Cationic Lipid Binding to DNA: Characterization of Complex Formation†

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ABSTRACT: We recently demonstrated that cationic lipids, added in monomer or micellar form, bind to DNA, resulting in the formation of a hydrophobic complex. This complex can serve as a well-defined intermediate in the preparation of DNA−lipid particles (DLPs) with many potential applications for delivery of polynucleotides *in vitro* and *in vivo*. To develop a better understanding of the factors governing complex formation, we have characterized the cationic lipid/DNA binding reaction. This was evaluated by measuring DNA and cationic lipid (DODAC) complex formation using the Bligh and Dyer extraction procedure. Efficient recovery of DNA (>95%) in the organic phase was achieved when sufficient monocationic lipids interact with DNA phosphate groups. The rate of binding depends on the amount of DNA or cationic lipid present in the system. The time required to generate the hydrophobic complex was increased when <10 μg of DNA or <40 nmol of DODAC was present. Surprisingly, the rate of complex formation was contingent on the incubation period after partitioning the DNA/lipid mixture into organic and aqueous phases. These results suggest that the cationic lipid/DNA complex forms at the aqueous/organic interface and that DNA/lipid binding is dependent on multivalent interactions at this interface. A Scatchard analysis of DNA/DODAC binding demonstrated that the binding reaction exhibits a high degree of positive cooperativity. The apparent dissociation constant (K_n), using data obtained under conditions where DODAC binding to DNA approached saturation, indicated a high-affinity reaction ($K_n > 10^{-11}$ mol L^{-1}). At this point, ~8400 mol of DODAC was bound per mole of DNA, which is equivalent to a charge ratio (+/−) of 0.585 for the 7.2 kb plasmid used and suggests that formation of the hydrophobic complex occurs at a stage prior to charge neutralization. The influence of other lipids on DNA/cationic lipid binding at the aqueous/organic interface was also studied. Cholesterol and DOPC had little effect on DNA/DODAC binding while the anionic lipids LPI, DOPS, and DMPG inhibited complex formation. The zwitterionic lipid DOPE, however, had a concentration-dependent effect on cationic lipid binding that was also dependent on the mixing order. We believe that this approach for evaluating lipid/DNA binding provides an effective procedure for assessing factors which control the dissociation of lipids from DNA and may be beneficial in the selection of lipids for effective use in gene transfection studies.

Synthetic nonviral vectors for the delivery of plasmid DNA are being developed for gene therapy applications based on the assumption that problems associated with viruses will be difficult to overcome. These problems include antigenicity and a relatively small capacity for carrying genetic information (Setoguchi *et al.,* 1994; Yang *et al.,* 1994, 1995). In addition, it will be difficult to target viruses to cells not expressing cell surface receptors which are required by the virus for binding and entry into the cell (Miller & Vile, 1995). Although the preparation of synthetic vectors has focused on the development of carriers which mimic many viral attributes such as cell binding (Chowdhury *et al.,* 1993), membrane fusion triggering (Wagner *et al.,* 1992), and DNA translocating peptides (Kato *et al.,* 1991), it would be surprising if these complicated synthetic vectors did not suffer problems similar to those of viral vectors.

One of the attractive features of lipid- and polymer-based systems for DNA delivery is their simplicity. Essentially all that is required is a cationic surface which can bind DNA. This binding results in the formation of a complex that is capable of transfecting cells [for a review, see Felgner (1990)]. The transfection efficiency using these complexes, however, is often lower than viral vectors (Behr, 1993; Mulligan, 1993) and depends on the chemical and physical characteristics of the complex (Gustafsson *et al.,* 1995), the design of the plasmid DNA (Ponder *et al.,* 1991), and the target cell population (Philip *et al.,* 1993). A variety of cationic surfaces have been shown to be capable of complexing with DNA and include linear and branched polymers [e.g., polylysine (Bogliolo *et al.,* 1986; Gershon *et al.,* 1993), glycopeptides (Wadhwa *et al.,* 1995), and dendrimers (Tomalia, 1995)] as well as lipid membranes [e.g., uni- and multilamellar liposomes (Smith *et al.,* 1993)]. Optimization of these systems for use in gene therapy is dependent on many parameters including particle size and charge (Gershon *et al.,* 1993), DNA structure (Sternberg *et al.,* 1994), and the ability of DNA to dissociate from the charged surface (Zabner *et al.,* 1995). The last characteristic is becoming increasingly important since it is thought that complexed DNA is not in a form which can be transcribed within the nucleus of a cell (Zabner *et al.,* 1995). Moreover, it is unclear whether dissociation of the complex must occur prior to or after delivery of the DNA to the nucleus.

