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Phosphatidylethanolamine mediated destabilization of lipid-based pDNA delivery systems

F.M.P. Wong [a,b,1], P. Harvie [a,2], Y.-P. Zhang [a,1], E.C. Ramsay [a,*], M.B. Bally [a,b]

[a] *Department of Advanced Therapeutics, British Columbia Cancer Agency, 601 West 10th Ave., Vancouver, BC, Canada V5Z 1L3*

[b] *Department of Pathology and Laboratory Medicine, University of British Columbia, 2211 Westbrook Mall, Vancouver, BC, Canada V6T 2B5*

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Abstract

We have previously reported the development of lipid–DNA particles (LDPs) formed, via a hydrophobic cationic lipid–DNA complex intermediate, when detergent-solubilized cationic lipids are mixed with DNA. This study investigates the influence of zwitterionic co-lipid headgroups on the formation and stability of this intermediate and the subsequent DNA protection and transfection properties afforded by the resultant LDPs. We report that inclusion of diacylphosphatidylethanolamines (diacylPE), but not diacylphosphatidylcholines (diacylPC), as co-lipids destabilizes and prevents the formation of the cationic lipid–DNA intermediate to an extent dependent on the concentration of diacylPE and its acyl chain characteristics. DNA formulated in LDPs containing cationic:zwitterionic lipids at a 1:1 ratio is not readily accessible to the intercalating fluorescent dye, TO-PRO-1. At a lipid ratio 1:4, diacylPC LDPs are associated with significantly greater TO-PRO-1 fluorescence than equivalent diacylPE formulations, a result believed to reflect lipid-dependent penetration of TO-PRO-1 through the supramolecular LDP assembly, rather than condensation and protection of the DNA per se. Transfection studies utilizing the in vitro murine B16/BL6 melanoma cell line and the in vivo intraperitoneal B16/BL6 mouse tumor model demonstrated that only diacylPE LDPs mediated gene transfer. This was found not to be a consequence of differences in DNA delivery or cell toxicity.

Keywords: Gene transfer; Non-viral gene therapy; Phosphatidylethanolamine; Lipid–DNA particles; Plasmid DNA; Headgroup interactions

1. Introduction

Non-viral gene therapy protocols require reproducible and efficient delivery systems for the introduction of plasmid DNA into specific cell types (Ledley, 1995; Tomlinson and Rolland, 1996). The most widely investigated systems are the synthetic cationic lipid-based vehicles and, in particular, cationic liposomes (Felgner et al., 1987; Gao and Huang, 1991; Rose et al., 1991). It is well known that these systems are capable of transfecting many cell types (Nabel et al., 1990; Fasbender et al., 1995; Meyer et al., 1995); however, the mechanisms by which these systems bypass the biological barriers in order to achieve transgene expression are not understood (Zabner et al., 1995; Reimer et al., 1997).

* Corresponding author. Tel.: +1-604-877-6098x3012; fax: +1-604-877-6011.
E-mail address: eramsay@bccancer.bc.ca (E.C. Ramsay).
[1] Present address: ALZA Corp., 1050 Hamilton Court, Menlo Park, CA 94025, USA.
[2] Present address: Targeted Genetics Corp., Suit 100, 1100 Olive Way, Seattle, WA 98101, USA.

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Formation of these systems is mediated through simple electrostatic interactions, typically between positively charged cationic liposomes and the phosphate groups of DNA (Zuidam and Barenholz, 1998). These systems are formulated in a manner whereby the resulting DNA/liposome aggregates exhibit a positive charge that facilitates association with a negatively charged cell surface (Stamatatos et al., 1988; Wong et al., 1999). Numerous structural changes are induced by electrostatic binding reactions, both in terms of DNA–liposome interactions and cationic liposome/DNA–cell interactions. These changes involve more than simple electrostatic crosslinking of components. It has been demonstrated, for example, that DNA binding to liposomes can engender significant disruption in liposome structure (Sternberg et al., 1994; Radler et al., 1997; Wasan et al., 1999). It is unclear whether further changes in the adopted structures occur as a consequence of cell binding.

Structure–activity relationships of cationic liposome–DNA complexes have been difficult to assess due to the diverse range of structures that are generated when using formulation strategies involving preformed cationic liposomes. Further, it is apparent that any single preparation can contain a wide range of structures in varying proportions and it is unknown which of these morphologically distinct structures contribute to transfection activity. We have suggested that following DNA addition to liposomes significant alterations in the liposome structure, which occur as a consequence of electrostatic and hydrophobic interactions, lead to transient generation of mixed lipid micelles and/or monomeric lipids (Wasan et al., 1999). In turn, this may facilitate fusion, hemifusion or other bilayer destabilization events. Alternatively, these intermediate lipid structures may be available to bind directly to DNA, leading to formation of a hydrophobic lipid–DNA complex (Wong et al., 1996; Zhang et al., 1997).

