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Mechanism of DNA Release from Cationic Liposome/DNA Complexes Used in Cell Transfection[†,‡]

Yuhong Xu[§] and Francis C. Szoka, Jr.*[,||]

Department of Biophysics, State University of New York, Buffalo, New York 14214, and Department of Biopharmaceutical Sciences and Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143-0446

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ABSTRACT: To understand how DNA is released from cationic liposome/DNA complexes in cells, we investigated which biomolecules mediate release of DNA from a complex with cationic liposomes. Release from monovalent[1,2-dioleoyl-3-(trimethylammonio)propane] or multivalent (dioctadecylamidoglycyl-spermine) lipids was quantified by an increase of ethidium bromide (EtBr) fluorescence. Plasmid sensitivity to DNAse I degradation was examined using changes in plasmid migration on agarose gel electrophoresis. Physical separation of the DNA from the cationic lipid was confirmed and quantified on sucrose density gradients. Anionic liposomes containing compositions that mimic the cytoplasmic-facing monolayer of the plasma membrane (e.g. phosphatidylserine) rapidly released DNA from the complex. Release occurred near a 1/1 charge ratio (−/+) and was unaffected by ionic strength or ion type. Water soluble molecules with a high negative linear charge density such as dextran sulfate or heparin also released DNA. However, ionic water soluble molecules such as ATP, tRNA, DNA, poly(glutamic acid), spermidine, spermine, or histone did not, even at a 100-fold charge excess (−/+). On the basis of these results, we propose that after the cationic lipid/DNA complex is internalized into cells by endocytosis it destabilizes the endosomal membrane. Destabilization induces flip-flop of anionic lipids from the cytoplasmic-facing monolayer, which laterally diffuse into the complex and form a charge neutral ion pair with the cationic lipids. This results in displacement of the DNA from the cationic lipid and release of the DNA into cytoplasm. This mechanism accounts for a variety of observations on cationic lipid/DNA complex−cell interactions.

Cationic liposomes are one of the more promising nonviral systems for use in gene therapy (Behr, 1994; Alton & Geddes, 1995). Clinical studies using cationic liposome/DNA complexes are proceeding (Nabel et al., 1993; Alton & Geddes, 1995) even though the biochemical and biophysical mechanisms of complex formation and action are unresolved (Legendre & Szoka, 1992; Smith et al., 1993; Zhou & Huang, 1994; Zabner et al., 1995). The steps involved in the process leading to transfection in vitro include the initial interaction of the cationic liposome with the DNA to form a complex (Gershon et al., 1993; Felgner et al., 1994), the delivery of the complex into the cell (Felgner et al., 1987; Legendre & Szoka, 1992; Zhou & Huang, 1994; Zabner et al., 1995), and the release of the DNA from the complex so it is accessible to the transcription apparatus. An understanding of each of these steps is necessary in order to explain the observed cellular toxicity, to improve the in vivo gene transfer capability, and to comprehend the limits to gene transfer of the cationic lipid/DNA delivery systems.

There is a consensus that cationic liposome/DNA complexes enter the cell via an endocytotic pathway (Legendre & Szoka, 1992; Zhou & Huang, 1994; Wrobel & Collins, 1995; Zabner et al., 1995). However, the exact mechanism of DNA release from the complex, DNA escape from the endosome, and DNA entry into the nucleus remains unknown. Plasmid DNA must dissociate from the cationic liposome (Zabner et al., 1995) and localize inside the nucleus in order to be expressed (Capecchi, 1980). Remy and colleagues speculated that the plasmid may dissociate from the complex in the nucleus due to displacement of the plasmid DNA from the cationic lipid by genomic DNA (Remy et al., 1995). However, the fact that cationic lipid/DNA complexes microinjected directly into the nucleus showed low transfection efficiency does not support a major role of genomic DNA in dissociation of the complex (Zabner et al., 1995) and implies that, in the normal course of cationic lipid-mediated transfection, plasmid DNA is released from the complex prior to entry into the nucleus. An alternative explanation for release of DNA from the complex is that certain ionic molecules found in high concentrations in the cell, such as ATP, polypeptides, RNA, spermine, histone, or anionic lipids, displace the ionic interaction between plasmid DNA and the cationic lipid. In this study, we tested these various possibilities and demonstrate that anionic lipids normally found on the cytoplasmic-facing monolayer of cell membranes can very potently displace DNA from the cationic liposome/DNA complex. On the basis of these results, we

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* To whom correspondence should be addressed. Telephone: 415-476-3895. Fax: 415-476-0688. E-mail: Szoka@cgl.ucsf.edu.

[§] State University of New York.

[||] University of California.

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propose a mechanism to explain release of DNA from the cationic liposome complex in cells.

MATERIALS AND METHODS

Materials. All the lipids were purchased from Avanti Polar Lipids Inc. (Alabaster, AL) except for DOGS.[1] DOGS was synthesized following the procedure of Behr (1994). The CMV-CAT plasmid was prepared using a standard protocol (Sambrook et al., 1989). Salmon sperm DNA (ssDNA), tRNA, and protease K were from Gibco BRL (Gaithersburg, MD). The salmon sperm DNA was exposed to sonication in a bath sonicator (Laboratory Supplies Co. Inc., Hicksville, NY) for 30 min under an argon atmosphere. It was then extracted with phenol chloroform and precipitated with ethanol. The resulting salmon sperm DNA fragments were about 0.5 kb in length as measured using agarose gel electrophoresis. Cholesterol, dextran sulfate, histone, poly(glutamic acid), spermidine, and spermine were purchased from Sigma (St. Louis, MO). Heparin was obtained from ESI, Elkins-Sinn, Inc. (Cherry Hill, NJ), and ethidium bromide (EtBr) was from Molecular Probes Inc. (Eugene, OR).