We have focused our studies on the development of lipid-based carriers since these synthetic formulations are the most

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pharmaceutically advanced (Mulligan, 1993). However, these systems are perhaps the most difficult to characterize (Felgner *et al.*, 1994; Sternberg *et al.*, 1994; Zabner *et al.*, 1995). Addition of DNA to preformed cationic liposomes has been shown to trigger significant structural changes in the liposomes as well as the DNA (Sternberg *et al.*, 1994). Depending on the liposomal lipid composition (cationic lipid and associated secondary lipids) and lipid concentration, DNA addition engenders liposomal aggregation, resulting in the formation of structures which are heterogeneous with respect to physical and chemical characteristics (Sternberg *et al.*, 1994). The structural changes in the complexes are also associated with changes in the DNA structure. DNA within the liposome aggregates is less sensitive to DNase I and is resistant to ethidium bromide binding (Gershon *et al.*, 1993). Although these changes to the DNA are consistent with polycation-induced DNA condensation (Bloomfield, 1991), it is unlikely that liposomes condense DNA in a manner similar to classical condensing agents such as polylysine. For example, multivalent cation-condensed DNA has been shown to generate toroid-like structures (Marx & Ruben, 1983; Bloomfield, 1991) which have not been observed with liposome-condensed DNA. The benefits associated with DNA structural changes induced by such polyvalent cations are comparable in that DNA adopts a structure that renders the molecule partially protected from extracellular and intracellular degradation.

We have recently demonstrated that hydrophobic lipid/DNA complexes can be prepared using monomeric lipids rather than preformed liposomes and that DNA in the resulting complex is not in a condensed state (Reimer *et al.*, 1995). This observation is important for a number of reasons. First, the complex can be used as a well-defined intermediate in the preparation of DNA−lipid particles (DLPs). Second, characterization of this binding reaction will provide information on the factors that control association, dissociation, and aggregation of lipids and DNA. Finally, the influence of bound lipid on the DNA structure can be assessed in the absence of a membrane structure. The objective of this study was to determine the binding characteristics of cationic lipid to plasmid DNA. We assessed the lipid/DNA binding by evaluating the hydrophobic cationic lipid/DNA complex extracted into organic solvents. It was demonstrated that the binding reaction was highly cooperative, and we propose that this reaction occurs at the interface between aqueous and organic phases. It is suggested that DNA-bound lipid in the organic phase adopts an inverted micelle-like structure.

MATERIALS AND METHODS

Materials

The monocationic lipids, dioleoyldimethylammonium chloride (DODAC)[1] and [^{14}C]DODAC (specific activity ~9.0 × 10^5 dpm/μg) were synthesized and supplied by Steven Ansell of INEX Pharmaceuticals Corp. (Vancouver, B.C.). Zwitterionic lipids (DOPE, DOPC) and anionic lipids (DOPS, DMPG, PEG-PE, and LPI) were obtained from Avanti Polar Lipids Inc. (Birmingham, AL). Cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO). Solvable was obtained from Dupont NEN (Boston, MA). The pCMVβ plasmid encoding *Escherichia coli* β-galactosidase was obtained from Clontech Labs (Palo Alto, CA) and was propagated and purified using standard techniques (Sambrook *et al.*, 1989). Methyl[^{3}H]thymidine 5′-triphosphate, obtained from Dupont NEN, was used to synthesize radiolabeled pCMVβ plasmid. A specific activity of ~5.0 × 10^4 dpm/μg was typically obtained. Solvents used were HPLC grade.

Methods

Bligh and Dyer Extraction. The monocationic lipid DODAC and the pCMVβ plasmid were solubilized in a Bligh and Dyer monophase consisting of chloroform/methanol/water (1:2.1:1) (Bligh & Dyer, 1959). DNA (1−160 μg) and DODAC (10−640 nmol) were combined to a total volume of 1 mL. The monophase mixture was subsequently partitioned into two phases by the addition of 250 μL each of chloroform and water. The samples were mixed vigorously by vortexing for 1 min and centrifuged at 2000 rpm (600*g*) for 5 min at room temperature. The upper aqueous phase (~1.0 mL) and the lower organic phase (~0.5 mL) were removed, and the amount of DNA in each phase was determined. The pH was estimated to be 6.5 in both aqueous and organic phases and was made using pH sticks obtained from EM Science (Cherry Hill, NJ).

Quantification of DNA and DODAC. One of two methods was used to quantify the amount of DNA recovered in the aqueous and organic fractions following Bligh and Dyer extraction. First, trace quantities of [^{3}H]pCMVβ were added to unlabeled plasmid DNA in the monophase such that each sample contained 3000−4000 dpm (~60 pg) of ^{3}H-labeled DNA. The lipid and DNA were mixed in the monophase and separated into organic and aqueous fractions as described. The organic phase was dried down using a stream of nitrogen gas prior to the addition of 1 mL of Solvable. This lipid−DNA film was incubated for 1 h at 50 °C to solubilize the complex. Subsequently, PicoFluor-40 scintillation cocktail was added to the aqueous and organic fractions, and the radioactivity was measured by a Packard TR 1900 scintillation counter. Alternatively, DNA in the aqueous phase was quantified by measuring the optical density at a wavelength of 260 nm using a Beckman UV spectrophotometer (DU-64). Data collected by this method were presented as a percentage of the DNA recovered in the organic phase. No differences were observed in the values when data were collected by the radioactive labeling method or by spectrophotometric analysis. Trace amounts of [^{14}C]DODAC (~8.0 pmol) were also evaluated (in the presence of 10 μg of DNA) in organic and aqueous phases by scintillation counting following Bligh and Dyer extraction.