Considering the incertitude of formulations relying on preformed cationic liposomes, our laboratory has established a method whereby cationic lipid and DNA are combined in a systematic manner in the presence of detergents (Bally et al., 1997; Zhang et al., 1997), thereby bypassing the steps involving liposome formation followed by DNA-mediated liposome destabilization. In this procedure, cationic lipids and any other lipid species of interest are solubilized as mixed lipid micelles prior to the addition of plasmid DNA. Ion-pairing between the DNA and cationic lipids is believed to generate a hydrophobic complex (Reimer et al., 1995). Additional lipid incorporation into the hydrophobic lipid–DNA complex is anticipated on the basis of hydrophobic interactions. Under appropriate conditions, these intermediates spontaneously form lipid–DNA particles (LDPs), where hydrophobic effects and solvent reorganization are the main driving forces promoting particle formation. The development of technologies that carefully control the cationic lipid–DNA interactions has enabled the generation of well defined stable particles that demonstrate extended circulation longevity suitable for the systemic delivery of pDNA (Tam et al., 2000; Fenske et al., 2001) and antisense oligonucleotides (Stuart et al., 2000).

This report demonstrates that it is possible to generate LDPs from a wide variety of lipid species, under well-defined conditions and in a stepwise manner. Studies with the resulting LDPs were developed in an attempt to expand the understanding of the interactions that govern lipid binding and dissociation. In particular, these studies correlate lipid headgroup and lipid–DNA interactions with formulation stability, destabilization and transfection activity both in vitro and in vivo.

2. Materials and methods

2.1. Materials

N-Octyl-β-D-glucopyranoside (OGP) was purchased from Sigma (St. Louis, MO). Steven Ansell at Inex Pharmaceuticals Corp. (Vancouver, BC) synthesized the cationic lipid, *N*-*N*-dioleyl-*N*-*N*-dimethylammonium chloride (DODAC). 1,2-Dimyristoyl-*sn*-glycerol-3-phosphoethanolamine (DMPE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were obtained from Northern Lipids Inc. (Vancouver, BC), while 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine (DLPE) and 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) were purchased from Avanti

Polar Lipids Inc. (Alabaster, AL). The DNA intercalating fluorescent dye, TO-PRO-1 was acquired from Molecular Probes (Eugene, OR). Radiolabeled [^{14}C]-chloramphenicol and methyl-[^{3}H]-thymidine-5′-triphosphate were from NEN Dupont (Boston, MA). The plasmid DNA pINEXCATv2.0 was obtained from Inex Pharmaceuticals Corp. (as previously described, Reimer et al., 1995). The plasmid DNA was isolated by standard molecular techniques (Sambrook et al., 1989) and purified using a Qiagen Plasmid DNA Purification Kit (Mississauga, ON). The nucleic acid concentration was measured by UV absorption at 260 nm and purity was verified by electrophoresis on a 0.8% agarose gel.

2.2. Bligh and Dyer extraction procedure

The monocationic lipid DODAC (40 nmol) and the pINEXCATv2.0 plasmid DNA (10 μg) were solubilized separately in a Bligh and Dyer monophase consisting of chloroform:methanol:water (1:2.1:1) (Bligh and Dyer, 1959). The effects of zwitterionic lipids (DLPE, DMPE, DSPE, DOPE, DLPC, DMPC, DSPC, DOPC) on the formation and/or dissociation of DODAC/pINEXCATv2.0 complexes were evaluated. The effects of the additional lipids on the formation of the complexes were evaluated by mixing them with DODAC prior to the addition of pINEXCATv2.0 (final 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 600 × *g* for 5 min at room temperature. In order to assess dissociation, the DODAC/pINEXCATv2.0 complexes were formed prior to the addition of other lipids. Zwitterionic lipids were injected directly into the organic phase following Bligh and Dyer extraction and formation of the two-phase system. For both procedures the upper aqueous phase (∼1.0 ml) was removed and the amount of DNA in the aqueous phase was determined. The estimated pH was found to be 6.5 as determined by using pH paper obtained from EM Science (Cherry Hill, NJ). DNA in the aqueous phase was quantified by measuring the optical density at a wavelength of 260 nm using a luminescence UV Spectrophotometer (DU-64) from Beckman Instrument Inc. (Fullerton, CA). Data collected by this method were presented as percentage of DNA recovered in the organic phase. As a control, DOPE was added to the DNA in the absence of DODAC to ensure that DOPE alone did not mediate extraction of the DNA into the organic phase.

