

# The Role of Helper Lipids in Cationic Liposome-Mediated Gene Transfer

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**ABSTRACT** In the procedure for cationic liposome-mediated transfection, the cationic lipid is usually mixed with a "helper lipid" to increase its transfection potency. The importance of helper lipids, including dioleoylphosphatidylcholine (DOPC) and phosphatidylethanolamine (dioleoyl PE), DO was examined. Freeze-fracture electron microscopy of DNA:cationic complexes containing the pSV- $\beta$ -GAL plasmid DNA, the cationic lipid dioleoyl trimethylammonium propane, and these helper lipids showed that the most efficient mixtures were aggregates of ensheathed DNA and fused liposomes. PE-containing complexes aggregated rapidly when added to culture media containing polyanions, whereas PC-containing complexes did not. However, more granules of PC-containing complexes were formed on cell surfaces after the complexes were added to Chinese hamster ovary (CHO) cells in transfection media. Pronase treatment inhibited transfection, whereas dilute poly-L-lysine enhanced transfection, indicating that the attachment of DNA:liposome complexes to cell surfaces was mediated by electrostatic interaction. Fluorescence spectroscopy studies confirmed that more PC-containing complexes than PE-containing complexes were associated with CHO cells, and that more PC-containing complexes were located in a low pH environment (likely to be within endosomes) with time. Cytochalasin-B had a stronger inhibitory effect on PC-containing liposome-mediated than on PE-containing liposome-mediated transfection. Confocal microscopic recording of the fluorescently label lipid and DNA uptake process indicated that many granules of DNA:cationic liposome complexes were internalized as a whole, whereas some DNA aggregates were left out on the cell surfaces after liposomes of the complexes fused with the plasma membranes. For CHO cells, endocytosis seems to be the main uptake pathway of DNA:cationic liposome complexes. More PC-containing granules than PE-containing granules were formed on cell surfaces by cytoskeleton-directed membrane motion, after their respective DNA:liposome complexes attached to cell surfaces by electrostatic means. Formation of granules on the cell surface facilitated and/or triggered endocytosis. Fusion between cationic liposomes and the cell membrane played a secondary role in determining transfection efficiency.

## INTRODUCTION

The use of cationic liposomes as a vector for gene transfer has caught much attention lately. Although cationic lipids have been synthesized and investigated for some time, and their properties of inducing liposome fusion are known (Martin and MacDonald, 1976), the use of liposomes containing these lipids as delivery vehicles is a rather recent idea. Felgner and colleagues (1987) reported the use of cationic liposome to transfect cells. Since then, a number of cationic lipids have been synthesized and used as vectors to transfect cells both *in vitro* and *in vivo* (Smith et al., 1993). These cationic liposomes are relatively effective and may be applied *in vivo*, and are biologically safe in comparison to viral vectors.

The mechanism of cationic liposome action is not exactly known. In a majority of reported studies, cationic liposomes function most efficiently when the cationic lipid is mixed with a helper lipid. Unsaturated phosphatidylethanolamines (PEs) such as dioleoyl-PE (DOPE) are the most commonly used helper lipid in applications (Farhood et al., 1995; Felgner et al., 1994; Legendre and Szoka, 1993). The ef-

fectiveness of unsaturated PEs, such as DOPE, is generally believed to rest on their propensity to form nonbilayer structures that are akin to membrane fusion intermediates (Hui et al., 1981). This property of helper lipids is thought to facilitate the fusion of cationic liposome in DNA:cationic liposome complexes to cell membranes, thus releasing the DNA into the cytoplasm. For most published protocols, the liposomes formed by cationic lipids and helper lipids are mixed with plasmid DNA at a certain ratio and added to cells that are placed in serum-free media. In at least one instance, helper lipids are used not only as mixing partners, but also as chasers added after the cationic liposomes are added to targeted cells (Farhood et al., 1995). Treatment time is usually 3–4 h. The cells are then washed and cultured in full media.

It seems that cationic liposomes form some types of complexes with plasmid DNA, either by encapsulating them or attaching to them (Gershon et al., 1993). During the treatment time, the cationic liposome-DNA is taken in by the cell. The mechanism of DNA intake is not exactly known but is believed to be related to endocytosis (Felgner et al., 1987; Zhou and Huang, 1994; Wrobel and Collins, 1995), although spontaneous fusion between cationic liposome and plasma membrane (Stamatatos et al., 1988; Duzgunes et al., 1989; Li and Hui, manuscript submitted for publication) cannot be ruled out. Once inside the cell, how and when DNA and cationic lipids become separate remain in question. Knowing the exact mechanism and the relative importance of endocytosis and fusion may help us to use

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cationic liposomes more effectively, especially for their in vivo applications when transfection efficiency is of paramount importance.

In this paper, we studied the role of helper lipids in transfection efficiency, especially in comparing phosphatidylethanolamine (PE) with phosphatidylcholine (PC), which is normally stable as bilayers. Their morphology, uptake route, and kinetics of uptake and transfection are investigated. The function of helper lipids in granule formation on cell surfaces, as found by this work, may be exploited to improve their transfection efficiency.