Effects of Other Lipids. The effects of zwitterionic (DOPE and DOPC), neutral (cholesterol), and anionic (DOPS, DMPG, PEG-PE, and LPI) lipids on the formation and/or dissociation of DODAC/pCMVβ complexes were evaluated using two methods. In one method, the DODAC/pCMVβ complexes were preformed prior to the addition of other lipids. These lipids were injected directly into the organic

[1] Abbreviations: DODAC, dioleoyldimethylammonium chloride; DOPE, dioleoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; PEG-PE, poly(ethylene glycol)-modified phosphatidylethanolamine; DOPS, dioleoylphosphatidylserine; DMPG, dimyristoylphosphatidylglycerol; LPI, liver phosphatidylinositol; K_n, apparent dissociation constant.

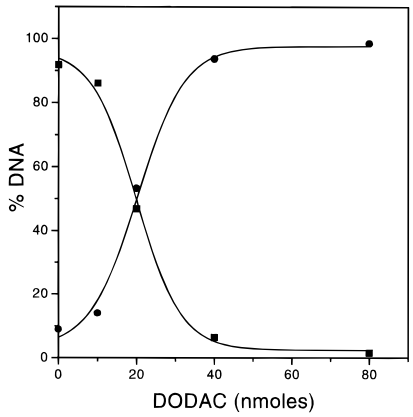

FIGURE 1: Effect of increasing amounts of DODAC on the recovery of pCMVβ plasmid DNA in the aqueous (■) and organic (●) phases following Bligh and Dyer extraction of the DNA/DODAC complexes. Ten micrograms of plasmid DNA was used, and recovery was expressed as a percentage.

phase following Bligh and Dyer extraction and formation of the two-phase system. The samples were mixed vigorously by vortexing and separated by centrifugation as previously described. The effect of these added lipids on the dissociation of the complex was evaluated by quantifying the DNA in the aqueous and/or organic phases. Alternatively, the effects of the additional lipids on the formation of the complexes were evaluated by mixing them with pCMVβ prior to the addition of DODAC. As a control, DOPE was added to the DNA in the absence of DODAC to ensure that DOPE did not mediate extraction of the DNA into the organic phase.

RESULTS

Evidence for the formation of a hydrophobic cationic lipid/DNA complex has recently been published (Reimer *et al.*, 1995) and is summarized in Figure 1. Specifically, when 40 nmol of DODAC, a monovalent cationic lipid, was added to 10 μg of plasmid DNA, >95% of the DNA initially present in the monophase was extracted into the organic phase. We concluded that the presence of DNA in the organic phase resulted from the formation of a hydrophobic complex generated through interactions between the cationic head group of the lipid and the negatively charged phosphate groups of the DNA backbone. In samples containing 40 nmol of cationic lipid and 10 μg of DNA, the charge ratio of lipid to nucleotide phosphate (+/−) was 1.0, and thus we further suggested that charge neutralization mediated the formation of this hydrophobic complex. One of the primary objectives of this study was to further characterize this binding reaction.

The significance of cationic lipid-dependent charge interactions with the DNA phosphate groups was evaluated over a broad range of DNA concentrations. The results presented in Figure 2 illustrate two important points regarding the binding reactions. First, formation of the hydrophobic complex, as measured by an increase in the proportion of DNA isolated in the organic phase, appears to be dependent on achieving charge neutralization when the amount of DNA in the system was in excess of 10 μg. Figure 2A shows that for 10, 20, and 40 μg of DNA >95% of the DNA was recovered in the organic phase when 40, 80, and 160 nmol of DODAC was added, respectively. Upon calculation of

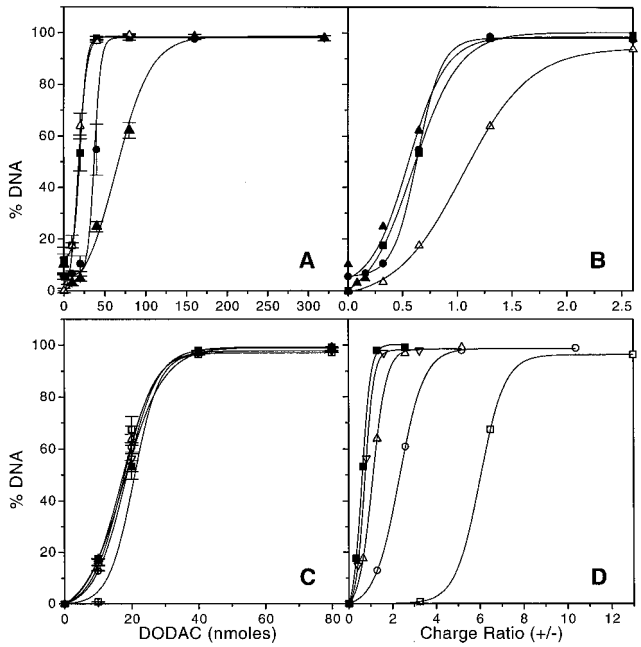

FIGURE 2: Effect of increasing amounts of DODAC on DNA recovery (%) in the organic phase following Bligh and Dyer extraction of the complexes (incubation time, 5 min) expressed as nanomoles of DODAC (A, C) and as calculated charge ratio (B, D). Amounts of DNA used in panels A and B were 5.0 μg (△), 10.0 μg (■), 20.0 μg (●), and 40.0 μg (▲). Amounts of DNA used in panels C and D were 1.0 μg (□), 2.5 μg (○), 5.0 μg (△), 8.0 μg (▽), and 10.0 μg (■). All data points are averaged from three replications and expressed ±SEM.