2.3. Preparation of lipid–DNA particles (LDPs)

Cationic and neutral lipids, at the desired lipid ratio, were dissolved in chloroform:methanol (1:1, v/v). Subsequently, the solvents were evaporated under a stream of N_2 gas to obtain a thin lipid film followed by lyophilization for 1 h at a pressure <50 mTorr in a freeze-dry system (LabConco, Kansas City, MO). The lipids were then solubilized with at least 35 mM OGP. To aid solubilization, the lipid solution was heated briefly at 50 °C. If the sample remained turbid, 1 M OGP was added dropwise until the solution was no longer visibly cloudy. The lipid solutions (3520 μM total lipid) were incubated on ice until ready for particle formation. The plasmid DNA, prepared in OGP (concentration equal to that used to prepare the mixed detergent–lipid micelles), was diluted to a concentration such that mixing equal volumes of the DNA (up to 280 μg/ml) and lipid solution would achieve the desired lipid–DNA ratio. Pre-chilled DNA was added in one addition to the lipid solution and the resulting solution immediately became turbid. Unless stated otherwise the final concentration of DNA and total lipid was 140 μg/ml and 1760 μM, respectively. The mixture was vortexed and incubated on ice for 30 min. (These temperature conditions were employed based on previous observations that the particle size of lipid–DNA complexes is significantly smaller when formed at 4 °C compared with 25 and 37 °C, unpublished observations.) Following incubation, the mixture was transferred to dialysis tubing (MWCO 12-14000). LDPs were dialyzed against sterile distilled water for 72 h at 4 °C with a complete change of water at 12 h intervals. Particle size distribution and homogeneity were evaluated after dialysis by QELS. The resulting LDPs, when prepared at 4 °C, had mean diameters ≤150 nm.

2.4. TO-PRO-1 dye exclusion assay

The dye, TO-PRO-1, fluoresces under conditions when it is intercalated to DNA (Crissman and Hirons, 1994). LDPs at charge ratios (+/−) of 2:1 and 4:1, and cationic lipid:neutral lipid ratios of 1:1 and 1:4 were

generated as described above. Two microgram of formulated DNA was added to a quartz cuvette containing water in a final volume of 500 μl. Subsequently, 1 μl of TO-PRO-1 was added to these samples to achieve a final concentration 1 μM. Fluorescence was measured at room temperature using a Luminescence Spectrophotometer 50B (Perkin-Elmer, Norwalk, CT) at an excitation wavelength of 509 nm, slit width of 2.5, and an emission wavelength of 533 nm, slit width of 5.0. All samples containing TO-PRO-1 were maintained in the dark to minimize photobleaching. Initial fluorescence was determined as (I). The maximum fluorescence intensity (I_F) was measured after solubilization of LDPs by 50 μl 100 mM OGP. Dye exclusion index was calculated as: $[(I_F - I)/I_F] \times 100$.

2.5. In vitro transfection

B16/BL6 murine melanoma cells were plated at 4×10^3 cells per well in a 96-well plate in DMEM supplemented with 10% fetal bovine serum (FBS). Twenty-four hours later, media was removed and 100 μl containing 2 μg of pINEXCATv2.0 of free DNA, or LDPs were added to the cells and incubated for 4 h. Subsequently, media was removed and replaced with 200 μl fresh media and incubated for another 48 h. The cells were analyzed for chloramphenicol acetyl transferase (CAT) activity as previously described (Seed and Sheen, 1988). After protein extraction, 25,000 dpm of [^{14}C]-chloramphenicol and *N*-butyryl-CoA (5 mg/ml) were added to each sample and incubated for 2 h at 37 °C. Mixed xylenes (Aldrich, Milwaukee, WI) were added followed by vigorous mixing and separation by centrifugation ($8000 \times g$). The upper aqueous phase was removed and washed with buffer. Subsequently, 100 μl of the aqueous phase was removed and Pico-fluor scintillant was added to determine the amount of radioactivity. CAT units were derived from a standard curve. Each assay was evaluated three separate times with at least three well replicates per sample and reported as ±S.E.M.

2.6. In vivo transfection and delivery

Seven week old C57/BL6 (Charles River, Montreal, PQ) female mice were injected intraperitoneally with 1×10^5 B16/BL6 cells. Seven days after tumor cell inoculation LDPs or free DNA was injected i.p. with 500 μl of a sample containing 70 μg/ml DNA. At 24 h post-injection the tumors (<100 mg) were excised, frozen at −70 °C for at least 24 h and subsequently analyzed for CAT activity. For delivery studies, [^{3}H]-DNA (∼25,000 dpm) was used to prepare the LDP formulations and tissues were harvested and processed by addition of Solvable (Packard Bioscience RV, Groningen, NL) and incubated overnight at 50 °C. Scintillation fluid was added to 200 μl homogenates and radioactivity was determined by scintillation counting using a Packard TR 1900 Scintillation Counter. Each group contained four animals and assay results were reported as ±S.E.M.