Sample Preparation. All liposomes were prepared by drying a chloroform solution of lipids on the sides of a glass vessel, exposing the dry film to high vacuum for at least 2 h, resuspending the lipids in water (cationic lipids) or 20 mM Hepes (pH 7.4) (anionic and neutral lipids), and sonication under argon until a translucent lipid suspension (<30 min) was obtained (Legendre & Szoka, 1992). Cationic liposome compositions examined included DOTAP, DOTAP/DOPE (1/1), and DOGS. The exact composition is indicated in the text or figure legend. The cationic liposome/DNA complexes in most of the experiments were made by mixing equal volumes of the lipid and DNA at a 4/1 charge ratio (+/−), which according to previous studies (Gershon et al., 1993; Xu et al., 1995) was a condition in which the majority of DNA molecules are complexed. When negatively charged complexes were tested, they were prepared by mixing a large excess of DNA with cationic liposome and separating the excess of DNA from the complex using centrifugation on sucrose gradients as described below. The ionic strength and ion type of the complex were adjusted to either 130 mM NaCl or 130 mM potassium acetate and 10 mM $MgCl_2$ and 10 mM Hepes (pH 7.4) where indicated. The radius of curvature of the anionic vesicles used for release was greater and of the opposite direction than that found in an endosomal membrane. This is not thought to be a significant factor because of the strong electrostatic forces involved in the interaction.

EtBr Intercalation Assay. EtBr fluorescence at 610 nm was continuously monitored in a Spex fluorimeter (excitation wavelength of 500 nm; 2.5 mm excitation and 5.0 mm emission slits) to measure the effect of various additives on the extent of EtBr intercalation into DNA. The cationic lipid/DNA complex was stirred with a magnetic stirrer, and a small volume (less than $^1/_{200}$ of total volume) of the test substance was quickly injected into plasmid DNA or cationic liposome/DNA complex solutions containing 0.2 μg/mL EtBr. The fluorescence signal usually stabilized in less than 5 min after the addition of the test compound and was reported as the fraction of the maximum fluorescence signal I_{max} obtained when EtBr was bound to DNA in the absence of other molecules. Light scattering resulting from the addition of liposomes or other test substances caused less than a 5% change in the fluorescence signal.

Complex Sensitivity to DNAse I Digestion. DNA plasmid (20 μg) or preformed cationic liposome/DNA complexes containing the same amount of DNA were mixed with a charge excess (10−100-fold) of the compounds tested and then incubated with 10 units of DNAse I in a total volume of 1 mL in 50 mM Tris buffer (pH 7.4) containing 0.9 mM Mn^{2+}. An aliquot (200 μL) of the reaction mixture was removed after 5, 30, and 90 min of digestion and the reaction stopped by adding 200 μL of phenol. The sample was extracted two times with chloroform to remove the bound lipids. In control experiments, more than 90% of the DNA was recovered in the aqueous phase using this protocol. The aqueous phase was loaded directly onto a 1% agarose gel (110 V, 2 h) to examine the integrity of the plasmid DNA. When spermidine was tested, a high salt concentration (1.5 M NaCl) was used to dissociate DNA/spermine binding before gel electrophoresis was performed. When histone was tested, protease K treatment was applied to digest the histone and eliminate its binding to DNA, which interfered with the migration of plasmid DNA into the agarose gel.

Quantification of Plasmid DNA Release. Cationic liposome/plasmid DNA complexes, in which 1% of Rh-PE was added as a marker for the lipid, were mixed with a charge excess (10−100-fold) of each molecule tested. The mixture containing 66 μg of plasmid DNA was loaded onto a 4 mL, 0 to 30% (w/w) linear sucrose gradient and centrifuged for 16 h at 40 000 rpm in a Beckman SW50.1 rotor (150000*g*). The gradient was then separated into fractions and each fraction assayed for lipid and DNA content. The fluorescent lipid label, Rh-PE, was used as a probe to determine the lipid concentration. The plasmid DNA concentration in each fraction was measured using the Hoescht dye 33258 (Polysciences, Inc., Warrington, PA) and a DNA fluorometer (TKO100, Hoeffer Scientific Instruments, San Francisco, CA) (sensitivity > 10 ng/mL DNA) after methanol/chloroform extraction except when salmon sperm DNA or tRNA was used to release DNA from the complex. In those cases, each fraction was extracted and separated by electrophoresis (100 V, 2 h) on a 1% agarose gel and plasmid DNA was detected by EtBr staining.

RESULTS

EtBr Intercalation. When EtBr is added to DNA in solution, it intercalates between the base pairs of the DNA double helix, emitting an intense fluorescence signal at 610 nm when excited at 500 nm. As reported for DOTMA/DOPE lipid−DNA interaction (Gershon et al., 1993), the addition of DOTAP liposomes or DOGS micelles (data not shown) results in an immediate decrease of the fluorescence signal to about 10% of the maximum value, suggesting the displacement of the intercalating dye from the DNA by the binding of cationic liposomes to the DNA (Figure 1, arrow at $T = 325$ s).