the charge ratio of cationic DODAC to anionic DNA, it was shown that efficient recovery of DNA in the organic phase was achieved only when a charge ratio (+/−) of 1.0 or greater was obtained (Figure 2B). The second point illustrated in Figure 2 is that the relationship between charge ratio and complex formation was no longer valid when the amount of DNA present in the assay was below 10 μg. Specifically, when using 5 μg of DNA, a charge ratio (+/−) of approximately 2.6 was necessary for efficient recovery of DNA into the organic phase (Figure 2B,D). At the lowest amount of DNA evaluated (1 μg), even a higher charge ratio was required (>12.0) to obtain complete recovery of DNA in the organic phase. As shown in Figure 2C, these results suggest that a minimum concentration of lipid (40 nmol per assay) is necessary to effect the formation of the hydrophobic complex when the DNA concentration is below 10 μg.

To elucidate the mechanisms governing the formation of the hydrophobic complex, we evaluated the rate of complex formation. The rationale for this study was that at low DNA concentrations (<10 μg per assay) the rate of complex formation may be slower than that observed at higher DNA concentrations. Time-dependent formation of the complex was evaluated in two ways. First, the DNA and cationic lipids were incubated for defined periods in the Bligh and Dyer monophase prior to the creation of the two-phase system. Alternatively, the amount of DNA recovered in the organic phase was measured as a function of time after formation of the two phases. The results demonstrated that DNA recovery in the organic phase was not affected by the length of time the samples were incubated in the monophase (results not shown). However, a time-dependent increase in the amount of DNA recovered in the organic phase was obtained after formation of the two-phase system. The

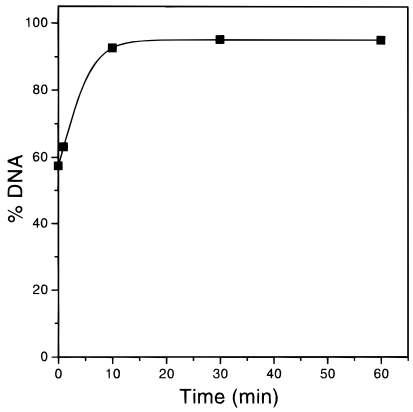

FIGURE 3: Percent recovery of plasmid DNA from the organic phase following Bligh and Dyer extractions of the complexes and expressed as a function of time following preparation of the two phases. Five micrograms of DNA and 20 nmol of DODAC were used. Data points were averaged from two replications.

results presented in Figure 3 suggest that for assays containing 5 μg of DNA and 20 nmol of DODAC the amount of DNA recovered in the organic phase increased as the incubation time following phase separation increased. Thus, for low amounts of DNA, a charge ratio of 1.0 was sufficient to mediate efficient extraction of DNA into the organic phase provided that the time after phase separation was ample. These results suggest that if the hydrophobic complex is formed within the monophase, it is not stable and easily dissociates and the process of DNA to DODAC binding and eventual complex formation occurs after preparation of the aqueous and organic phases.

A second experiment evaluating the formation of the hydrophobic complex assessed the partitioning behavior of the cationic lipid. It is believed that extraction of DNA into an organic phase is dependent on lipid binding. When sufficient amounts of lipids are bound, the DNA exhibits hydrophobic characteristics. If this binding reaction is strong enough to mediate the transformation of a hydrophilic molecule into a hydrophobic complex, the reverse may also be true. For this reason, the effect of trace amounts of cationic lipid binding to excess DNA on the partitioning characteristics of the lipid was studied. The results shown in Figure 4 demonstrate that with excess DNA >90% of the cationic lipid was always recovered in the organic phase (Figure 4C). The results conclusively demonstrate that the binding reaction was not strong enough to mediate partitioning of the lipid into the aqueous phase.

The data presented thus far strongly suggest that the formation of the hydrophobic complex between DNA and the monovalent cationic lipid DODAC occurs at the aqueous/organic interface after phase separation. The binding of cationic lipid and DNA at this interface was evaluated and is shown in Figure 5. In this analysis, an estimate of bound lipid is plotted as a function of added lipid for a system that contains 40 μg of DNA. The estimated bound lipid was calculated from the amount of DNA recovered in the organic phase after addition of various amounts of lipid, where the amount of bound lipid was calculated using the assumption that each anionic phosphate charge on DNA recovered in the organic phase was complexed to one cationic lipid. Under conditions where >95% of the DNA (40 μg) was recovered in the organic phase, for example, it was estimated that 160 nmol of lipid was bound. The sigmoidal curve

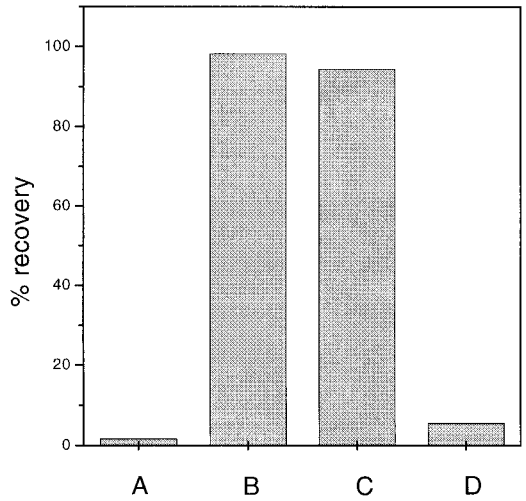

FIGURE 4: Percent recovery of plasmid DNA in organic (A) and aqueous (B) phases following Bligh and Dyer extraction in the absence of DODAC. Percent recovery of trace amounts of [^{14}C]-DODAC (~5.0 pmol) in organic (C) and aqueous (D) phases following Bligh and Dyer extraction in the presence of excess (10 μg) DNA.

