Potentiation of Cationic Liposome-Mediated Gene Delivery by Polycations†

Xiang Gao‡ and Leaf Huang*

Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

Received October 12, 1995⊗

ABSTRACT: We discovered that several high molecular weight cationic polymers, such as poly(L-lysine) and protamine, can enhance the transfection efficiency of several types of cationic liposomes by 2-28-fold in a number of cell lines *in vitro*. Small polycations such as spermine and a cationic decapeptide derived from SV40 T-antigen were only moderately active. The addition of poly(L-lysine) and protamine dramatically reduced the particle size of the complex formed between DNA and cationic liposomes and rendered DNA resistant to the nuclease activity. The complexes composed of DNA, poly(L-lysine), and cationic lipids were purified from an excess of free liposomes with sucrose gradient ultracentrifugation. Purified complex formed at low cationic liposome ratio was poor in lipid content and only had weak transfection activity. Addition of free liposome to the purified complex significantly enhanced the transfection activity. In contrast, complexes formed at a higher initial ratio of liposome to DNA had a higher lipid content and were highly active in transfection; the activity was about 3−9-fold more active than the corresponding complex before purification. Negative stain EM studies revealed that the most active complexes prepared from 40 nmol of lipid, 0.5 μg of poly(L-lysine), and 1 μg of DNA and purified by gradient ultracentrifugation were spherical, electron dense, small (<100 nm in diameter) particles, and some of them were associated with lipid membranes. These highly active, stable, small-sized lipid/poly(L-lysine)/DNA complexes represent a new class of nonviral gene delivery vehicles that might be useful in gene therapy.

Cationic liposomes recently have become a popular gene transfer reagent and have been used as an alternative nonviral DNA delivery vector for gene therapy (Nabel et al., 1993; Caplen et at., 1995). We have developed a cationic liposome formulation, DC-chol/DOPE,[1] that contains a novel cationic cholesterol derivative [3β-[*N*-[(*N*′,*N*′-dimethylamino)ethane]-carbamoyl]cholesterol (DC-chol)] and a neutral lipid [dioleoylphosphatidylethanolamine (DOPE)]. This formulation is more efficient and less toxic than Lipofectin, the first cationic liposome of this class and a widely used reagent (Gao & Huang, 1991). The efficiency and safety of the *in vivo* use of DC-chol liposomes have been demonstrated in studies of experimental animals (Stewart et al., 1992) and two separate human gene therapy clinical trials (Nabel et al., 1993; Caplen et al., 1995). However, on the basis of the data collected so far, the need remains for further improvement of the transfection efficiency of this system.

Current efforts to improve the transfection efficiency of cationic liposomes focus on the synthesis of new cationic lipids and on the search for better cationic liposome formulations. Cationic liposomes that contain multivalent cationic lipids have better transfection activity than those composed of monovalent cationic lipids (Behr et al., 1989; Hawley-Nalson et al., 1993). However, research in this direction largely relies on trial and error with a slow rate of progress. Further improvement in the transfection activity of currently available cationic liposomes thus is desirable.

One of the drawbacks of DC-chol liposomes is their tendency to form large DNA/liposome complexes. At an optimal DNA to liposome ratio at which maximal transfection activity occurs, large complexes (about 0.6-1 μm) are formed. This creates difficulties for *in vivo* studies and clinical trials in which a high concentration of the complex is often used (Nabel et al., 1993; Caplen et al., 1995). DMRIE/DOPE, a cationic liposome that does not form large complexes with DNA at high concentrations, has recently been used for direct intratumor DNA transfer in a phase I/II gene therapy clinical trial for the treatment of malignancy (San et al., 1993). In the recent phase I clinical trial for the treatment of cyclic fibrosis conducted in London, a modified protocol was developed by Caplen and colleagues (Caplen et al., 1995), which used a high-pH diluent (pH 9) to prepare a DNA/liposome complex to minimize the problem of aggregation.

Freeze-fracture electron micrographs showed that some freshly prepared DNA/DC-chol/DOPE liposome complexes contained "spaghetti and meatballs" structures (Sternberg et al., 1994), suggesting that in the complexes the DNA molecules are not well condensed, but may exist in an extended conformation covered by lipids. In contrast to the DC-chol liposomes, cationic polymers such as poly(lysine), histone, and protamine are known to complex and condense DNA from an extended conformation to highly compact structures of about 30−100 nm in diameter (Wagner et al., 1991). Multivalent cationic lipids, such as several lipospermine derivatives, and liposomes composed of lipopoly-

† Supported by National Institutes of Health Grants HL 50256, CA 59327, and DK 44935 to L.H.

‡ Current address: Center for Lung Research, Vanderbilt University Medical Center, Nashville, TN 37232.

⊗ Abstract published in *Advance ACS Abstracts,* January 1, 1996.

[1] Abbreviations: DC-chol, 3β-[*N*-[(*N*′,*N*′-dimethylamino)ethane]carbamoyl]cholesterol; DMEM, Dulbecco's Modified Eagle medium; DMRIE, 1,2-bis(myristyloxy)-3-propyldimethylhydroxylammonium chloride; DOPE, dioleoylphosphatidylethanolamine; DOSPA, 2,3-bis(oleoyloxy)-*N*-[2-(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; LPD, liposome/poly(lysine)/DNA; NLS, nuclear localization signal; PLL, poly(L-lysine).

0006-2960/96/0435-1027$12.00/0

(lysine)/DOPE can also condense DNA into complexes of 80−300 nm (Behr et al., 1989; Hawley-Nalson et al., 1993; Behr, 1994; Zhou & Huang, 1994). We hypothesized that the introduction of cationic polymers at appropriate ratios to the DC-chol/DOPE cationic liposomes and DNA mixture may alter the overall structure of the liposome/DNA complexes and, thus, change the biological activity of the complexes. We report here the effect of polycations on complex formation and on the potentiation of transfection activity of cationic liposome-mediated DNA delivery.

MATERIALS AND METHODS

Chemicals. 1,2-Dioleoyl-3-phosphatidylethanolamine (DOPE) was purchased from Avanti Polar Lipids, Inc. 3β-[*N*-[(*N*′,*N*′-dimethylamino)ethane]carbamoyl]cholesterol (DC-chol) was synthesized as described (Gao & Huang, 1991). Poly(L-lysine) (PLL) hydrobromide (MW 3400, 25 600, 200 000), poly(D-lysine) hydrobromide (MW 14 800), poly(L-ornithine) hydrobromide (MW 28 000), protamine sulfate from salmon sperm, histone from calf thymus, and Pro-Lys-Lys-Lys-Arg-Lys-Val-Glu-Asp-Pro-Tyr-Cys, a synthetic decapeptide derived from SV40 T-antigen that encodes a nuclear localization sequence, were supplied by Sigma. Hexadimethrine bromide (polybrene) was purchased from Aldrich. Polyamidoamine dendrimer (generation 6, MW 58 000) was supplied by Polysciences. Luciferase assay kit was provided by Promega. Dulbecco's Modified Eagle medium (DMEM), F12 nutrient mixture, CHO-S-SFM medium, and Hank's balanced salt solution (HBSS) were from Life Technologies, Inc. Fetal bovine serum was from Hyclone Laboratories, Inc. All other chemicals were reagent grade. Lipofectin and LipofectAMINE were obtained from Life Technologies, Inc. [^{3}H]Cholesteryl hexadecyl ether was supplied by Amersham and was purified by TLC before use. DC-chol/DOPE liposomes were prepared in filtered distilled water by sonication as described (Gao & Huang, 1991).