[1] Abbreviations: DOGS, dioctadecylamidoglycylspermine; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOTAP, dioleoyl(trimethylammonio)propane; EtBr, ethidium bromide; PA, phosphatidic acid; PG, phosphatidylglycerol; PI, phosphatidylinositol (bovine liver); PS, phosphatidylserine (bovine brain); Rh-PE, rhodamine-labeled phosphatidylethanolamine; ssDNA, salmon sperm DNA.

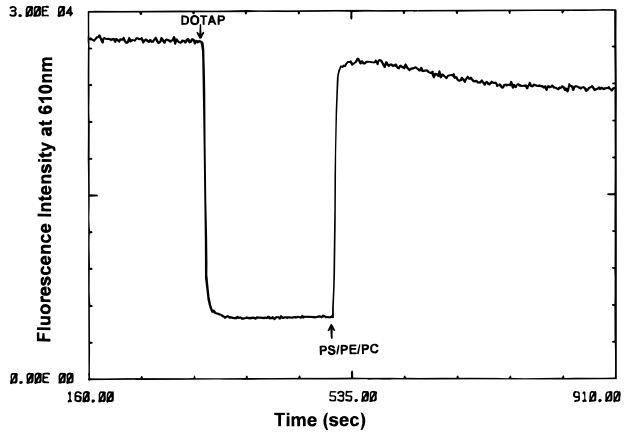

FIGURE 1: Effect of anionic liposomes on DNA accessibility in cationic liposome/DNA complexes. Plasmid DNA (15 μg) was preincubated with EtBr (0.6 μg). DOTAP liposomes (200 nmol) were injected at $T = 325$ s, indicated by the arrow. The signal stabilized within 100 s. PS/PE/PC (1/2/1 mole ratio) liposomes (containing 1000 nmol of PS) were then injected ($T = 515$ s) in 5× excess over the cationic lipids. The fluorescence signal increased and stabilized within 300 s. Duplicate experiments agreed to with ±5% of the fluorescence intensity.

The complexation is reversed by the addition of certain ionic agents both in 20 mM Hepes (pH 7.4) and in physiologic buffer compositions. Upon addition of the anionic liposomes, a significant amount of the EtBr fluorescence is recovered, suggesting the displacement of DNA from the cationic lipids and reexposure of the EtBr intercalation site (Figure 1, arrow at $T = 515$ s). The interaction between anionic liposomes and the complex is surprisingly fast, since the process is usually complete within 50 s (Figure 1). The anionic liposomes, containing either PS, PG, PI, or PA, are among the more efficient substances to reverse the EtBr fluorescence signal.

The reversal of the cationic liposome/DNA complex is dependent on the ratio of negative charge added to the positive charge in the complex (Figure 2A). In the vicinity of a 1/1 charge ratio, greater than 60% of the EtBr fluorescence intensity is usually recovered. A maximum recovery of 80−90% is obtained by adding a 2-fold excess of anionic to cationic charge. Varying the anionic lipid type does not affect the final extent of DNA release, although a slight difference of DNA-displacing efficiency (ratio of anionic/cationic charge needed to reach the same fractional DNA displacement) is observed among liposomes containing PG, PS, PA, or PI. Likewise, changing the molar fraction of the anionic lipid in the anionic liposome from 10 to 50% does not affect the final extent of DNA released (data not shown). Including DOPE in the cationic liposome formulation at a 1/1 mole ratio slightly reduces the DNA-displacing efficiency of the anionic liposome at the same charge ratio (Figure 2B), but the final extents are similar and occur within a 50% lipid concentration value. Likewise, including cholesterol at a 33 mol % ratio in the anionic liposome resulted in the same release of DNA from the complex (Figure 2B). Thus, cholesterol at ratios normally found in endosomal membranes does not interfere with DNA release. In limited experiments with the multivalent lipid, DOGS, anionic liposomes reversed the complex but ATP did not. When the ionic strength and ion type more closely approximated that found in cells, the final release of DNA in the potassium acetate buffer by PS/PE/PC was 82 ± 4.5%,