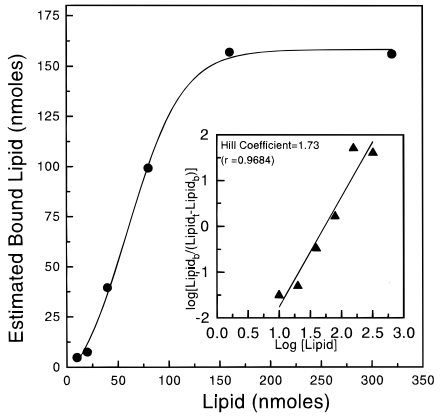

FIGURE 5: Correlation between estimated lipid bound to 40 μg of DNA and added lipid. The estimate of bound lipid was calculated from the amount of DNA recovered in the organic phase after Bligh and Dyer extraction assuming that each nucleotide phosphate bound one cationic lipid. Inset: Corresponding Hill plot.

obtained is indicative of a reaction which exhibits positive cooperativity. The corresponding Hill plot of the data (Figure 5 inset) is linear ($r = 0.97$) with a slope of 1.73, which is also consistent with positive cooperativity. Figure 6 shows DNA bound plotted as a function of DNA added. Over a large range of DNA concentrations, the amount of DNA in the organic phase correlated directly with the amount of DNA added to the system, provided that the amount of lipid present was not limiting. A Scatchard analysis of these data exhibited a bell-shaped curve, which is characteristic of reactions exhibiting positive cooperativity (results not shown). It has been suggested that for highly cooperative binding reactions the apparent dissociation constant (K_n) can be estimated within regions where near-saturation was achieved. For this system, the estimated K_n was 2.03×10^{-11} mol L^{-1}. In addition, at saturation, n (picomoles of DNA per nanomole of lipid) was estimated to be 0.1188. The inverse of this value ($1/n$) was the estimate of the amount of lipid bound to each DNA molecule (8417 mol of lipid bound per mole of DNA). Therefore, the charge ratio (+/−) at saturation was estimated to be 0.585 based on these binding curves. It is important to note that the Scatchard

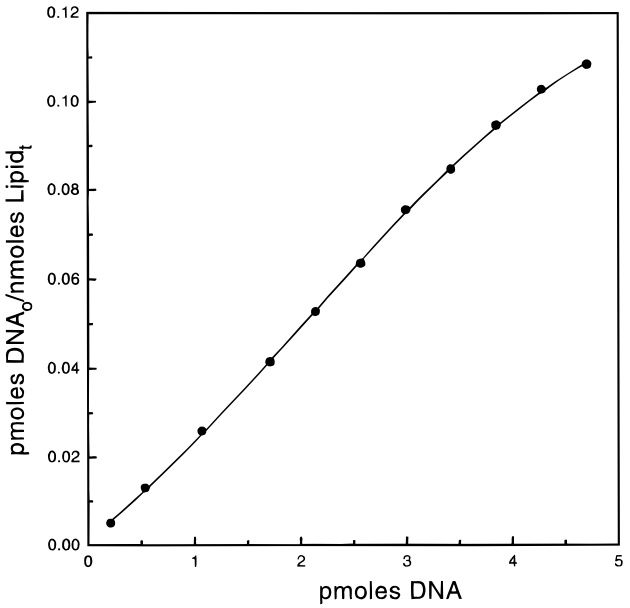

FIGURE 6: Amount of DNA bound per amount of lipid as a function of DNA added and extracted using the Bligh and Dyer method.

analysis required no assumptions regarding the number of lipid monomers bound to the DNA; however, the method employed was not based on direct measurement of free lipid.

Our interest in the binding reactions that resulted in formation of a hydrophobic cationic lipid/DNA complex has focused on methods for preparing well-defined DNA−lipid particles (DLPs) for use in gene therapy. As indicated in the study by Reimer *et al*. (1995), two approaches could be considered. First, particles could be prepared from solutions where cationic lipids, secondary lipids, and DNA were mixed in the presence of detergent. Second, solvent-based approaches could be considered where hydrophobic cationic lipid/DNA complexes were mixed with selected lipids prior to solvent removal (lyophilization or rotoevaporation) and subsequent hydration to achieve particle formation. If the latter approach is to be viable, then it is important to assess complex formation and stability in the presence of other lipids. The presence of secondary lipids can affect lipid/DNA complex formation in two ways. In a similar manner to DODAC, added amphipathic lipids can arrange themselves at the interface such that the head groups would be oriented toward the aqueous phase. Since the interface represents a finite surface area, these added lipids would displace cationic lipids at the interface and effectively reduce the amount of DODAC at the interface. Alternatively, the added amphipathic lipid may interact directly with the cationic lipid, preventing complex formation. For these reasons, secondary lipids may affect both complex formation as well as complex stability. Studies evaluating complex destabilization, where 40 nmol of neutral or anionic lipids was added to preformed DODAC/DNA complexes, are summarized in Figure 7. The results in Figure 7A indicate that zwitterionic lipids such as DOPE and DOPC and the neutral lipid cholesterol had little or no impact on the stability of the DODAC/DNA complex. The anionic lipid PEG-PE also did not affect complex stability (Figure 7B). In contrast, the presence of anionic lipids such as DOPS, DMPG, and LPI destabilized the complex (Figure 7B). This was most evident when DMPG was the secondary lipid which, when present in equimolar amounts to DODAC, completely dissociated the complex