2.7. Statistical analysis

All data values for transfection studies are presented as the mean ± standard error of the mean. A Student's *t*-test was performed on treated and untreated samples comparing independent or dependent results using Statistica software. Differences were considered significant if the P-value <0.05 and P-values are reported.

3. Results

Our laboratory has previously demonstrated that the formation of lipid–DNA particles (LDPs) is mediated through the generation of a hydrophobic cationic lipid–DNA complex intermediate (Reimer et al., 1995; Wong et al., 1996). The formation and destabilization of this hydrophobic intermediate provides a useful tool to assess interactions between the lipids and DNA. This is demonstrated by the representative data shown in Fig. 1, where the influence of defined diacylphosphatidylethanolamines (diacylPE: DLPE, DMPE, DSPE, DOPE) and diacylphospahtidylcholines (diacylPC: DLPC, DMPC, DSPC, DOPC) on formation (Fig. 1A) and destabilization (Fig. 1B) of the hydrophobic cationic lipid–DNA intermediate was studied. Regardless of whether PC-containing lipids were added before or after lipid–DNA complex formation, all the DNA was recovered in the organic phase, even at greater than eight-fold molar excess of PC-lipids over cationic lipids. This result is consistent with our previous reports (Wong et al., 1996; Harvie et al., 1998) and clearly demonstrates that diacylPC

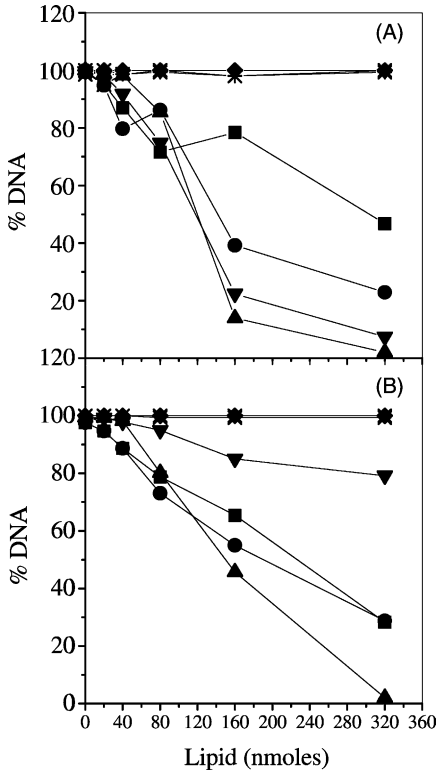

Fig. 1. Effect of various helper lipids added before (formation) (A) and after (dissociation) (B) addition of cationic lipid to DNA via the Bligh and Dyer extraction procedure (see Section 2). The % DNA (10 μg initial) recovered in the organic phase was determined in the presence of increasing amount of lipids: DLPE (■), DMPE (●), DSPE (▲), DOPE (▼), DLPC (◆), DMPC (+), DSPC (×), DOPC (∗).

have no impact on formation or destabilization of hydrophobic cationic lipid–DNA complexes under these conditions.

When similar studies were completed in the presence of diacylPEs, prevention of formation as well as dissociation of the preformed lipid–DNA complex was observed. For example, when there was an eight-fold molar excess of DSPE, there was greater than 95% inhibition of complex formation (Fig. 1A, ▲) and complete destabilization of pre-formed complexes (Fig. 1B, ▲). Interestingly, the effect of diacylPEs was dependent on the acyl chain composition. Specifically, inhibition of complex formation by these lipids was greater as acyl chain length increased; e.g. inhibition with DMPE (C14, ●) was greater than that observed with DLPE (C12, ■), an observation that was found to be independent of acyl chain saturation (cf. DOPE (C18:1, ▼) and DSPE (C18:0, ▲)). In contrast, measurements of complex destabilization (Fig. 1B) indicated that DOPE (▼) was the least effective diacylPE in terms of facilitating dissociation of the hydrophobic complex. This suggests saturation of the acyl chain may be an important parameter in complex destabilization, with the saturated diacylPEs demonstrating a trend of increased destabilization with increasing acyl chain length in a manner similar to the results illustrated in Fig. 1A.