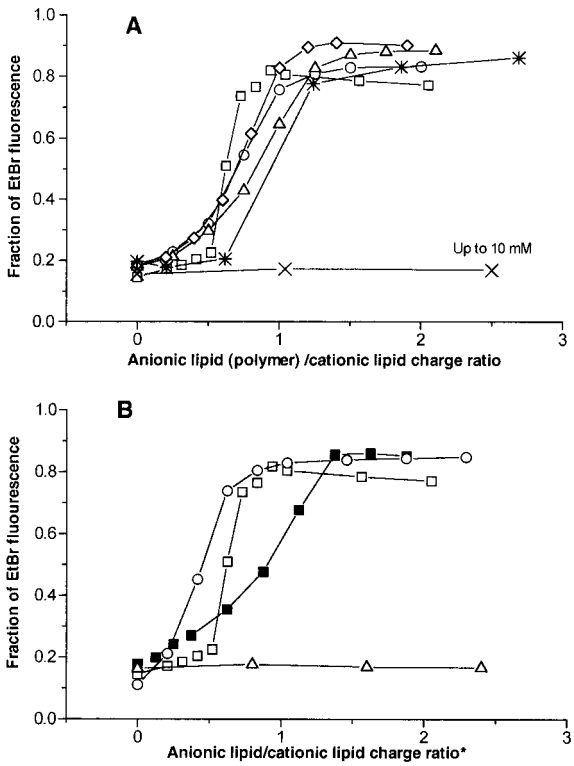

FIGURE 2: Effect of various substances on DNA accessibility in cationic liposome/DNA complexes. DOTAP liposomes (0.6 μM) were mixed with plasmid DNA (5 μg/mL) at a 4/1 lipid/DNA charge ratio to form complexes in the presence of EtBr (0.2 μg/mL) in 20 mM Hepes (pH 7.4). From 0- 4-fold charge excess of the releasing agents was used, except with ATP where the final concentration was 10 mM. The values are expressed as the fraction of the maximum fluorescence signal when EtBr bound to DNA in the absence of other materials. Each data point is from triplicate experiments and had a standard deviation no greater than ±0.05. (A) DNA release from DOTAP/DNA complexes: (□) PS/PE/PC (1/2/1), (○) PA/PE/PC (1/2/1), (△) PG/PE/PC (1/2/1), (◇) PI/PE/PC (1/2/1), (*) dextran sulfate, and (×) ATP. (B) DNA release from complexes induced by various liposomes: (□) effect of PS/PE/PC (1/2/1) liposomes on DOTAP/DNA complexes, (■) effect of PS/PE/PC (1/2/1) liposomes on DOTAP/DOPE(1/1)/DNA complexes, (○) effect of PS/PE/PC/Chol (1/2/1/2) liposomes on DOTAP/DNA complexes, and (△) effect of PE/PC (1/1) liposomes on DOTAP/DNA complexes (*lipid/cationic lipid ratio was used instead of anionic lipid/cationic lipid ratio with PE/PC liposomes).

by dextran sulfate was 64 ± 6.0%, or by ATP was 2.0 ± 2.3%. Release of DNA in the NaCl buffer by PS/PE/PC was 86 ± 8.8%, by dextran sulfate was 60 ± 7.3%, or by ATP was 1.0 ± 1.5%.

Dextran sulfate, a highly charged anionic polysaccharide, displayed an efficiency similar to that of the anionic lipids in displacing DNA from the cationic liposome (Figure 2). As indicated above, at physiologic ionic strength, the anionic liposomes are even more effective than dextran sulfate at exposing DNA to EtBr intercalation. Other water soluble anions such as ATP, poly(glutamic acid) (data not shown), and neutral liposomes (PE/PC) had no effect on the fluorescence signal even at a 100-fold excess over the cationic lipid in the complex (Figure 2).

Complex Sensitivity to DNAse I Digestion. Cationic liposomes protect DNA from DNAse I digestion. Such protection is abolished when the complex dissociates. Thus, the sensitivity of DNA in the complex to DNAse I digestion has been used as an assay for exposure of DNA in the complex. Here again, among the anionic compounds tested,