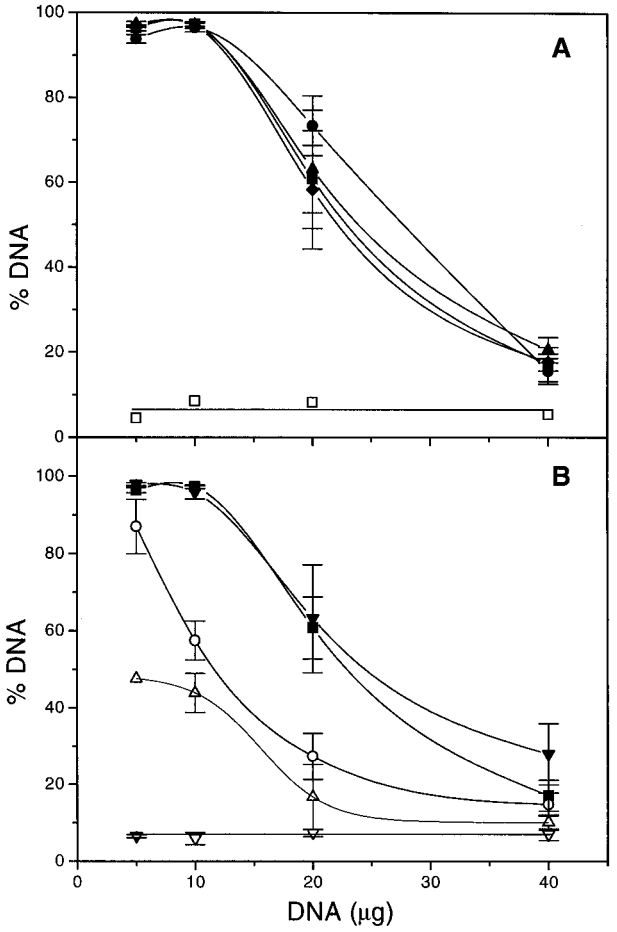

FIGURE 7: Effect of neutral lipids (A) and anionic lipids (B) on the recovery of pCMVβ DNA from the organic phase following formation of DNA/DODAC complexes. Varying amounts of DNA were added to 40 nmol of DODAC prior to addition of other lipids. Neutral lipids used were DOPE (●), DOPC (▲), and cholesterol (◆). As a control, DOPE was added to DNA in the absence of DODAC (□). Anionic lipids used were DOPS (○), DMPG (▽), PEG-PE (▼), and LPI (△). DODAC was also added to DNA in the absence of all other lipids (■). Data points were averaged from three replications and expressed ±SEM.

even when prepared at a cationic lipid to DNA charge ratio (+/−) of 2.0.

A second approach assessing the effect of added lipids on cationic lipid/DNA complex formation and/or destabilization was based on a lipid titration where the secondary lipid amount was increased in a system containing 10 μg of DNA and 40 nmol of DODAC (Figure 8). The second lipid, either DOPE or DOPC, was added either before or after complex formation. These lipids were selected on the basis of results shown in Figure 7 which indicate that DOPC and DOPE have little effect on complex stability. Three points are evident from these studies and are summarized in Figure 8. First, as shown in Figure 8A, the addition of DOPC had no impact on formation or stability of the DODAC/DNA complex even at levels approaching 10-fold molar excess relative to DODAC. Second, the cationic lipid/DNA complex was destabilized by the addition of increasing amounts of DOPE (Figure 8B). When DOPE was added in amounts that were >2-fold molar excess relative to DODAC, a reduction in DNA recovered in the organic phase was observed. Vigorous mixing of this sample in the two-phase system lead to near-complete dissociation of the complex (results not shown). Third, complex formation was inhibited

when DOPE was added prior to complex formation (Figure 8B). These results strongly indicate that DOPE influences DODAC/DNA binding, an effect that is likely a consequence of direct DOPE/DODAC interactions.

DISCUSSION

We have recently demonstrated that cationic lipid binding to DNA engenders formation of a hydrophobic complex which can be isolated in organic solvents (Reimer *et al.*, 1995). This previous study suggested that the complex could be formed in the presence of neutral detergents and dissociated after the addition of NaCl at concentrations as low as 1 mM. DNA in this complex was shown to be sensitive to DNase I and susceptible to intercalation of small DNA binding fluorescent probes, suggesting that it was not in a form that was condensed. We concluded by suggesting that this hydrophobic complex, prepared under well-defined conditions, can serve as an intermediate in the preparation of DNA−lipid particles (DLPs) useful for *in vitro* and *in vivo* delivery of plasmid DNA. The studies described here further characterize binding reactions between cationic lipids and DNA in an aqueous/organic system where the lipids exist as free monomers or in micellar form.