Purification and Iodination of Plasmid DNA. Plasmid pRSVL, which contains the luciferase cDNA under the control of the Rous sarcoma virus long terminal repeat, has been described (De Wet et al., 1987). Plasmid was isolated from *Escherichia coli* by alkaline lysis and purified by cesium chloride density gradient ultracentrifugation (Sambrook et al., 1989). Plasmid DNA was labeled with ^{125}I by using a published method (Prensky, 1976). Labeled DNA was purified from free iodine by a spin column (Bio-Spin-P30) and two consecutive ethanol precipitations. The purified ^{125}I-labeled DNA was >95% precipitable by cold trichloroacetic acid and contained roughly equal amounts of supercoiled and relaxed closed circular DNA, as examined by gel electrophoresis and autoradiography (data not shown).

DNA Gel Retardation and Protection Assay. Two micrograms of pRSVL (0.5 mg/mL in 20 mM Tris-HCl, pH 7.4) was mixed with an equal volume of a solution (6 μL, in distilled water) containing the indicated amounts of liposomes, 1 μg of PLL, or the indicated amounts of liposomes plus 1 μg of PLL. After a 30 min incubation at 4 °C, sampels were mixed with 2 μL of sample loading buffer containing 40% sucrose, 0.25% bromophenol blue, and 200 mM Tris−acetate buffer containing 5 mM EDTA (pH 7.8) and analyzed by electrophoresis on a 0.6% agarose gel in 40 mM Tris−acetate/1 mM EDTA buffer. Some of the DNA/liposome or DNA/liposome/PLL complexes was incubated with an equal volume of fetal bovine serum at 37 °C for the indicated time. After the incubation SDS was added to the samples to a final concentration of 1% to release DNA from cationic liposomes and PLL. Samples were analyzed by gel electrophoresis as before.

Synthesis and Iodination of Poly(L-lysine) Modified by Bolton−Hunter Reagent. Fifty milligrams of PLL (MW 26 500) was reacted with Bolton−Hunter reagent (Pierce) at a 4/1 ratio (w/w) in 50 mM sodium bicarbonate at room temperature for 2 h. Excess Bolton−Hunter reagent was removed by dialysis against distilled water at 4 °C for 48 h. The resulting modified PLL was lyophilized and redissolved in 20 mM sodium acetate buffer (pH 5.0) at a concentration of 1 mg/mL. Concentration of hydroxyphenyl groups in the modified PLL was estimated by a UV spectrophotometric assay at 278 nm. Final modified PLL had an amine to hydroxyphenyl ratio of about 8/1. One milligram modified PLL was iodinated with 200 μCi of ^{125}I by using IODOGEN (Pierce) according to the manufacturer's protocol. Labeled PLL was separated from free iodine by a Sephadex G25 column (1.6 × 15 cm), using 20 mM Tris-HCl (pH 7.4) as eluent.

Preparation and Purification of Liposome/Poly(L-lysine)/DNA Complex. Twenty micrograms of pRSVL plasmid DNA diluted in 0.5 mL of 20 mM Tris-HCl buffer (pH 7.4) was added to a 0.5 mL solution containing 10 μg of PLL (MW 25 600) and the indicated amount of DC-chol/DOPE (2/3, mol/mol) liposomes. Trace amounts of ^{125}I-labeled DNA and ^{3}H-labeled DC-chol/DOPE liposomes were included for quantitation. The two solutions were quickly mixed and incubated at room temperature for 30 min. The resulting mixture was loaded on top of a 3 mL 5−30% linear sucrose gradient and centrifuged at 100000*g* at 20 °C for 30 min. Fractions were collected from top to bottom of the gradient. Both DNA and lipid contents were measured for each fraction on the basis of the radioactivity. The amount of PLL in fractions was measured in some experiments by using a trace amount of ^{125}I-labeled PLL.

Cell Culture and in vitro Transfection. Chinese hamaster ovary (CHO) cells and HeLa cells were from American Type Culture Collection. 2008, a human ovarian carcinoma cell line, was a kind gift from Dr. P. A. Andrews (Georgetown University, Washington, DC). 293, a human embryonic kidney carcinoma cell line, was kindly provided by Dr. R. J. Samulski (University of North Carolina, Chapel Hill, NC). K562, a human chronic myeloleukemia cell line, was kindly provided by Dr. J. Yalowich (University of Pittsburgh, Pittsburgh, PA). Mouse lung cells, primary cells derived from Balb/c mice, were kindly provided by Dr. S. J. Kennel (Oak Ridge National Laboratory, Oak Ridge, TN). A431, a human epidermoid carcinoma cell line, was a kind gift from Dr. G. Carpenter (Vanderbilt University, Nashville, TN). All cells were maintained in medium containing 10% fetal bovine serum supplemented with antibiotics. HeLa, A431, 293, K562, and mouse lung cells were cultured in DMEM medium. CHO cells were cultured with F12 medium, and 2008 cells were cultured with RPMI 1640 medium.

All transfections were performed on cultures of about 80% confluency. Cells grown in a 48-well plate were washed once with Hank's balanced salt solution (HBSS) and incubated with either DNA/liposome complex or unpurified or purified liposome/poly(L-lysine)/DNA (LPD) complex for 4 h. DNA/liposome complex or unpurified LPD complex

was prepared as follows: DNA diluted in CHO-S-SFM (2−4 μg/mL) was added in equal volumes to a solution containing the indicated amount of liposomes with or without PLL, mixed, and incubated at room temperature for 10 min. One-half milliliter of the mixture containing 0.5−1 μg of total DNA was added to each well. For transfection with the purified LPD complex, the complex containing 0.25−0.5 μg of DNA was diluted with 0.5 mL of HBSS/CHO-S-SFM (1/1, v/v) and added to each well. After transfection, cells were returned to culture with 10% featal bovine serum containing medium. Thirty-six hours after transfection, cells were washed with saline and lysed for 5 min at room temperature with 200 μL of lysis buffer containing 200 mM Tris-HCl (pH 7.8), 2 mM EDTA, and 0.05% Triton X-100. Cell lysates were then transferred to microcentrifuge tubes. After brief centrifugation, 10 μL of the supernatant was measured for protein concentration with Coomassie Plus protein assay reagent according to the manufacturer's protocol, using BSA as a standard. A 10 μL sample containing 2 μg of protein was assayed for luciferase activity by injecting 100 μL of reconstituted luciferase assay solution using an Autolumat LB 953 luminometer equipped with an automatic injector (Berthold). Counts were measured over a period of 20 s.

Negative Staining Electron Microscopy. For negative staining, 10 μL of the sample was applied to a copper grid covered with a Formvar support for 5 min. The adsorbed samples were washed and stained with a solution of 1% phosphotungstic acid. Electron micrographs were taken on a Jeol 100B instrument operating at 80 kV.