The results presented thus far demonstrate differences in cationic lipid–phospholipid–DNA interactions that depend primarily on whether the phospholipid has a choline or ethanolamine headgroup. In order

Table 1
TO-PRO-1 dye exclusion assay

Lipid ratio (DODAC:phospholipid)	Charge ratio (+/−)	Phospholipid used in combination with DODAC in the preparation of LDPs			
		DOPC	DOPE	DLPC	DLPE
1:1	2:1	86	87	78	93
1:1	4:1	90	88	87	97
1:4	2:1	33	61	25	77
1:4	4:1	34	63	26	85

TO-PRO-1 binding to DNA formulated in LDPs prepared at 4 °C with DODAC and the indicated phospholipids. LDPs were formulated with DNA at charge ratios (+/−) of 2:1 and 4:1 and with cationic lipid to phospholipid ratios of 1:1 and 1:4. The LDP formulations were equilibrated to 25 °C before addition of TO-PRO-1 and the resultant fluorescence, I, was determined as described in Section 2. Fluorescence was also measured after addition of OGP to a final concentration of 100 mM and this value was recorded as I_F. Dye exclusion indices were calculated as $[(I_F - I)/I_F] \times 100$. For reference, poly-L-lysine condensed DNA typically shows a dye exclusion index of >95.

to assess how these interactions affect the attributes of LDPs, particles were generated from hydrophobic lipid–DNA complex intermediates prepared from lipid–detergent mixtures as previously described (Zhang et al., 1997; Harvie et al., 1998).

The ability of the fluorescent intercalating probe, TO-PRO-1, to access the DNA incorporated in LDPs was investigated as a function of both the cationic lipid:phospholipid ratio and the cationic lipid:DNA charge ratio (+/−). Table 1 indicates that all LDP formulations prepared at a lipid ratio of 1:1 show high dye exclusion indices (>77). However, formulations prepared with a four-fold molar excess of phospholipids, exhibited dye exclusion indices that were not only lower, but were also dependent on the phospholipid present, with LDPs prepared with diacylPEs exhibiting dye exclusion indices (>63) that were substantially greater than those observed for LDPs prepared with diacylPCs (<35). These observations were shown to be independent of charge ratio at the charge ratios tested.

To determine whether the phospholipid composition affects LDP-mediated gene transfer, the delivery and expression of a plasmid DNA encoding the CAT protein was assessed using both in vitro and in vivo assays. Fig. 2 presents the results of an in vitro transfection study. DNA, in the absence of any carrier, was not able to significantly transfect B16/BL6 cells. The inclusion of DNA in LDP formulations comprising

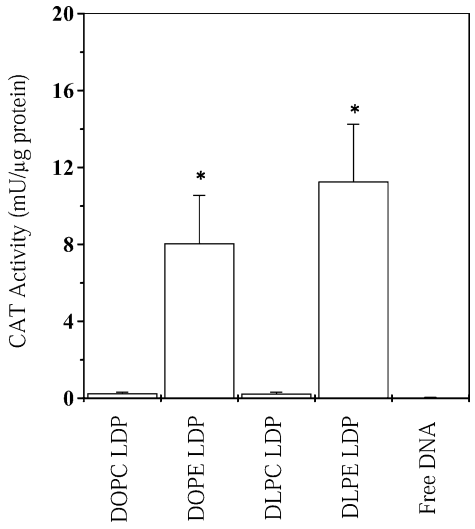

Fig. 2. In vitro transfection of B16/BL6 cells. B16/BL6 melanoma cells were plated at 4×10^3 cells/well in a 96 well plate containing DMEM and 10% FBS and grown up overnight. Lipid-based formulations containing the cationic lipid DODAC and various phospholipids were made up at charge ratios (+/−) of 2:1 and DODAC to phospholipid ratios of 1:1. LDPs (containing helper lipids DOPC, DOPE, DLPC, or DLPE) or free DNA were added and incubated in DMEM and 10% FBS for 4 h. Media was removed and replaced with fresh media for a further 48 h. The level of chloramphenicol acetyltransferase (CAT) activity was measured as described in Section 2. Values were determined from three replications and expressed as mean ± S.E.M. Statistical tests as described in Section 2. (*)$P < 0.00005$ when comparing DOPC to DOPE LDPs and $P < 0.005$ when comparing DLPC to DLPE LDPs.