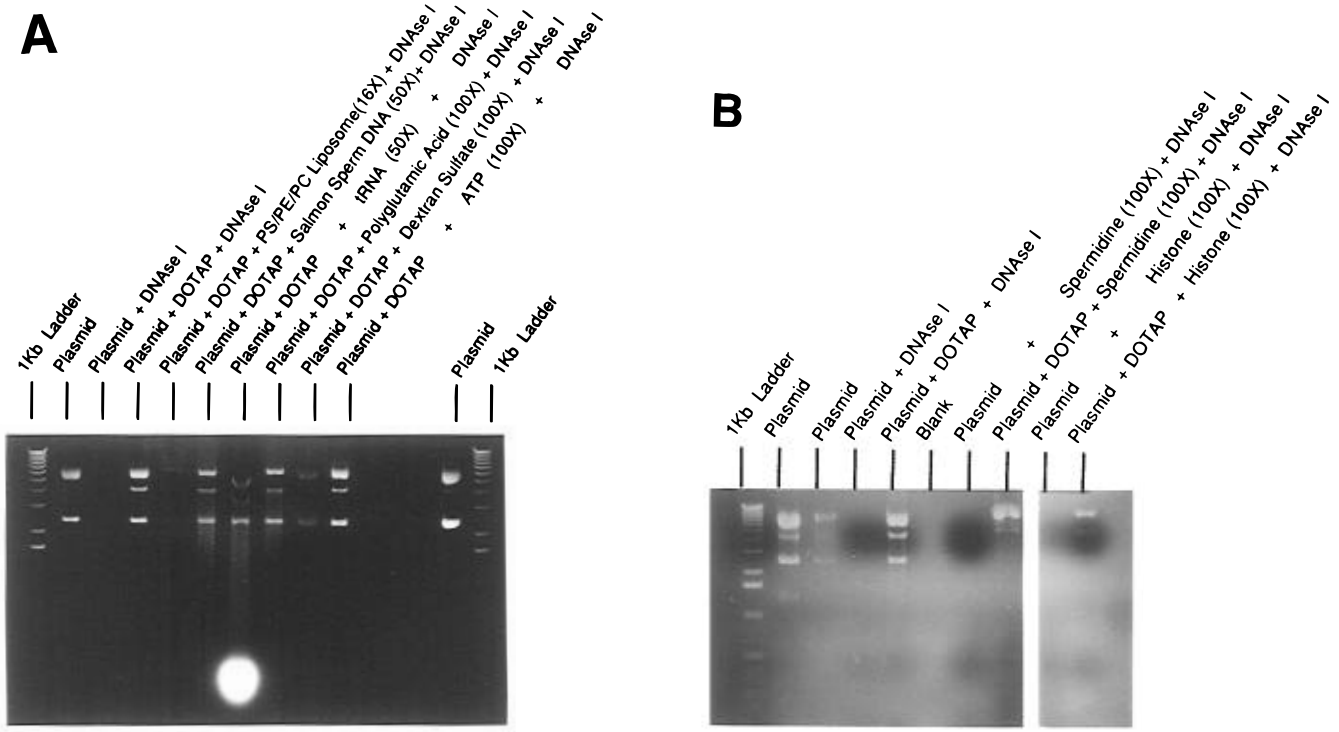

FIGURE 3: Digestion of plasmid DNA by DNAse I in complexes treated with various agents. Preformed DOTAP/DNA complexes (formed at a 4/1 lipid/DNA charge ratio) were mixed with the indicated material and then subjected to DNAse I digestion (2 units/μg of plasmid DNA) for 90 min. (A) The effect of anionic material: lane 1, 1 kb DNA ladder; lane 2, undigested plasmid DNA; lane 3, plasmid DNA alone digested with DNAse I; lane 4, DOTAP/DNA complexes; lane 5, 16× charge excess of PS/PE/PC (1/2/1) liposomes; lane 6, 50× charge excess of ssDNA; lane 7, 50× charge excess of tRNA; lane 8, 100× charge excess of poly(glutamic acid); lane 9, 100× charge excess of dextran sulfate; lane 10, 100× charge excess of ATP. (B) The effect of cationic material: lane 1, 1 kb DNA ladder; lanes 2 and 3, undigested plasmid DNA; lane 4, plasmid DNA digested with DNAse I; lane 5, DOTAP/DNA digested with DNAse I; lane 7, plasmid DNA digested with DNAse I in the presence of spermidine; lane 8, DOTAP/DNA digested with DNAse I after incubation with 100× charge excess of spermidine; lane 9, plasmid DNA digested with DNAse I in the presence of a 100× charge excess of histone; lane 10, DOTAP/DNA digested with DNAse I after incubation with a 100× charge excess of histone.

only anionic liposomes (1/2/1 PS/PE/PC) and dextran sulfate resulted in degradation of DNA by DNAse I digestion, after mixing with the complex at a 10-fold excess. The majority of the plasmid DNA is degraded after 5 min (Figure 3A). Incubation of the complex with other anionic molecules, including salmon sperm DNA, tRNA, poly(glutamic acid), and ATP, resulted in no significant change to the DNA even when added at a 100-fold excess. The majority of the plasmid DNA was intact after exposure to DNAse I for up to 90 min under these conditions (Figure 3A). When plasmid DNA by itself is incubated with DNAse I in the presence of these reagents, but in the absence of cationic lipids, all of the DNA is degraded within 5 min (data not shown).

The cationic compounds spermidine and histone also were unable to increase the DNAse I sensitivity of DNA when complexed to cationic liposomes (Figure 3B). A substantial amount of intact plasmid DNA is still visible after exposure to these cationic water soluble compounds. To rule out the possibility that cationic lipids are in fact displaced but the plasmid DNA is protected by the spermidine or histone, DNA was mixed with the cationic compound and subjected to DNAse I digestion. The DNA was completely degraded. This indicates that protection of plasmid DNA in the cationic lipid complex from DNAse I in the presence of the water soluble cations occurs because the complex remains intact (Figure 3B).

Quantification of Release of Plasmid DNA. The EtBr and DNAse I assays indicate that DNA becomes accessible to either EtBr or DNAse I but do not necessarily mean the DNA has become physically detached from the complex. To demonstrate physical detachment of plasmid DNA from cationic liposome upon interaction with the ionic substances, and to quantify the amount of plasmid released, a sucrose gradient ultracentrifugation method was used to separate liposomes, the liposome/DNA complexes, and free DNA according to their density. DNA sediments to the bottom of the 0 to 30% sucrose gradient after ultracentrifugation, whereas cationic liposomes and complexes equilibrate at around 2 and 15% sucrose concentrations (w/w), respectively.

When positively charged DOTAP/DNA complexes formed at a 4/1 charge ratio are loaded onto the gradient, more than 95% of the DNA and most of the lipid are found in complexes in a band appearing at a 12% sucrose concentration. Small amounts of lipids are detected in a liposome band at the top of the gradient. The same amount of the DOTAP/DNA complex was then incubated with various ionic compounds. After gradient separation, the lipids remained at either the top or the upper portion of the gradient (sucrose concentration < 20% w/w), while various amounts of DNA sediment to the bottom of the gradient (sucrose concentration > 20% w/w). The DNA found in the lower portion of the gradient (sucrose concentration > 20% w/w) was no longer bound to the cationic liposomes. Quantifying the amount of DNA in the upper (<20% w/w) and lower (>20% w/w) portion of the gradient yields an estimation of the amount of DNA released from the complex after interaction with the various charged molecules (Figure 4).