RESULTS

Potentiation of Cationic Liposome-Mediated Transfection in Vitro by Polycations. CHO cells were transfected with 1 μg of pRSVL plasmid DNA complexed with various amounts of cationic liposomes alone or cationic liposomes plus various amounts of PLL (MW 25 600). When PLL was not included, the transfection efficiency of three cationic liposome formulations followed the order of LipofectAMINE > DC-chol > Lipofectin. The peak transfection activities of LipofectAMINE, which contained a multivalent cationic lipid, 2,3-bis(oleoyloxy)-*N*-[2-(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate, (DOSPA), and DOPE were about 3.5- and 8.8-fold more efficient than those of DC-chol and Lipofectin liposomes, respectively (Figure 1a−c). For 1 μg of DNA, the optimal dose of cationic liposome that gave maximal activity of LipofectAMINE, Lipofectin, and DC-chol/DOPE was 12, 9, and 10 nmol of total lipids, respectively. When suboptimal doses of liposome were used, the DNA/liposome complex was less active regardless of the liposome formulation used.

In the presence of PLL (PLL to DNA ratio of 0.5−1/1, w/w), the level of luciferase gene expression was significantly enhanced in cells transfected with DNA and suboptimal doses of DC-chol liposomes (3.3 and 6.5 nmol of total lipid) (Figure 1a) and Lipofectin (4.5 and 6 nmol of total lipid) (Figure 1b). In both cases, the peak expression was 10−30-fold higher than that of the cells transfected with the same amount of DNA and liposomes without PLL. More importantly, these levels were 3−10-fold higher than the maximal level achieved with a DNA/liposome complex at optimal ratio (Figure 1a,b). These levels approached or exceeded

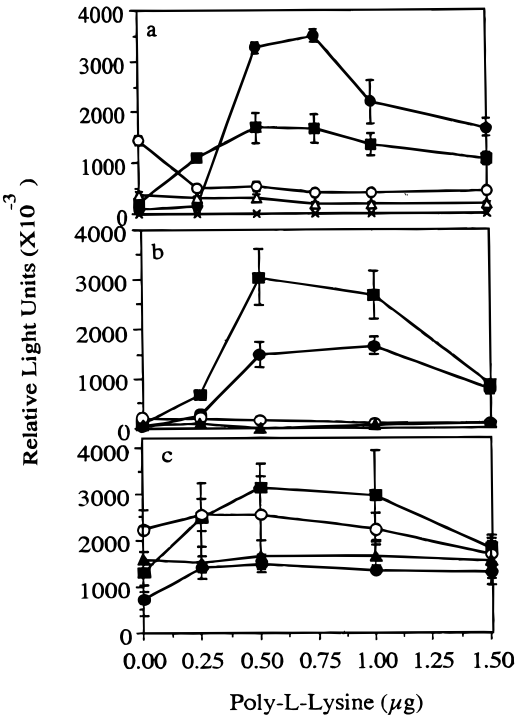

FIGURE 1: Potentiation of cationic liposome-mediated transfection by poly(L-lysine) on CHO cells. CHO cells (a−c) grown in a 48-well plate were transfected for 4 h with 1 μg of pRSVL plasmid complexed with the indicated amount of DC-chol/DOPE (a), Lipofectin (b), and LipofectAMINE (c) cationic liposomes and with the indicated amount of poly(L-lysine). Thirty-six hours after the transfection, luciferase activity was measured over a period of 20 s using 2 μg of cellular proteins from each cell lysate. Symbols: (a) ● 3.3 nmol, ■ 6.6 nmol, ○ 10 nmol, ▲ 12.5 nmol, × no DC-chol/DOPE (4.5/5.5, mol/mol) liposomes; (b) ● 4.5 nmol, ■ 6.0 nmol, ○ 8.5 nmol, ▲ 10 nmol Lipofectin, (c) ● 4 nmol, ■ 6 nmol, ○ 8.5 nmol, ▲ 10 nmol LipofectAMINE.

the maximal expression level obtained using the DNA/LipofectAMINE complex. A similar but moderate potentiation effect was also observed in cells treated with DNA and LipofectAMINE (Figure 1c). In the absence of liposomes, PLL at the doses tested caused only a background level of transfection, indicating that high transfection activity was absolutely dependent on the presence of cationic liposomes (Figure 1a). At higher doses of liposomes, combination of PLL and liposomes caused apparent toxicity to CHO cells (data not shown), and the potentiation effect by PLL was largely diminished. However, this was not the case for mouse lung cells; the best transfection occurred at a ratio of DNA/PLL/DC-chol liposome equal to 1 μg/0.5 μg/10 nmol (Figure 2). The difference between CHO cells and MLC may be due to the different level of tolerance for the DC-chol liposomes. Thus, additional PLL at a ratio of PLL/DNA = 0.5−1/1 (w/w) enhanced the transfection efficiency of all three cationic liposome formulations at suboptimal or optimal doses.

Table 1 showed that several other types of cell lines were transfected 2−5-fold more efficiently by the complex of DNA, liposomes, and polycations, such as PLL or protamine, than the conventional DNA/liposome complex without polycations. In one extreme case, the mouse lung cells, which were poorly transfected by the DNA/DC-chol complex, were readily transfected with DNA/DC-chol/PLL complex, and an up to 28-fold increase in the transgene expression was observed (Figure 2).

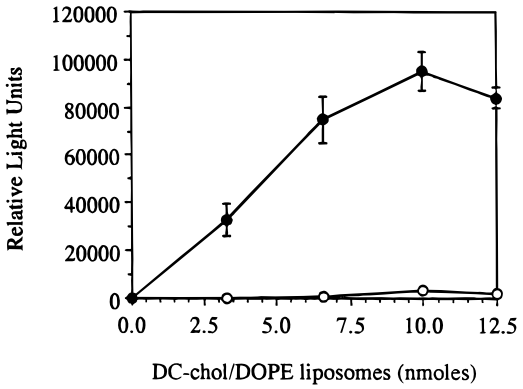

FIGURE 2: Potentiation of DC-chol liposome-mediated transfection by poly(L-lysine) on mouse lung cells. Mouse lung cells grown in a 48-well plate were transfected for 4 h with 1 μg of pRSVL plasmid complexed with the indicated amount of DC-chol/DOPE (3/2, mol/mol) (○) or 0.5 μg of poly(L-lysine) (MW 25 600) and DC-chol/DOPE liposomes (3/2, mol/mol) (●).

Table 1: Potentiation of Cationic Liposome-Mediated Transfection of Different Cell Lines by Polycations

	liposome/DNA[b]		liposome/polycation/DNA[c]	
cell lines[a]	liposomes (nmol)	luciferse activity counts (SD) × 10^6	luciferase activity counts (SD) × 10^6	fold increase[e]
293	10.0	27.00 (0.9)	107.50 (3.0)	4.0
BHK	10.0	1.90 (0.3)	4.10 (0.4)[d]	2.0
			0.87 (0.0)	
K562	12.5	0.79 (0.1)	6.16 (0.7)	7.8
A431	10.0	0.58 (0.1)	1.30 (0.1)	2.2
2008	10.0	0.23 (0.0)	0.63 (0.1)	2.7
CV-1	10.0	0.12 (0.0)	0.55 (0.1)[d]	4.6
			0.20 (0.0)	1.7
He La	5.0	0.12 (0.0)	0.36 (0.0)	3.0

[a] Cells were cultured in 48-well plates until about 80% confluency. [b] Cells were transfected with 1 μg of pRSVL plasmid DNA complexed with the indicated amount of DC-chol/DOPE liposomes (3/2, mol/mol) for 4 h. Cells were further cultured for 36 h in serum-containing medium. Luciferase expression was measured by using 2 μg of cellular protein as described in the text. [c] Cells were transfected for 4 h with 1 μg of pRSVL plasmid DNA complexed with 6 nmol of DC-chol/DOPE liposomes and 0.5 μg of poly(L-lysine) or [d] with 10 nmol of DC-chol/DOPE liposomes and 1 μg of protamine. Luciferase expression was measured as in ***b***. [e] Fold of increase of luciferase activity of cells transfected with liposome/polycations/DNA over that of cells transfected with liposome/DNA complexes.