Other investigators have characterized the binding of mono- and divalent cations to anionic lipids using a two-phase partitioning assay similar to the one used in this report (Sokolove *et al.*, 1983; Brenza *et al.*, 1985). Briefly, binding parameters were obtained by determining the anionic lipid-dependent movement of cations from an aqueous phase to an organic phase. These previous studies suggested that the binding reaction involved formation of an inverted micelle where the cation was sequestered within a structure where the hydrophobic moieties were oriented toward the organic phase. Although the number of cationic lipids affecting redistribution of DNA into the organic phase is significantly greater than would be expected for a small cation like Ca^{2+}, an inverted micelle-like structure can also be envisioned for the hydrophobic lipid/DNA complex. DNA in the organic phase will be surrounded by lipids which are bound to the nucleotide polymer through ionic as well as hydrogen bonding interactions.

It is evident from the results presented here that hydrophobic cationic lipid/DNA complex formation is achieved through a binding reaction that occurs at the aqueous/organic interface following Bligh and Dyer extraction. This likely involves DNA, a multivalent anion, binding to cationic lipids which are arranged at the interface with their acyl chains extending into the organic phase and their ionic head groups orienting toward the aqueous phase. DNA binding to lipids at the interface is illustrated by the reaction scheme shown in Figure 9. This reaction involves a binding reaction between two multivalent systems, DNA (which carries 14 400 negative charges) and the charged surface at the aqueous/organic interface. The formation of the hydrophobic cationic lipid/DNA complex would progress *via* a reaction that exhibits positive cooperativity, a conclusion supported by sigmoidal lipid binding curves with a Hill coefficient of 1.73 (Figure 5) and a bell-shaped Scatchard plot. It should be noted that binding curves obtained for multivalent interactions exhibiting positive cooperativity are difficult to analyze. Our results, however, indicate that the formation of the hydrophobic complex requires lipid binding, partial charge neutralization, and hydrophobic interactions.

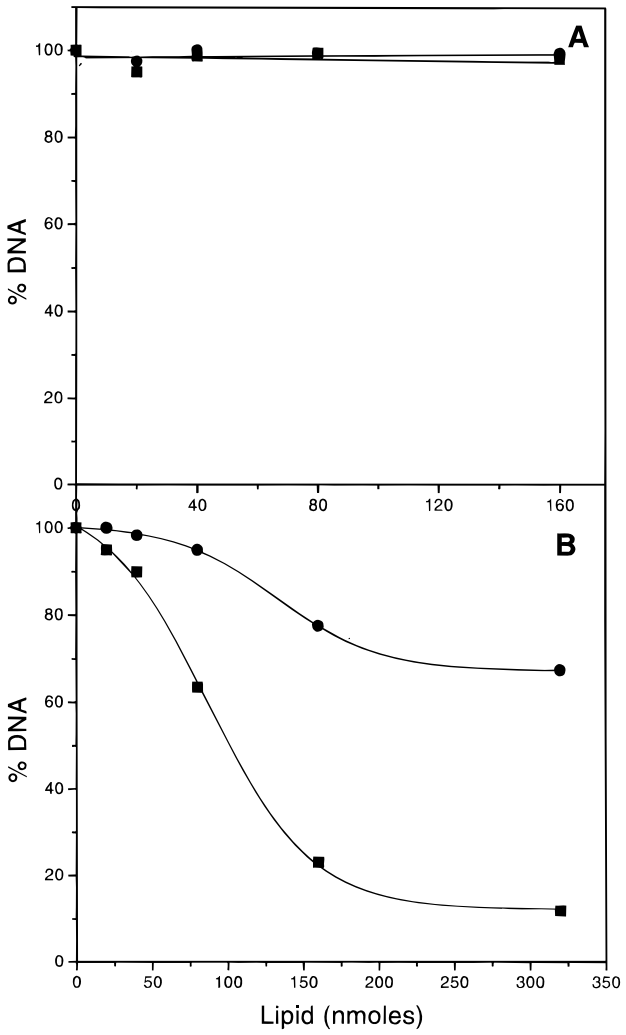

FIGURE 8: Effect of increasing amounts of DOPC (A) and DOPE (B) on the recovery of plasmid DNA from the organic phase following Bligh and Dyer extraction under conditions where the neutral lipids were added before (■) or after (●) formation of the complexes.

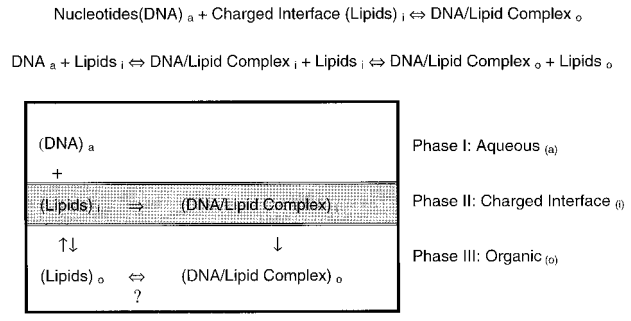

FIGURE 9: Reaction scheme proposed for the DNA/DODAC binding reaction that occurs at the aqueous/organic phase interface.