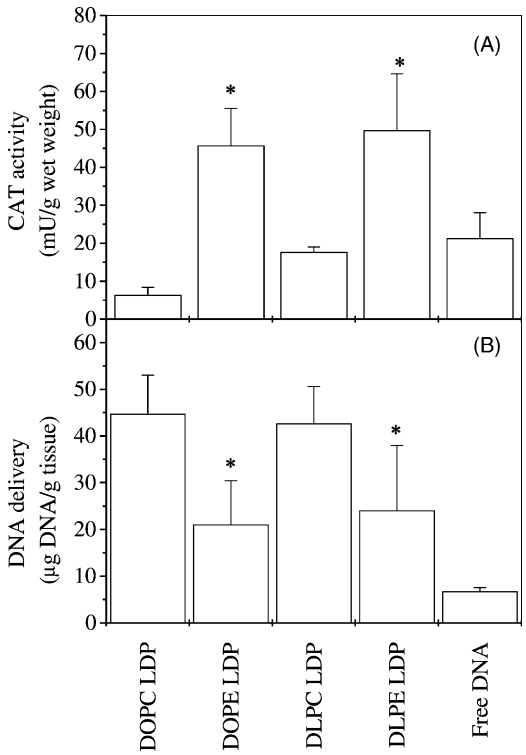

Fig. 3. In vivo transfection of B16/BL6 tumors (A) and delivery of [^{3}H]-DNA to B16/BL6 tumors (B) following i.p. administration of LDPs in C57/BL6 female mice inoculated with B16/BL6 cells (i.p.) 7 days previously. LDPs or free DNA was injected at a dose of 35 μg pINEXCATv2.0 per animal. LDP formulations used were at a charge ratio (+/−) of 2:1 and DODAC to phospholipid (DOPC, DOPE, DLPC, DLPE) ratio of 1:1. Tumors were harvested 24 h post-injection and analyzed for chloramphenicol acetyltransferase (CAT) activity (A) or radioactivity (B). There were four animals per group and data were expressed as mean activity ±S.E.M. Statistical tests as described in Section 2. (*)$P < 0.05$ when comparing DOPC to DOPE, or DLPC to DLPE LDPs.

DLPC or DOPC did promote marginally greater expression than DNA alone. However, LDPs prepared using either DLPE or DOPE increased the level of CAT expression, by at least an order of magnitude, to values in excess of 8 mU/μg protein. The superior transfection efficiency mediated by diacylPE-based LDPs was not found to be the result of differences in cell toxicity (data not shown).

Fig. 3 extends the transfection studies by investigating the relationship between the amount of DNA delivered to, and the degree of CAT expression in an in vivo peritoneal B16/BL6 tumor mouse model (Reimer et al., 1997). The CAT expression mediated by the LDPs is shown in Fig. 3A and follows a similar pattern to the in vitro data, where LDPs prepared with diacylPEs showed significantly increased transfection activity (two- to four-fold) in comparison to those prepared with diacylPCs. In contrast, the levels of tumor-associated plasmid DNA (Fig. 3B) were found to be significantly greater when LDPs were prepared using diacylPCs as the secondary lipid compared to equivalent formulations comprising diacylPEs.

4. Discussion

The ability to establish structure–function relationships for cationic liposome formulations designed to bind, protect and deliver plasmid DNA expression vectors has been severely hampered by the heterogeneous structures that arise from cationic lipid–DNA interactions. Many investigators have begun to develop formulation approaches that rely on the use of a well-defined hydrophobic lipid–DNA complex intermediate which, in aqueous solutions, spontaneously adopt a heteromolecular structure (Bally et al., 1997; Hara et al., 1997; Zhang et al., 1997; Wheeler et al., 1999). This study characterizes these formulations with the aim of developing a greater understanding of how lipid–lipid and lipid–DNA interactions can influence transfection activity.

Under optimal conditions, LDPs exhibiting comparable mean size distributions of less than 150 nm can be formulated using the cationic lipid, DODAC and a variety of lipid components. This allows questions, such as what is the role of ‘helper’ lipid headgroup in governing transfection activity, to be addressed in a methodical fashion. In previous studies we focused on the lipid-mixing behavior of LDPs in comparison to cationic liposome/DNA aggregates (Harvie et al., 1998). In this report, an assessment of changes in DNA protection and transfection was made as a function of simple changes in phospholipid headgroup and acyl chain length. This discussion will extend our argument that the ethanolamine headgroup helps to destabilize cationic lipid–DNA interactions, a process that involves cationic/phospholipid lipid interactions as well as phospholipid/DNA interactions.

Results in Fig. 1 demonstrate that phospholipids with an ethanolamine headgroup, in contrast to a choline headgroup, can inhibit formation and cause dissociation of the hydrophobic cationic lipid–DNA complex. The efficacy of the diacylPE-mediated inhibition of complex formation was found to be dependent on acyl chain length, where DSPE (C18) = DOPE (C18:1) > DMPE (C14) > DLPE (C12). This observation was independent of acyl chain saturation, demonstrated by comparable degrees of inhibition with the monounsaturated DOPE and its saturated equivalent, DSPE. These results may be explained by the schematic shown in Fig. 4A. Based on the model of complex formation previously described (Wong et al., 1996), the headgroups of the cationic lipids are aligned along the aqueous–organic interface. When modulating phospholipids are added in combination with DODAC prior to the addition of DNA, this interface will consist of a monolayer matrix of cationic lipid and zwitterionic lipid. The presence of the larger choline headgroups at this interface does not interfere with the formation of the hydrophobic cationic lipid–DNA complex indicating that steric effects do not appear too important in disrupting complex formation. In contrast, the smaller ethanolamine headgroup actively interferes with complex formation. The potency of this disruption is dependent on acyl chain length, with the diacylPEs that have the same acyl chain length as DODAC (C18) proving the most disruptive. This suggests that the packing parameter of zwitterionic lipid at this interface reflects acyl chain miscibility (Slater et al., 1993).