Since both cationic liposomes and PLL will compete for binding with DNA, the protocol of complex preparation may affect the structure and function of the complex. To test whether this is the case, DNA was mixed with either cationic polymer or liposomes first. After incubation for 10 min, the second component was then added. After another 10 min incubation, the mixture was used to transfect cells. Results shown in Figure 3 indicate that, if DNA was incubated with liposomes first and then with PLL there was no potentiation effect, whereas incubation of DNA with PLL first and then with DC-chol liposomes resulted in about 80% of the transfection activity of the DNA/liposome/PLL complex prepared by simultaneously mixing all three components. Thus, the order of addition in complex formation was important for the optimal activity of the resulting complex.

PLL (MW 25 600) was not the only cationic polymer that could potentiate the transfection of cationic liposomes. Figure 4a,b shows the effect of a panel of such polycations on the liposome-mediated transfection of CHO cells. These

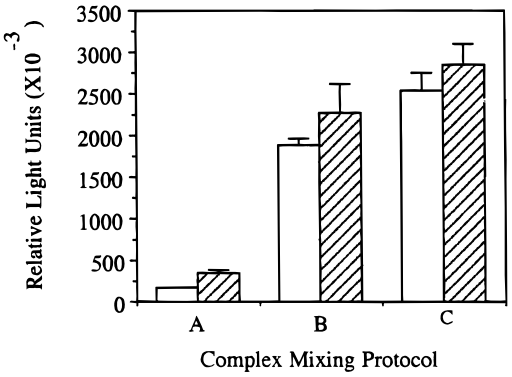

FIGURE 3: Transfection activity of liposome/poly(lysine)/DNA complexes. CHO cells were treated for 4 h with 1 μg of pRSVL complexed with 6 nmol of Lipofectin (open bar) or DC-chol/DOPE liposomes (slashed bar) for 5 min, (A) followed by incubation with 0.5 μg of poly(L-lysine) (MW 25 600) for another 5 min, (B) preceded by complexation with 0.5 μg of poly(L-lysine), or (C) complexed simultaneously with 0.5 μg of poly(L-lysine). Luciferase activity was measured after 36 h.

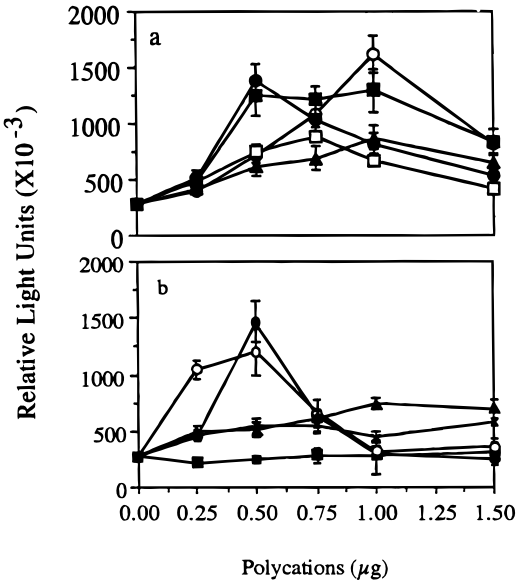

FIGURE 4: Effect of polycations on the potentiation of DC-chol/DOPE liposome-mediated transfection. CHO cells in a 48-well plate were transfected for 4 h with 1 μg of pRSVL and 6.6 nmol of DC-chol/DOPE liposomes (4.5/5.5, mol/mol) together with the indicated amount of the following: (a) □ poly(L-lysine) (MW 3400), ● poly(L-lysine) (MW 200 000), ■ poly(L-ornithine) (MW 28 000), ▲ calf thymus histone, ○ protamine (free base); (b) ● poly(D-lysine) (MW 14 800), ○ polybrene, × spermine, ▲ Pro-Lys-Lys-Lys-Arg-Lys-Val-Glu-Asp-Pro-Tyr-Cys, a synthetic decapeptide derived from SV40 T-antigen that encodes a nuclear localization sequence, ■ dendrimeric polymer (generation 6). Luciferase activity was measured after 36 h posttransfection.

polymers can be classified into three groups on the basis of their activity of potentiation. High molecular weight cationic polypeptides, such as PLL (MW 3400, 25 600, 200 000), poly(D-lysine) (MW 14 800), poly(L-ornithine) (MW 28 000), polybrene, histone, and protamine, were among the active polymers tested. Oligocations such as spermine and Pro-Lys-Lys-Lys-Arg-Lys-Val-Glu-Asp-Pro-Tyr-Cys, a synthetic decapeptide derived from SV40 T-antigen that encodes a nuclear localization sequence, were moderately active. A polyamidoamine dendrimeric polymer (generation 6, MW 58 000) was inactive.

Formation of the DNA/Cationic Liposome/Polycation Complex. We examined the complex by several physical

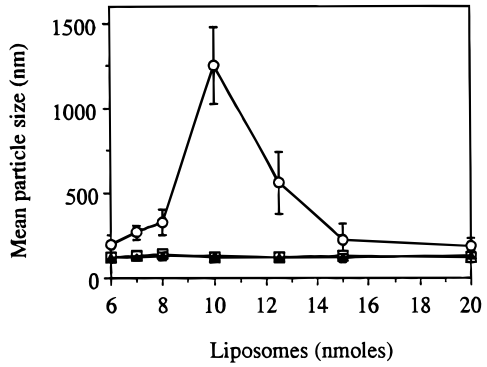

FIGURE 5: Effect of poly(L-lysine) and protamine on the particle size of a complex of DNA and DC-chol/DOPE liposomes. Two micrograms of pRSVL plasmid DNA in 200 μL of 20 mM Tris-HCl (pH 7.4) was mixed with (○) the indicated amount of DC-chol/DOPE liposomes (4.5/5.5, mol/mol) diluted in the same buffer (control) or with the same amount of liposomes containing (□) 1 μg of poly(L-lysine) (MW 25 600) or (▲) 2 μg of protamine. The mixtures were incubated at room temperature for 10 min. The size of the complex was measured by laser dynamic light scattering.

methods to gain insight into the nature of the interaction among the components and the mechanism of the enhanced transfection activity. We first looked at the effect of polycations on the formation of DNA/liposome complex. Figure 5 shows that the size of the DNA/liposome complex was dependent on the ratio of DNA to DC-chol liposomes. At a ratio of 1 μg of DNA/10 nmol of total lipid, at which maximal transfection activity occurred, the size of the complex reached a maximum (average mean diameter = 1200 nm). The size of the complex was significantly reduced by the addition of PLL. In the presence of PLL at a ratio of 1 μg of DNA/0.5 μg of PLL (MW 25 600), the size of the complex remained small at all doses of DC-chol liposomes added. Addition of protamine at 1 μg/μg of DNA had a similar effect of reducing the complex size. Thus, PLL and protamine prevented the formation of large complexes, possibly by direct participation in the process of complex formation.