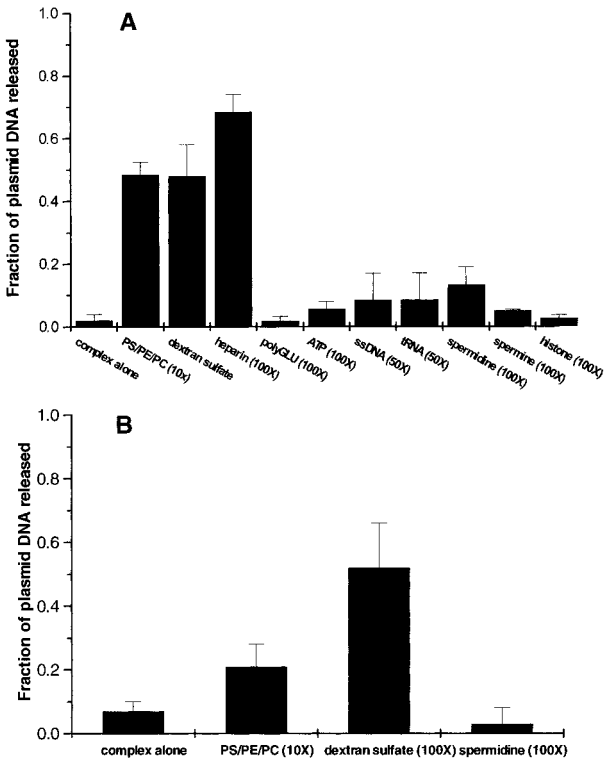

FIGURE 4: Fraction of plasmid DNA physically detached from the complexes by various substances. DOTAP/DNA complexes were treated with various test materials, and the released plasmid DNA was separated from the complexes as described in Materials and Methods. (A) Plasmid DNA release from positively charged complexes. PS/PE/PC (1/2/1) liposomes were added at a 10× charge excess over cationic lipids; ssDNA and tRNA were added at a 50× charge excess. All the other molecules were added at a 100× charge excess. (B) Plasmid DNA release from negatively charged complexes. PS/PE/PC (1/2/1) liposomes were added at a 10× charge excess over cationic lipids, and all the other molecules were added at a 100× charge excess.

This assay confirms that a charge excess of anionic liposomes, dextran sulfate, and heparin causes a substantial release of the plasmid molecules (∼50%) from cationic liposomes. Whereas other ionic molecules, including poly(glutamic acid), spermidine, spermine, and histone, induced a less than 10% release of DNA.

When salmon sperm DNA and tRNA were tested, gel electrophoresis was used to estimate the amount of plasmid DNA released from the complex. After the preformed DOTAP/plasmid DNA complexes were incubated with a 50× charge excess of ssDNA or tRNA and subjected to sucrose gradient ultracentrifugation, the nucleic acid components from the upper and lower portions of the gradients were extracted and separated in a 1% agarose gel. Plasmid DNA was not detected from the lower portions of the gradient, whereas a definitive plasmid band is clearly visible in extracts from the upper portion of the gradient. Assuming a detection limit for gel electrophoresis of 100 ng of DNA in 20 μL of sample, this observation means that less than 5 μg plasmid DNA is released from the complex by either DNA or tRNA. This is less than 10% of the plasmid DNA initially present in the complexes. This finding is consistent with results in the DNAse I degradation assay.

The cationic liposome/DNA complexes tested above were positively charged, with ζ potentials in the vicinity of 20 mV. The strong interaction observed between the complexes and the anionic substances that release DNA could be facilitated by electrostatic interactions. To examine the release of DNA from negatively charged complexes, such complexes were prepared and purified by centrifugation on a sucrose gradient. The purified complexes had a ζ potential of -25 mV. The negatively charged complexes were mixed with an excess of either anionic liposomes, dextran sulfate, or spermine and assayed after gradient separation (Figure 4B). In the absence of added substances, the negatively charged complexes released about 7% of the complexed DNA. A 10-fold charge excess of anionic PS/PE/PC liposomes increased the percentage of DNA released to 21%, while a 100-fold charge excess of dextran sulfate mediated a 50% release of DNA from the negatively charged complex. This is in spite of the electrostatic barrier between the complex and the releasing agent. When a cationic agent such as spermidine was added to the negatively charged complexes, visible aggregates were observed but there was no significant increase in plasmid DNA release from the negatively charged complexes.

DISCUSSION

Cationic liposomes are widely used to transfect DNA into cells both in culture and in animals, but their mechanism of action is poorly understood. The formation of cationic lipid/DNA complexes facilitates association of the plasmid DNA with the cell membrane and entry of the complex into the cell primarily via the endocytotic route (Felgner et al., 1987; Legendre & Szoka, 1992; Zhou & Huang, 1994; Wrobel & Collins, 1995; Zabner et al., 1995). The strong interaction between the cationic lipid and DNA (Gershon et al., 1993) creates a conundrum relating to how the complex dissociates within the cell. Indeed, Zabner and colleagues (Zabner et al., 1995) showed that complexes that are microinjected directly into the nucleus do not induce high expression levels. This implies that, during the normal transfection process, plasmid DNA is released before entering the nucleus and raises the question of which biomolecules are involved in release of DNA from the complex.

On the basis of the biomolecules found to release DNA from the complex, we propose the following model for DNA release during transfection (Figure 5). First, the cell surface-associated complex is internalized into an endosome (step 1). The complex initiates a destabilization of the endosome membrane that results in flip-flop of anionic lipids that are predominately located on the cytoplasmic face of the membrane (step 2). The anionic lipids laterally diffuse into the complex and form charge-neutralized ion pairs with the cationic lipids (step 3). This displaces the plasmid DNA from the complex and permits DNA entry into the cytoplasm (step 4).