Based on the reaction mechanism proposed in Figure 9, we believe that the presence of secondary lipids can affect lipid/DNA complex formation and/or stability in several ways. Added amphipathic lipids could displace cationic lipids at the interface, ultimately reducing the amount of cationic lipid to DNA binding. This effect would be most pronounced when using a lipid such as the PEG-PE, where the large hydrophilic head group would orient at the interface as predicted and would also provide a charge shielding effect (Kenworthy *et al.*, 1995). Alternatively, the added lipids may directly interact with DODAC, preventing and/or

inhibiting this cationic lipid from binding to the nucleotide phosphate groups. Under the conditions employed here, where the interfacial area and lipid concentration at the interface are not controlled, it is likely that lipid−lipid interactions rather than interfacial displacement would be the most significant factor affecting complex formation. Thus, the charge shielding effect of PEG-PE at the interface likely does not prevent complex formation. Instead, the charge shielding reduces the ionic interactions between the PEG-PE anionic head group and DODAC. In contrast, anionic lipids that lack the PEG head group will bind DODAC and interfere with formation and stability of the complex. This is apparent when using the anionic lipids DOPS, DMPG, and LPI, which destabilize the complex (Figure 7). Ionic interactions between DODAC and the added anionic lipid would result in competitive inhibition of DODAC binding to DNA.

It is of interest that DMPG is a more effective inhibitor of complex stability then DOPS, a result that suggests that DODAC interaction with the phosphoglycerol head group is stronger than the interaction of DODAC with phosphoserine. The reduced effect of DOPS and LPI on the inhibition of DODAC/DNA complexes compared with that of DMPG may be explained by differences in the ability of these anionic lipids to form intermolecular hydrogen bonding [see Boggs (1987) for a review]. DMPG exhibits little intermolecular hydrogen bonding; therefore, DMPG may have an increased propensity to interact with the cationic DODAC through charge−charge interactions. DOPS and LPI, alternatively, exhibit higher levels of intermolecular bonding, reducing the propensity to interact with the cationic lipid, DODAC.

Zwitterionic lipids (such as DOPE and DOPC), cholesterol, and the anionic PEG-PE are less effective in terms of destabilizing preformed cationic lipid/DNA complexes. When evaluating the effects of the zwitterionic lipid DOPE, inhibition of complex formation was observed if the amount of DOPE present in the system described above increased above a DODAC/DOPE mole ratio of 2.0. This result suggests that there is an interaction between the cationic lipid DODAC and DOPE. This interaction may be due to hydrogen bonding between the phosphoethanolamine head group (Boggs, 1980, 1987) and the cationic head group of DODAC. Alternatively, since the ethanolamine group of DOPE has a titratable amine function, there may be sufficient quantities of the anionic DOPE to affect DODAC binding to DNA. The latter is unlikely since the pH of the assay system used here is approximately 6.5 for the organic and aqueous phases and the pK_a of the ethanolamine group is 9.5. If the pH of the system is increased, one might expect an increase in the capacity for the ethanolamine head group of DOPE to interact with DODAC through ionic interactions. Regardless of the mechanisms controlling the DODAC/DOPE interaction, these results clearly demonstrate the potential for DOPE to interact with cationic lipids. This interaction may be relevant in liposomal systems used for complexing polynucleotides. The importance of DOPE/cationic lipid interactions with respect to the development of transfection systems based on liposomes prepared from DOPE and cationic lipids is equivocal.

The ability to study DNA/lipid binding reactions using the systems described here provides a reasonable approach for characterizing and selecting novel cationic lipids that are being considered for use in gene transfer applications. Furthermore, factors that can alter DNA binding to cationic lipid surfaces can be easily identified without generating large heterogeneous aggregates that typically occur when mixing DNA with cationic liposomes. Reimer *et al*. (1995), for example, have shown that DNA does not appear to condense as a consequence of charge interactions or cationic lipid binding. Research programs synthesizing novel cationic lipids (Felgner *et al.,* 1994) could use this assay to characterize differences in DNA binding to these cationic lipids in well-defined systems. Additionally, the presence of contaminants (such as endotoxins) in plasmid DNA preparations may be detectable through subtle changes in cationic lipid binding as measured by the procedure described. A major advantage of this assay system is its reproducibility and predictability in forming lipid/DNA complexes under controlled conditions. Our interest in the binding reaction, however, is based on the fact that a hydrophobic lipid/DNA complex can be isolated in organic solvents and used subsequently for the preparation of DNA−lipid particles (DLPs). It will be possible to make use of the hydrophobic nature of this complex to generate novel systems that may, in turn, be designed to promote gene transfer. These systems can be generated with the use of secondary lipids added to stabilize the complex following removal of solvents and/or addition of water or buffer.

In summary, we cannot preclude the possibility that DODAC/DNA hydrophobic complexes are formed when the cationic lipid is added to DNA in a Bligh and Dyer monophase. However, following phase separation of the monophase into an organic and an aqueous phase, this complex must dissociate with DNA being retained in the aqueous phase, and lipid being extracted into the organic phase. These results indicate that after phase separation DNA binds cationic lipids at the interface between the organic and aqueous phases. This binding reaction is highly cooperative and is likely a consequence of multivalent interactions between DNA and the cationic interface consisting of a specifically oriented monolayer of cationic lipids. Complex formation can be effectively inhibited when adding secondary lipids that bind DODAC. Such effects were anticipated for the anionic lipids such as DMPG, LPI, and DOPS. However, these results show that the zwitterionic lipid DOPE also affects complex formation, perhaps through an interaction with DODAC.

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