The complex was further characterized by gel retardation and degradation assays. Plasmid DNA was completely retarded by DC-chol liposomes at a ratio of 1 μg of DNA/6.6 nmol of lipid (total charge ratio of about 1/1.2) or by PLL (MW 25 600) at a weight ratio of DNA/PLL equal to 1/0.5 (charge ratio of 1/0.8). A combination of 3.3 nmol of DC-chol liposomes and 0.5 μg of PLL also completely retarded 1 μg of DNA (Figure 6). Addition of PLL also rendered DNA resistant to nuclease activity. Figure 7 shows that free DNA and DNA/liposome complex formed at suboptimal ratios were sensitive to the DNase activity present in the fetal bovine serum, which converted the plasmid from the supercoiled form to the open circle form. DNA/liposome complexes formed at optimal ratio were more resistant to the DNase activity; however, the protection was not complete. About 50% of the supercoiled DNA was converted to the open circle form after 1 h of incubation with 50% fetal bovine serum at 37 °C. Only 0.5 μg of PLL (MW 25 600), alone or in combination with 3.3 nmol of DC-chol liposomes, provided complete protection of 1 μg of plasmid DNA against DNase activity.

Fractionation and Analysis of Liposome/Poly(L-lysine)/DNA Complexes. The formation of complexes among liposomes, PLL, and DNA was demonstrated directly by sucrose density gradient ultracentrifugation. Under conditions of 100000*g* for 30 min, free DNA, PLL, or liposomes remained on top of the linear 5−30% sucrose gradient (data not shown). In comparison, the mixture of DNA/PLL/cationic liposomes formed more dense materials, which sedimented as a broad band in the middle of the gradient. The exact position of the DNA peak was influenced by the ratio of liposome to DNA. At high liposome to DNA ratios, the peak DNA content migrated to the upper portion of the gradient, whereas at low liposome to DNA ratios, the complex tended to form a more dense structure and migrated to the lower portion of the gradient (Figure 8a). In contrast to DNA, the majority of the liposomes remained above the sucrose gradient. These were possible free liposomes since very little DNA was found in these fractions. However, a small but significant amount of liposomes entered the gradient and colocalized with DNA (Figure 8b). The final amount of liposomes that associated with DNA was proportional to the initial amount of liposomes used to form the complex. The ratio of lipid to DNA varied among the fractions collected along the gradient. Fractions located on the upper portion of the gradient were more enriched with lipids than the ones located on the lower portion of the gradient. This uneven distribution of lipids in the gradient was not due to the contamination of free liposomes from the top of the gradient during the process of fraction collection, but was due to the difference in the density of the complexes as a result of different amounts of lipid associated with them. When fractions were collected from bottom to top, the distribution pattern of lipid content remained the same (data not shown). Moreover, when fractions collected from a given gradient were diluted and separated on a second gradient of identical composition, distribution patterns of DNA and lipids similar to that of the first separation were obtained (data not shown). Unlike liposomes, the majority of PLL added associated with DNA, suggesting that PLL binds DNA with a higher affinity than DC-chol cationic liposomes (Figure 8c). These results indicate that mixing of liposomes, PLL, and DNA produces stable complexes that can be separated and purified from the starting components by sucrose density gradient centrifugation. We name the purified complex as a liposome/PLL/DNA (LPD) complex. To distinguish different LPD complexes containing different amounts of lipids, the number in nanomoles of initial liposome used in the preparation of the complex is also listed. Thus, for example, LPD6.6 means that 6.6 nmol of total lipid was used to prepare this particular complex.

All LPD complexes apparently contained cationic charges in excess. For example, LPD13.2 fraction number 5 contains about 3 μg of DNA (∼9 nmol of negative charges), 13 nmol of total lipids (∼5 nmol of cationic charges), and 1.4 μg of PLL (∼7 nmol of cationic charges). Table 2 lists the composition of fraction number 5 from samples prepared from various liposome to DNA ratios. These fractions were chosen because, in general, high transfection activity was associated with these particular fractions (see the following). The same fractionated samples were also examined morphologically with electron microscopy (the following). These purified complexes appeared to be physically stable; no increase in size distribution was observed after storage at 4 °C for a period of 3 months (data not shown).

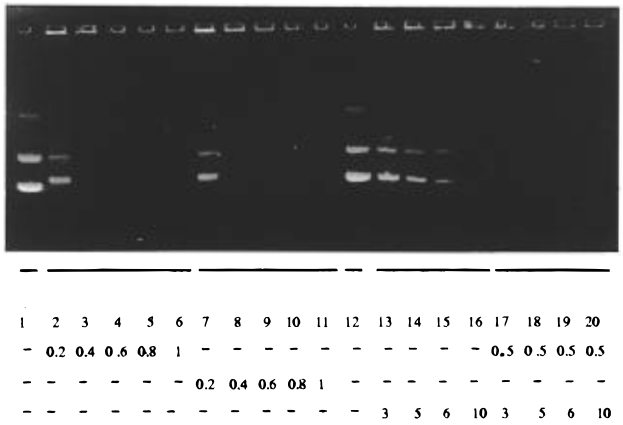

FIGURE 6: Gel retardation assay of DNA complexed with PLL and DC-chol liposomes. One microgram of pRSVL plasmid DNA was complexed with the indicated amounts of poly(lysine) (lanes 2−4), indicated amounts of DC-chol/DOPE liposomes (3/2, mol/mol) alone (lanes 5−8), or a mixture of 0.5 μg of PLL and indicated amounts of DC-chol/DOPE liposomes (lanes 9−11). The same amount of free DNA was loaded on lanes 1 and 12. DNA was run on 0.6% agarose gel in Tris−acetate−EDTA buffer.

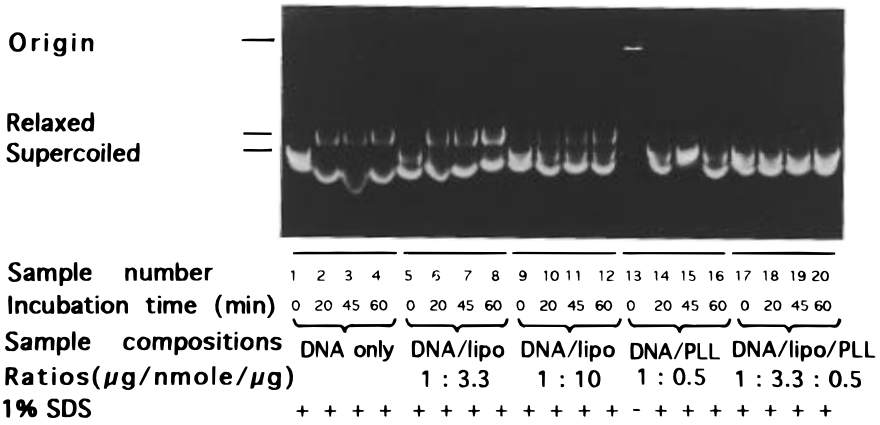

FIGURE 7: DNA degradation protection assay. Two micrograms of pRSVL plasmid was first incubated on ice for 30 min with 5 μL of water (lanes 1−4) or 5 μL of solution containing 6.6 nmol of liposomes (lanes 5−8), 20 nmol of liposomes (lanes 9−12), 1 μg of poly(lysine) (lanes 13−16), or 6.6 nmol of liposomes and 1 μg of poly(lysine) (lanes 17−20). At time 0, 5 μL of fetal bovine serum was added to the DNA solutions and incubated at 37 °C. At an indicated period of time, all samples (except lane 13) were withdrawn and mixed with 1.3 μL of 10% SDS and 2 μL of loading dye. Treated samples were incubated on ice until gel electrophoresis on 0.6% agarose gel in Tris−acetate−EDTA buffer. DC-chol/DOPE liposomes (3/2, mol/mol) and poly(L-lysine) (MW 25 600) were used for the experiment.