Anionic liposomes demonstrated a strong ability to displace bound DNA. In the presence of an equal molar charge ratio of anionic to cationic lipids in the complex, the DNA becomes accessible to both small intercalating agents such as EtBr (≥80% intercalation) and macromolecules such as DNAse I (≥80% degradation). Nearly 50% of the plasmids are released from the complex by the anionic liposomes as measured by centrifugation on sucrose gradients. The discrepancy between the EtBr or DNAse I results and the centrifugation assay may be due to the ability of even a few interacting regions to keep DNA attached to the liposome during the centrifugation since DNA can interact with liposomes with only a 5% positive charge (Koiv &

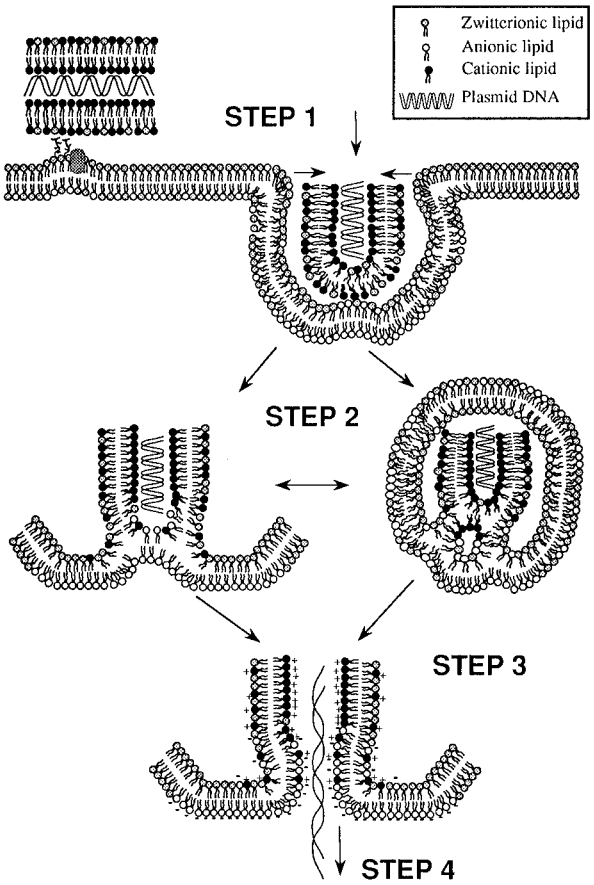

FIGURE 5: Mechanism of uptake and release of plasmid DNA from the complex. (Step 1) After electrostatic interaction with the cell membrane, cationic liposome/DNA complexes are endocytosed. (Step 2) In the early endosome, membrane destabilization results in anionic phospholipid flip-flop. (Step 3) The anionic lipids diffuse into the complex and form a charge neutral ion pair with cationic lipids. (Step 4) The DNA dissociates from the complex and is released into the cytoplasm.

Kinnunen, 1994).

The release of DNA from the complex by anionic lipids is likely due to a combination of electrostatic and hydrophobic factors. These include the multivalent nature of the anionic lipid surface, diffusion of anionic lipids into the complex, formation of a charge neutral ion pair with the cationic lipid, and possibly the sealing of hydrophobic defects in the complex. The formation of ion pairs within the lipid monolayer and their diffusion away from the DNA would allow the anionic lipid to effectively compete with the DNA for the cationic lipid. Contrary to previous speculation (Remy et al., 1995), excess DNA did not mediate release of the plasmid DNA from the complex and neither did other cellular polyanions such as ATP or tRNA. Polycations such as spermidine or histone also did not bring about dissociation of the preformed complex.

The proposed model is consistent with the cell biological data as well as the biophysical aspects of membrane fusion. First, this model accounts for the observations that plasmid DNA and RNA delivered by the cationic complexes are accessible to the transcription and translation apparatus (Felgner et al., 1987; Malone et al., 1989), including studies in which plasmids containing a T7 promoter were accessible to cytoplasmic T7 polymerase (Rose et al., 1991; Gao & Huang, 1993) and those in which cationic liposomes delivered RNA to its cytoplasmic site of translation (Malone et al., 1989). Several groups have indicated that less than 15% of the cell-associated DNA is released into the cell (Felgner et al., 1987; Legendre & Szoka, 1992); the physical separation experiments show that anionic lipids are capable of releasing the requisite amount of DNA to account for these observations. Our model also would explain the low expression levels observed by Zabner and colleagues (Zabner et al., 1995) for complexes directly injected into the nucleus because the complexes were not uncoated by biomolecules in the nucleus. The proposed ion pair formation between the cellular anionic lipids and the cationic lipids from the complex may also explain reports of cationic lipids inhibiting protein kinase C activity (Bottega & Epand, 1992; Farhood et al., 1992), as ion pair formation would inhibit interactions between the anionic lipids and protein required for activation (Buser et al., 1995). The lipid-mixing hypothesis also suggests that intermixing of the cationic lipids with other cell organelle membranes, such as the mitochondrial membranes, could lead to toxicity via ion pair formation. In mitochondria, cardiolipin is the major anionic lipid. Ion pair formation between cardiolipin and the cationic lipid might explain some of the toxicity associated with the transfection complexes (Felgner et al., 1987; Legendre & Szoka, 1992; Felgner et al., 1994).