We believe that this observation provides further evidence that the ethanolamine headgroup mediates its effect via electrostatic mechanisms. The titratable amine group of ethanolamine is believed to stabilize bilayer formation by interaction with the non-esterified

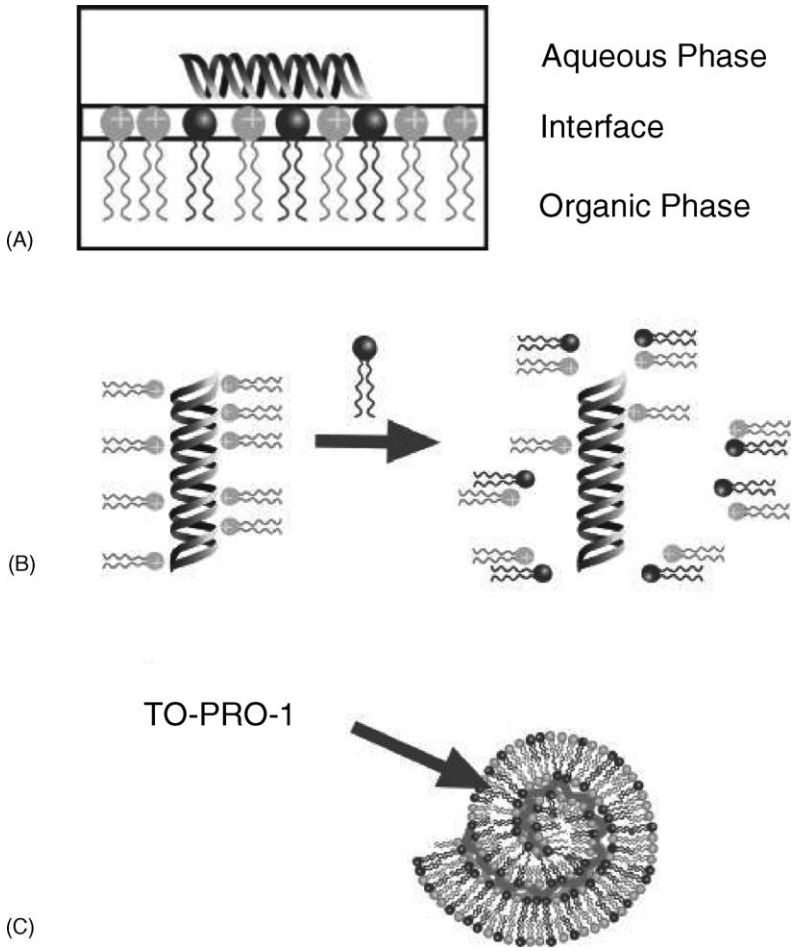

Fig. 4. A model of the putative mechanism of diacylPE-mediated inhibition of cationic lipid/DNA complex formation (A). Diacylphosphatidylethanolamines have the ability to interfere with cationic lipid/DNA interaction at the interface when diacylPEs and cationic lipids are mixed prior to the addition of DNA (B). After hydrophobic complex formation, diacylPE can disrupt the hydrophobic cationic lipid/DNA complex (C). TO-PRO-1 demonstrates differential access to DNA.

oxygen of phosphate groups within and between lipid bilayers (Damodaran and Merz, 1997). Potentially, the amine group could also interact with DNA phosphates thereby weakening the cationic lipid–DNA interaction.

The disruption of the preformed cationic lipid–DNA hydrophobic complex by subsequent addition of zwitterionic lipid (putative mechanism illustrated in Fig. 4B) follows a similar pattern to the inhibition of complex formation discussed above, with a notable exception (Fig. 1B). The behavior of the diacylPC mirrored that observed in Fig. 1A where an eight-fold excess of phospholipid over cationic lipid had no effect on complex stability. Similarly, the ability of diacylPE to disrupt the complex appeared to be influenced by acyl chain length, with addition of DSPE again resulting in the maximum loss of DNA from the hydrophobic complex. The major difference in behavior, by a zwitterionic lipid, between the experimental conditions described in Fig. 1 is observed with DOPE. Addition of DOPE after complex formation resulted in recovery of ~80% DNA in the organic phase compared with <10% if DOPE is mixed with DODAC prior to interaction with DNA. These observations suggest that subsequent to formation of the cationic lipid–DNA hydrophobic complex, the ability of PE to

mediate its effect is influenced both by the length of the acyl chain and its saturation characteristics. In an organic environment the prominent feature that differentiates DSPE (C18:0) and DOPE (C18:1) is the molecular shape of the lipid. DSPE adopts a cylindrical shape in contrast to DOPE, which is cone shaped due to the monounsaturated oleoyl chains. The large area occupied by the acyl chains of DOPE may sterically hinder the ability of the ethanolamine headgroup to access the site of cationic lipid–DNA interaction.