To test the biological activity of these LPD complexes, LPDs prepared from fixed amounts of DNA and PLL (weight ratio 1/0.5) and various amounts of DC-chol liposomes were separated by using sucrose density gradient centrifugation. CHO cells were transfected by using a portion of each fraction that contained 0.25 μg of DNA. Of all the fractions from the LPD complexes prepared by using 6.6 nmol of DC-chol liposome/μg of DNA (LPD6.6), only one fraction located close to the top of the gradient showed transfection activity. Other fractions located at lower portions of the gradient, although they contained more DNA (Figure 8a), had very weak transfection activities (Figure 9a). However, the fractions of LPD prepared by using 13.2, 25, or 40 nmol of DC-chol liposome/μg of DNA (LPD13.2, LPD25, LPD40, respectively) had higher transfection activities. The active fractions were mostly located in the middle or bottom of the gradient (Figure 9b−d) and were not the ones that contained the most DNA (compare with Figure 8a).

To test whether the poor transfection activities of fractions of LPD6.6 and some fractions of LPD13.2 and LPD25 located at the bottom of the gradient were due to the lack of a sufficient amount of lipid in these fractions, additional free DC-chol liposomes (3.3 nmol of lipid/μg of DNA) were added to each fraction and used to transfect cells. The fractions of LPD6.6 that previously showed weak activity became highly active in the presence of additional free liposomes. Additional free liposomes also significantly enhanced the transfection activity of the less active fractions of LPD13.2 and LPD25 at the bottom of the gradient, such as fraction number 6 in Figure 9b and fraction number 7 in Figure 9c. Additional free liposomes did not further enhance the transfection activity of the fractions located in the upper portion of the gradient, LPD13.2, LPD25, and most fractions of LPD40, suggesting that a sufficient amount of lipid was already present in these fractions. When test with CHO cells with 0.25 μg of DNA per well, purified LPD complexes showed approximately 9−22-fold and 3−9-fold higher activities than the freshly prepared DNA/liposome complexes of the same ratio and the unpurified LPD complexes, respectively (Figure 9a−d).

The various DNA-containing complexes were negatively stained and visualized by transmission electron microscopy. The DNA/PLL complex formed at weight ratio 1/0.5 appeared to be a rod- and donut-shaped structure, which was been previously described (Wagner et al., 1991). The purified LPD complexes showed various shapes of electron dense structures, ranging from elongated rod-shaped to ball-shaped particles. Figure 10 shows ultrastructural features

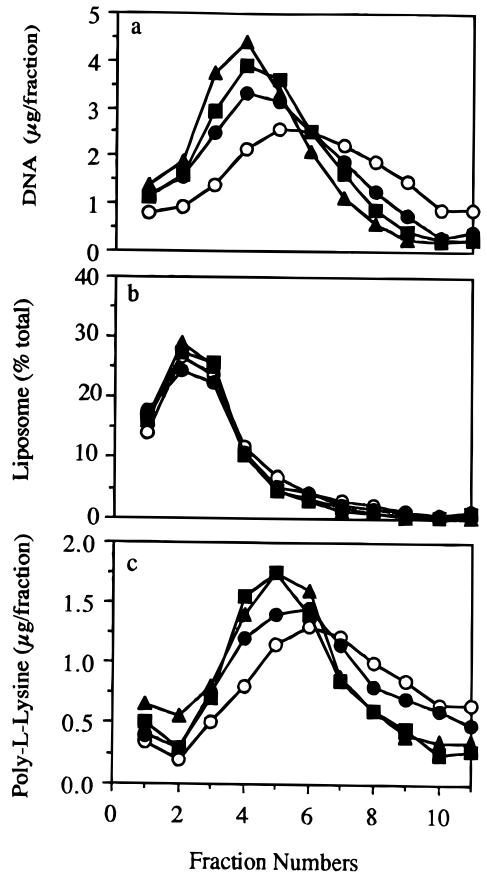

FIGURE 8: Analysis of LPD complexes using sucrose density gradient ultracentrifugation. Twenty micrograms of pRSVL was diluted in 500 μL of 20 mM Tris-HCl (pH 7.4) and added to 500 μL of solution containing liposomes (DC-chol/DOPE, 4.5/5.5, mol/mol) and 10 μg of poly(L-lysine) (MW 25 600) in 20 mM Tris-HCl buffer (pH 7.4). Final ratio of DNA to liposome was (○) 6.6, (●) 13.2, (■) 25, and (▲) 40 nmol of total lipid/μg of DNA. After 30 min, samples were loaded onto the top of a 3 mL 5−30% linear sucrose density gradient. After centrifugation for 30 min at 20 °C at 100000*g*, fractions were collected from the top to the bottom of the gradient. Amounts of DNA, liposomes, and poly-L-lysine) were estimated by using trace amounts of (a) ^{125}I-labeled DNA, (b) ^{3}H-labeled liposomes, or (c) ^{125}I-labeled PLL.

of the purified LPD40. Small, ball-shaped particles (with a diameter of about 52 nm) and small dense particles surrounded by a lightly strained, ring-shaped structure (with a diameter of about 79 nm) were seen in the LPD40 preparation. Some of the ring structures had characteristics of a typical membrane staining pattern (see arrows in Figure 10). The complex prepared at lower initial ratios of liposome to DNA, such as LPD6.6 and LPD13.2, showed more elongated structures and an absence of any lipidic structure. All of the LPD complexes, however, were under 100 nm in size (data not shown).

DISCUSSION

We present the development and characterization of a new strategy to prepare small-sized and more active liposome/PLL/DNA (LPD) complexes as an effort to further improve the performance of the DC-chol/DOPE cationic liposome formulation. The addition of a polycation has a dramatic effect on the size and activity of the DNA/lipid complex. The transfection activity of the resulting LPD complex issignificantly higher than that of the freshly prepared liposome/DNA complex.

Potentiation of Lipofection by Polycations Is a General Phenomenon. The reporter DNA pRSVL complexed with PLL and cationic liposomes, including Lipofectin, LipofectAMINE, and DC-chol/DOPE at various molar ratios, had a higher transfection activity in CHO cells than the corresponding DNA/liposome complexes (Figure 1a−c). These liposome formulations are composed of cationic lipids of very different structures and contain different amounts of helper lipid DOPE. Table 1 also shows that most of the cell lines tested were transfected more efficiently with a DNA/liposome/PLL mixture than a DNA/liposome complex. This indicates that PLL has general activity to potentiate cationic liposome-mediated transfection of mammalian cells *in vitro*.

