structure so that a correlation between proteoglycan-mediated transfection and precise structural features can be elucidated.

In conclusion, we suggest that the variable expression of proteoglycans among tissues may explain why some cell types are more susceptible to transfection than others. Although all tissues express proteoglycans to some level (27), proteoglycan expression is regulated by both the state of differentiation and growth of the cell. Immature myeloid and lymphoid cells express the hematopoietic proteoglycan core protein (HpPG) at low levels only but up-regulate the HpPG gene 10-fold upon differentiation (28). Recently, the undifferentiated erythroleukemia cell line, K562, was shown to express 2–3 times more of the basement membrane proteoglycan, perlecan, if the cells were artificially stimulated to differentiate with 12-*O*-tetradecanoylphorbol-13-acetate, a phorbol diester (29). Incidentally, K562 cells are one of the most difficult cell lines to transfect by cationic methods but yield high levels of expression by transferrin-PLL transfection (30). This implies that the susceptibility of a cell type to transfection may partially depend on the mechanism of entry and not entirely on intracellular factors. The development of methods to increase expression in these hematopoietic cell lines is important for *ex vivo* applications of gene replacement therapy. Experiments that attempt to modulate proteoglycan expression as a means to increase transfection by cationic methods are currently in progress.

Finally, these experiments bring to light the possible barriers that might be encountered by gene delivery *in vivo*. The inhibition of transfection by heparin and other charged components in serum has been established (31). This problem is avoided *in vitro* by conducting transfection experiments in serum-free media. Although typical concentrations of heparin in blood are low, the presence of heparin combined with other charged serum components might seriously affect transfection *in vivo*. Gene delivery might also be inhibited by wasteful binding of DNA complexes to the extracellular matrix and to basement membranes of tissues in the body. If these problems prove to be significant *in vivo*, then alternative gene delivery vectors that are optimized at more neutral charge ratios may have to be developed.

1. Behr, J. P. (1994) *Bioconjugate Chem.* **5**, 382–389.

2. Ruoslahti, E. (1988) *Annu. Rev. Cell Biol.* **4**, 229–255.
3. Kjellen, L. & Lindahl, U. (1991) *Annu. Rev. Biochem.* **60**, 443–475.
4. DeLuca, S., Caplan, A. I. & Hascall, V. C. (1978) *J. Biol. Chem.* **253**, 4713–4720.
5. Upholt, W. B., Vertel, B. M. & Dorfman, A. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4847–4851.
6. Vertel, B. M., Walters, L. M., Flay, N., Kearns, A. E. & Schwartz, N. B. (1993) *J. Biol. Chem.* **268**, 11105–11112.
7. Sugumar, G. & Silbert, J. E. (1990) *J. Biol. Chem.* **265**, 18284–18288.
8. Lindahl, U., Feingold, D. S. & Roden, L. (1986) *Trends Biochem. Sci.* **11**, 221–225.
9. Lindahl, U. & Kjellen, L. (1987) in *Biology of Proteoglycans*, eds. Wight, T. N. & Mecham, R. P. (Academic, Orlando, FL), pp. 59–104.
10. Timpl, R. (1993) *Experientia* **49**, 417–428.
11. Conget, P. & Minguell, J. J. (1994) *Br. J. Haematol.* **89**, 1–7.
12. Faasen, A. E., Schragar, J. A., Klein, D. J., Oegema, T. R., Couchman, J. R. & McCarthy, J. B. (1992) *J. Cell Biol.* **116**, 521–531.
13. Bernfield, M., Kokenyesi, R., Kato, M., Hinkes, M. T., Spring, J., Gallo, R. L. & Lose, E. J. (1992) *Annu. Rev. Cell Biol.* **8**, 365–398.
14. Shieh, M. T., WuDunn, D., Montgomery, R. I., Esko, J. D. & Spear, P. G. (1992) *J. Cell Biol.* **116**, 1273–1281.
15. Baeuerle, P. & Huttner, W. (1986) *Biochem. Biophys. Res. Commun.* **141**, 870–877.
16. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K., eds. (1987) *Current Protocols in Molecular Biology* (Wiley, New York), pp. 17.13.17–17.13.32.
17. Esko, J. D., Stewart, T. E. & Taylor, W. H. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 3197–3201.
18. Sambrook, J., Fritsch, E. F. & Maniatis, R. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
19. Glazer, A. N. & Rye, H. S. (1992) *Nature (London)* **359**, 859.
20. Rye, H. S., Yue, S., Wemmer, D. E., Quesada, M. A., Haugland, R. P., Mathies, R. A. & Glazer, A. N. (1992) *Nucleic Acids Res.* **20**, 2803.
21. Kabanov, K. A. & Kabanov, V. A. (1995) *Bioconjugate Chem.* **6**, 7–20.
22. Rapraeger, A. C., Krufka, A. & Olwin, B. B. (1991) *Science* **252**, 1705–1708.
23. Reiland, J. & Rapraeger, A. C. (1993) *J. Cell Sci.* **105**, 1085–1093.
24. Hortin, G. L., Schilling, M. & Graham, J. P. (1988) *Biochem. Biophys. Res. Commun.* **150**, 342–348.
25. Busch, S. J., Martin, G. A. & Barnhart, R. L. (1992) *J. Cell Biol.* **116**, 31–42.
26. Xu, Y. H. & Szoka, F. C. (1996) *Biochemistry* **35**, 5616–5623.
27. Varma, R. S. & Varma, R. (1982) *Glycosaminoglycans and Proteoglycans in Physiological and Pathological Processes of Body Systems* (Karger, Basel).
28. Stellrecht, C. M., Mars, W. M., Miwa, H., Beran, M. & Saunders, G. F. (1991) *Differentiation* **48**, 127–135.
29. Grassel, S., Cohen, I. R., Murdoch, A. D. & Eichstetter, I. (1995) *Mol. Cell. Biochem.* **145**, 61–68.
30. Zenke, M., Steinlein, P., Wagner, E., Cotten, M., Beug, H. & Birnstiel, M. L. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3655–3659.
31. Felgner, P. & Ringold, G. (1989) *Nature (London)* **337**, 387–388.