To further our investigations into the influence of zwiterionic phosopholipids on the stability of LDPs, we formulated LDPs comprising DODAC and diacylPE or diacylPC using the optimized detergent dialysis conditions and investigated the ability of the intercalating fluorescent dye, TO-PRO-1 to access the base pairs of DNA. Dye binding data (Table 1) confirms that upon formation of LDPs, TO-PRO-1 is excluded to some degree from intercalating DNA base pairs. Dye exclusion indices are similar for both PE and PC formulations when the DODAC:phospholipid ratio is 1:1, whereas DODAC:PE (1:4) excludes TO-PRO-1 from the plasmid DNA to a greater degree than for DODAC:PC at the same lipid ratio. The acyl chain composition had no effect on these observations. These results contradict the hypothesis that the presence of diacylPE destabilizes the cationic lipid–DNA binding.

Previous studies in our laboratory have shown that TO-PRO-1 binds to pDNA under conditions where the pDNA is protected from DNase I degradation (Zhang et al., 1997; Harvie et al., 1998 and unpublished observations). We believe that the fluorescence assay, particularly when the phospholipid is in excess, reflects phospholipid-dependent access of the small hydrophobic TO-PRO-1 molecules to the pDNA contained within supramolecular LDP assemblies. Therefore, TO-PRO-1 binding may reflect differences in the organization of the lipids coating the pDNA rather than condensation and protection of the DNA per se (Fig. 4C).

Transfection efficiencies achieved with lipid-based transfer vehicles have been attributed to the nature of the neutral lipid used (Campbell, 1995; Hui et al., 1996). Previous reports, for example, have emphasized the role of DOPE in mediating fusion with either the target cell plasma membrane or the endocytic vesicle membrane via the inverted hexagonal phase preferentially adopted by DOPE (Wimley and Thompson, 1991; Bailey and Cullis, 1997). Formulations containing lipids that include DOPC induce lower transfection activity than those containing DOPE as the headgroup. These observations are consistent with what we report here, but the increases in transfection are not solely observed with DOPE, contradicting the inverted hexagonal phase theory. Indeed, in both in vitro and in vivo models, the degree of transgene expression mediated by DLPE (C12:0) as the ‘helper’ lipid was not significantly different to that observed when DOPE (C18:1) was employed as the ‘helper’ lipid. Therefore, based on these observations, enhanced transfection can be attributed to any phospholipid containing the PE headgroup, including the bilayer-forming saturated phospholipids. These results are consistent with the proposed hypothesis that headgroup interactions are an important determinant for efficient LDP-based transgene expression.

Based on the results in Fig. 3A and B (and in vitro observations, data not shown), differences in transfection efficiency observed between the diacylPCs or diacylPEs formulated LDPs were not a consequence of DNA delivery. This suggests that the influence of the ‘helper’ lipid headgroup is subsequent to DNA delivery to the cell and is the rate limiting step determining transgene expression.

It should be noted that in comparison to data obtained using CAT plasmid DNA formulated with pre-formed liposomes, the level of DNA delivery to the B16/BL6 tumors was at least two- to three-fold less. Although the liposome/DNA aggregates and LDPs result in gene transfection systems that work equally well in vitro (Harvie et al., 1998) these LDP formulations are much less effective in terms of the in vivo transfection model used here (Reimer et al., 1999). It is argued that the reduced DNA delivery observed with LDPs is due in part to their enhanced stability, both in terms of physical and chemical attributes.

These studies have defined some of the basic interactions that are involved in formation of lipid–DNA particles and subsequent DNA release from bound cationic lipids. More specifically, these results further define the role of diacylPEs, suggesting that headgroup interactions with cationic lipids and DNA are distinct from properties promoting fusion. The use of LDPs, generated from mixed detergent/lipid micelle interaction with DNA, results in a more

versatile system for preparing plasmid DNA expression vector delivery systems. Based on the methodology described here, basic interactions involved in formation, delivery and lipid-destabilization of lipid-based delivery systems due to individual lipid components can be systematically assessed.

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