Biodegradable poly(L-lysine), poly(L-ornithine), and cationic proteins (Figure 4a) and non-degradable poly(D-lysine) and polybrene (Figure 4b) were equally active in potentiating the lipofection process. However, treatment with liposomes and high doses of poly(D-lysine) or polybrene had resulted in less extractable cellular proteins (data not shown), indicating that the transfection using liposomes and non-degradable polymers was more toxic than that of liposomes and poly(L-lysine) or protamine. It is surprising that a cationic polyamidoamine dendrimer (generation 6) did not show any potentiation effect, although it has been reported that the polymer by itself can complex with DNA and mediate DNA delivery (Haensler & Szoka, 1993). One possible explanation could be that the polymer contains shielded tertiary amino groups with a p$K_a \sim 6$ (Tomalia et al., 1990). These groups may prevent the acidification process inside the endosomes, a critical step that leads to the maturation of the endosomes to lysosomes. Although such an activity has been suggested to contribute to some degree its capability of DNA delivery (Haensler & Szoka, 1993), it may inhibit the transfection activity of certain types of cationic liposomes. Agents with such lysosomotropic activity, such as chloroquine or ammonium chloride, are known to inhibit the transfection activity of DC-chol liposomes (Farhood et al., 1995). In contrast to these high molecular weight polymers, simple polycations such as spermine and a synthetic cationic decapeptide derived from SV40 T-antigen, which carries three positive charges, were weakly active. Although spermine and spermidine are known to condense DNA, the spermine/DNA complex is not stable at physiological ionic strength, such as the one in the culture medium used for transfection (Milson & Bloomfield, 1979). This may explain the weak potentiation activity of polycations of small molecular weight. These results indicate that, with the exception of dendrimer, polycations of high molecular weight are generally active in potentiation of the transfection activity of several different cationic liposome formulations.

Possible Mechanism of PLL-Mediated Potentiation of Lipofection. There are several possibilities that may explain the potentiation effect of PLL on the transfection activity of liposomes. First, on the basis of the current endocytosis model, there is a size limitation for particles to be taken up efficiently by cells (Machy & Leserman, 1983). Direct size measurement of liposome/PLL/DNA mixtures showed that PLL significantly reduced the size of the complex formed over a wide range of liposome to DNA ratios (Figure 5). We have also obtained electron microscopic evidence that LPD particles were smaller than or close to 100 nm in

Table 2: Compositions of LPD Complexes Fractionated by Sucrose Density Gradient Ultracentrifugation

complex[a]	liposome/DNA (nmol/μg)	DNA[b] (μg/fraction)	liposome[b] (nmol/fraction)	poly(L-lysine)[b] (μg/fraction)	charge ratio[c] (negative/positive)
LPD6.6	6.6	2.5	7.0	1.21	1/1.16
LPD13.2	13.2	3.1	12.8	1.40	1/1.44
LPD25	25.0	3.5	24.1	1.75	1/1.65
LPD40	40.0	3.1	38.5	1.71	1/2.63

[a] Liposome/poly(L-lysine)/DNA (LPD) complexes were prepared as described in Figure 8. [b] Concentrations of DNA, liposome, and poly(L-lysine) were measured in fraction number 5 of each DC-chol/DOPE liposomes, and ^{125}I-labeled poly(L-lysine), respectively. [c] Charge ratios were calculated using an average molecular weight of 330 for each nucleotide and 200 for each lysyl residue (in HBr salt form). Liposomes were composed of DC-chol/DOPE (2/3, mol/mol).

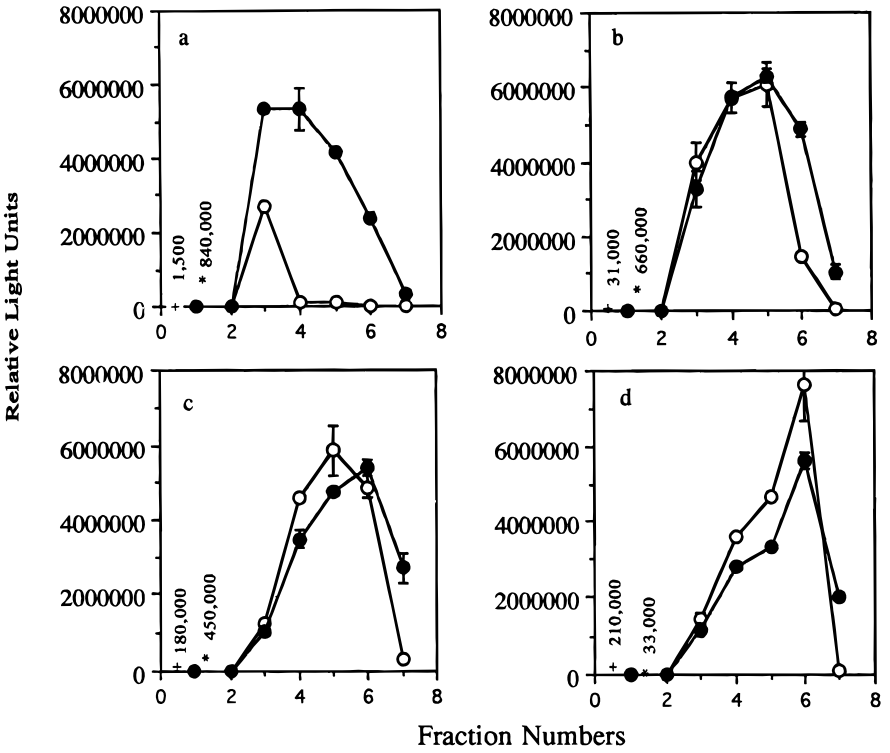

FIGURE 9: Transfection of CHO cells with density gradient-fractionated LPD alone or supplemented with free DC-chol liposomes. CHO cells grown in a 48-well plate were transfected with 0.25 μg of DNA of fractionated LPD complex alone or fractionated LPD complex plus 0.8 nmol of liposomes (DC-chol/DOPE, 4.5/5.5, mol/mol). LPD complexes were prepared from a liposome to DNA ratio of (a) 6.6, (b) 13.2, (c) 25, or (d) 40 nmol of total lipids/μg of DNA and fractionated in a 5−30% linear sucrose density gradient. Cells transfected with freshly prepared DNA/liposome complex or with unpurified LPDs served as controls.

diameter (see the following). These particles of small size should be more favorable to enter the cells via an endocytosis pathway than the larger ones, such as the conventional DNA/liposome complex.

Since DNA encodes linear information, any damage in the coding region would inactivate the entire molecule. Other physical properties such as the configuration of the plasmid DNA may affect the expression of the delivered DNA inside the cell. Supercoiled plasmids, for example, were more active in transgene expression after intramuscular injection than plasmids in the linear form (Wolff et al., 1992). DC-chol liposomes at a suboptimal ratio did not protect the DNA from nuclease activity in the FBS, and even at optimal ratio DNA was not fully protected. In contrast, PLL has superior ability to protect DNA. PLL by itself at a ratio of PLL to DNA equal to 0.5/1 (w/w) offered complete protection of the supercoiled conformation of the plasmid DNA. Addition of liposomes did not alter the capability of DNA protection by PLL (Figure 7). Since DNA is more well protected in LPDs than in DNA/liposome complexes, the chance of delivering an undamaged, functional DNA into the cell might be higher with LPDs than with DNA/liposome complexes.