In terms of the biophysical aspects of the model, anionic lipids are known to be located primarily on the cytoplasmic-facing monolayer of the plasma and endosomal membranes (Devaux, 1992). Although the model does not attempt to explain the initial membrane destabilization step, it is known that during fusion or membrane disruption membrane asymmetry is lost (Schroit et al., 1990; Bevers et al., 1994; Fattal et al., 1994). When cationic lipid/DNA complexes come into close contact with negatively charged phospholipid membranes, membrane destabilization and lipid mixing are initiated (Stamatatos et al., 1988; Duzgunes et al., 1989; Leventis & Silvius, 1990; Bentz, 1992; Bailey & Cullis, 1994; Koiv & Kinnunen, 1994). In this regard, phosphatidylethanolamine which increases transfection efficiencies (Leventis & Silvius, 1990; Farhood et al., 1995) promotes membrane mixing and fusion (Wilschut & Hoekstra, 1990). The membrane destabilization may be triggered by lipid structures with a high curvature such as can be induced by phosphatidylethanolamine (Wilschut & Hoekstra, 1990). Structures with high curvature are observed in electron micrographs of DNA/cationic lipid complexes (Gershon et al., 1993; Sternberg et al., 1994; Xu et al., 1995). Indeed, fusion between the cationic liposomes and endocytotic vesicles has been observed in cultured cells (Wrobel & Collins, 1995). The fusion process once initiated would be autocatalytic, since bilayer destabilization would result in additional anionic lipids appearing at the site of interaction. This would increase adhesion of the cationic complex to the endosomal membrane. As shown in Figure 5, the DNA could be released into the cytoplasm at the site of membrane fusion, although as discussed below, this is not necessary.

The model is also consistent with electron microscopy observations on complexes in cells that show electron dense structures appearing in disrupted coated vesicles close to the plasma membrane (Zhou & Huang, 1994). It also accounts for the observation that fluorescent lipids associated with the complex are found in punctuate cytoplasmic structures (Felgner et al., 1987; Wrobel & Collins, 1995). It is likely that cationic liposome-mediated oligonucleotide delivery follows a similar pathway. Fluorescence microscopic studies have shown that fluorescent oligonucleotides redistribute

from initial punctuate cytoplasmic regions into the nucleus (Bennett et al., 1992) and the physical separation of fluorescent oligonucleotides from fluorescent lipids in the complex occurs early in endocytosis (Zelphati and Szoka, unpublished observation).

The mechanism illustrated in Figure 5 can explain all of the published data about nucleic acid delivery by cationic liposome complexes. It does not, however, explain why endocytosis appears to be required, since destabilization and/or fusion of the complex with the plasma membrane would permit the same anionic lipids to flip to the surface as would fusion with the endosomal membrane (Devaux, 1992). One possibility is that internalization of the complexes into a compartment with a high radius of curvature promotes close contact between the two surfaces that is conducive for fusion (Lentz et al., 1992). Alternatively, compartmentalization may be the key; release of the compacted DNA (Gershon et al., 1993) from the cationic lipid in the endosome could generate mechanical or osmotic stress that ruptures the endosomal bilayer and releases DNA into the cytoplasm. In contrast, release of the DNA from complexes on the cell surface might be unable to stress the membrane to a degree sufficient for rupture. Finally, the loss of clathrin and the accessory proteins during the uncoating of the vesicle might make the uncoated vesicles more prone to rupture than the plasma membrane. Choosing among these alternatives requires further cell biology experiments.

Two of the highly charged anionic polymers, dextran sulfate and heparin, release DNA from the complex as efficiently as anionic liposomes. This is probably due to the greater linear negative charge density of these polymers compared to those of the anionic liposomes. Dextran sulfate and heparin also have a 2.0-fold greater charge density than DNA, which might explain why these molecules can release DNA from the complex whereas a 100-fold excess of RNA or DNA cannot reverse the preformed complex. At the other extreme, ATP which has a high charge density cannot displace DNA, suggesting that a minimum number of charges must be broken to disrupt the cationic lipid/DNA complexes. This result is reminiscent of findings on electrostatic interactions between proteins and membranes (McLaughlin, 1989). Thus, both the charge density and polyvalent nature of dextran sulfate and heparin allow them to overcome the interactions between the cationic lipid and the DNA.

In the case of cationic molecules such as spermine or polylysine, the complex is also positively charged so electrostatic interactions between the complex and the polycation would impede close approach. Electrostatic repulsion between the surface of the complex and the displacing molecule is obviously not sufficient to block displacement if the charge density of the displacing molecule is high enough, since dextran sulfate and anionic lipids were effective at releasing DNA from negatively charged complexes.

Finally, although complex formation between the cationic liposomes and DNA is crucial to the transfection system (Gershon et al., 1993), it is equally important for the dissociation step to occur at the correct site to obtain transfection. The poor correlation between in vitro and in vivo transfection activity (Yoshimura et al., 1992; Meyer et al., 1995; Solodin et al., 1995) may in part be due to premature release of DNA in vivo; our observation that heparin is a potent releasing agent raises the interesting possibility that endogenous glycosaminoglycans such as heparin may interfere with transfection in vivo by releasing DNA from cationic lipid/DNA complexes before they reach their target cell.

In summary, we propose that DNA is released from the cationic lipid/DNA complexes in cells by the action of anionic lipids initially located in the cytoplasmic face of the endosome (Figure 5). The model is based upon the ability of these anionic phospholipids to release DNA from the complex and accounts for the known biophysical and cell biological data relating to cationic lipid/nucleic acid complexes.

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