To function inside the cell, a DNA molecule must be delivered to the nucleus and released from the carrier. PLL may act on both steps and contribute to the potentiation of lipofection. Earlier work indicated that nuclear localization is a major rate-limiting step for the naked DNA microinjected into the cytoplasm (Capecchi, 1980). A plasmid may be relocated into the nucleus passively during the process of cell division, or alternatively DNA may be bound by newly synthesized host nuclear proteins in the cytoplasm, which may provide a nuclear localization signal (NLS) and facilitate the entry of DNA into the nucleus via an active process. The nuclear localization signals generally are a cluster of positively charged amino acid sequences or two clusters of positive amino acid sequences separated by a spacer (Dingwell & Laskey, 1991). Particles as large as 20 nm, such as NLS-containing peptide-coated colloidal gold, can be targeted to the nucleus efficiently (Richardson et al., 1988). Since protamine contains a NLS and PLL resembles a NLS, there is a possibility that these molecules might be able to

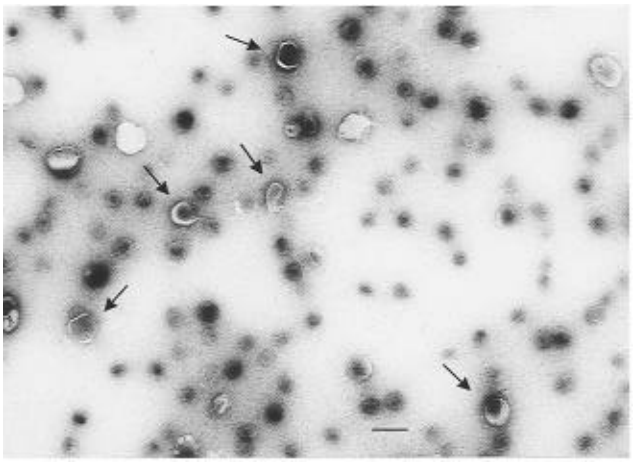

FIGURE 10: Electronic micrographs of free liposomes, DNA/liposome, DNA/PLL, and LPD complexes. Purified LPD complexes were examined with negative stain EM. The complexes were prepared from 20 μg of DNA, 10 μg of PLL (MW 26 500), and 800 nmol of DC-chol/DOPE liposomes (2/3, mol/mol) and purified with sucrose gradient ultracentrifugation. The samples were stained with 1% phosphotungstic acid. Arrows show particles with membranous structures. Bar = 100 nm.

facilitate the active nuclear uptake of the LPD complex into the nucleus. Finally, once the complex enters the nucleus, DNA may have to dissociate from the cationic carriers in order to be read by the host transcription machinery. Protamine or PLL, which are biodegradable and structurally more similar to other nuclear proteins than cationic liposomes, might be more readily dissociated from DNA. Thus, polycations may play several roles in the process of potentiation of lipofection, such as reducing the complex size, providing an excess of cationic charges and therefore enhancing the cellular uptake by the endocytosis pathway, protecting DNA from damage during this process, and possibly helping the release and relocalization of the delivered DNA.

The finding of potentiation of polycations on the transfection activity of cationic liposomes is interesting because it demonstrates for the first time that, in the presence of an appropriate amount of polycations, successful transfection can be achieved with suboptimal ratios of cationic liposomes. It has become clear that cationic liposome plays several roles in the process of transfection, such as condensing (Gershon et al., 1993) and protecting DNA, binding to the cell surface (Felgner et al., 1987), triggering endocytosis (Zhou & Huang, 1994), and releasing the DNA/lipid complex from endosome (Zhou & Huang, 1994; Farhood et al., 1995). Most of the complexation functions can be achieved by a simple polycation such as PLL through charge interactions. At an appropriate ratio, cationic liposomes may participate in the process of complex formation, resulting in structures such as those of purified LPD40 as shown in Figure 10, and presumably provide an efficient endosome-rupturing function to the complex. The combination of these functions provided by PLL and liposomes may contribute to the enhanced activities of the LPD complexes in transfection.

Our data also showed the importance of cationic liposome for a high level of transfection. We have shown that PLL/DNA complex did not transfect cell significantly unless a small amount of cationic liposome was added (Figure 1a). It is interesting to note that, while unpurified LPD6.6, which contains free liposomes, is very active in transfection, once the LPD6.6 was purified from the free liposomes, the fractions that contained most of the DNA lost most of their activity except for one fraction collected near the top of the gradient (Figure 9a). Since most of the purified LPD6.6 contained a low lipid content, the weak transfection activity might be due to the lack of a lipid component in the final complex. If this is the case, the activity of the inactive fractions could be restored by adding free liposomes. The results in Figure 9a−c indeed show that there was a significant increase in transfection activity in many fractions after additional liposomes were added. We hypothesize that LPDs and free liposomes might enter into the same endosome compartment. A subsequent fusion or disruption function of DC-chol/DOPE liposomes caused the rupture of the endosome and the release of LPD complex into the cytoplasm. Consistent with these data, successful transfection of CHO cells was observed with complexes prepared by adding DNA to PLL first and then to liposome, in which the DNA/PLL complex coexists with free liposomes, but not with complexes prepared from the same components but mixed on the reverse order, in which free liposomes were presumably consumed in the complexation with DNA (Figure 3). A similar "helper" function of cationic liposomes has been described for the delivery of DNA/liposome complexes (Farhood et al., 1995). Addition of free liposomes failed to enhance the transfection activity of most fractions of LPD40, suggesting that, at this ratio of DNA to liposome, the purified LPD40 contained a sufficient amount of lipids for endosome rupture. This is confirmed by EM studies as demonstrated in Figure 10, where membranous structures are clearly seen.

In this work, a new type of condensed DNA delivery vector has been described. We have demonstrated that small and highly active LPD complexes can be prepared by a simple procedure. Like other conjugated PLL/DNA complexes, the LPD complexes contain features of DNA condensation, excellent DNA protection, cell surface binding, and perhaps endosome-escaping activities. Liposomes are generally regarded as safe and nonimmunogenic. Results from recent clinical trials have confirmed the safety aspects of using DC-chol/DOPE liposomes in humans (Nabel et al., 1993; Caplen et al., 1995). PLL is not known to be immunogenic (Sela et al., 1962). The LPD complex therefore is likely to be non- or low immunogenic as well. The small particle size and high transfection activity of these LPD particles suggest that these complexes may be superior vectors for some applications of gene delivery compared to the regular DNA/liposome complexes. One example might be for *in vivo* transfection of hepatocytes, in which particle size is a dominant factor, to determine whether a particle can gain access to the surface receptors of hepatocytes (Perales et al., 1994).

In conclusion, the LPD complexes have good safety features and improved delivery efficiency. These stable and small-sized LPD complexes represent a new class of nonviral gene delivery vehicles that might be useful in gene therapy.

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BI952436A