## MATERIALS AND METHODS

### Cell culture

Chinese hamster ovary (CHO) cells from the American Type Culture Collection (Rockville, MD) were grown on 15% newborn calf serum, 85% F10 nutrient mixture, and 1% PSN antibiotic mixture (GIBCO-BRL, Grand Island, NY) and passaged every other day. Trypsinized cells in serum were seeded in Corning 24-well plates at a concentration of 20% of that of passaged cells. The cells were allowed to grow for 24 h at 37°C before transfection.

The dependence of transfection rates on confluence of cells was examined. Cells were seeded to 24-well plates at approximately 12, 25, 50, 100, 200, and 400% of the original seeding density, which resulted in 80–90% confluence at 24 h after seeding. Those wells seeded with 200 and 400% of the original cell concentrations were completely confluent in 24 h, and their transfection rates were very low. Therefore, all transfection studies were done using the original cell seeding density.

### Transfection procedures

The cationic lipid, dioleoyl trimethylammonium propane (DOTAP), as well as dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylethanolamine (DOPE), were purchased from Avanti Polar Lipids (Alabaster, AL). Other chemicals were obtained from Sigma (St. Louis, MO).

To prepare cationic liposomes, DOTAP and a helper lipid, either DOPC or DOPE, were mixed at 1:1 molar ratio in chloroform. Mixtures were dried by blowing nitrogen gas for 20 min, and the remaining solvent was removed by vacuum for 30 min. One milliliter of sterile deionized water was added to each micromole of DOTAP, and the tube was vortexed to suspend the lipid at room temperature, which was above the gel-fluid phase transition temperatures of these lipids. The suspension was sonicated until clear for 10 min in a Laboratory Supply bath sonicator. The suspension was diluted to a final concentration of 0.133  $\mu\text{mol}$  DOTAP/ml.

Plasmids coding for pSV- $\beta$ -galactosidase were extracted from transfected *Escherichia coli*. The plasmid contains a 6821-bp segment which comprises a 3737-bp  $\beta$ -galactosidase gene and a 3084-bp pSV promoter, as verified by excision with BAM-H14151 and Hind-H1414 restriction enzymes. The stock solution was diluted to 20  $\mu\text{g}/\text{ml}$  before use.

Equal volumes of cationic liposome suspension and plasmid DNA were mixed immediately before addition to the cells. The mixture corresponds to a 2:1 lipid:DNA charge ratio. Normal cell culture medium containing serum was removed by aspiration, and cells were washed twice with 0.5 ml F10 medium and kept in 0.7 ml of F10 medium per well. The lipid vesicle-DNA mixture was added at 0.3 ml per well. The cells were incubated for 4 h, unless specified, at 37°C. The transfection mixture was then removed, and 0.5 ml of medium containing serum was added to each well. The cells were incubated for 48 h, fixed, and stained by the Promega (Madison, WI)  $\beta$ -galactosidase staining protocol. The number of stained clones per well was counted on an Olympus IMT-2 inverted microscope. In parallel experiments, the transfection efficiency was also determined by using the Promega enzyme assaying kit and measured by spectrophotom-

etry. The results by this method always confirmed and are often more distinctive than those obtained by clone counting.

### Lipid adhesion/uptake and granule formation observed by fluorescence microscopy

*N*-(Lissamine rhodamine B sulfonate)-dihexadecanoyl PE (Rh-PE) (Molecular Probes, Eugene, OR), at 1 mol%, was used in the preparation of cationic liposomes to monitor the association of DNA:cationic liposome complexes with cells and granule formation by fluorescence microscopy. Cells were treated with DNA:cationic liposome complexes containing the fluorescent lipid label and washed after given treatment times. The number of fluorescent granules on cells and substrate were counted from the screen of video images recorded by an intensified CCD camera (ITT-Fairchild, IL) attached to an Olympus IMT-2 fluorescence microscope. The total number of cells, the number of fluorescent cells, and cells with fluorescent granules within a field were likewise counted, using both phase contrast and fluorescence images.

### Measurement of lipid environment by fluorescence probes

Dithionite ion, a strong fluorescence quencher, is not permeable through cell membranes (McIntyre and Sleight, 1991). The degree of susceptibility of fluorescent lipid label to dithionite quenching is an indication of the fraction of lipids exposed to the extracellular environment, i.e., on cell surfaces or in the outer leaflet of the plasma membrane (Langner and Hui, 1993). In this experiment, the susceptibility indicated the amount of lipids in DNA:cationic liposome complexes not yet internalized by cells at given treatment time points after 7-nitrobenz-2-oxa-1,3-diazol (NBD)-labeled DNA:cationic liposome complexes were added to cells. DNA:cationic liposome complexes containing DOPC or DOPE as helper lipids and 2 mol% of NBD-PE as fluorescent quenching substrate were used to transfect cells. Cells were washed and trypsinized at given time points during treatment and transferred to fluorimeter cuvettes. The NBD fluorescence was measured while 5  $\mu\text{l}$  of 1 M stock solution of sodium dithionite was added to the cuvette containing 3 ml of cells. The quenching of fluorescence was measured in a SLM 8000 fluorimeter. In general, a major portion of the fluorescence, including those from lipid labels on the outer half of the plasma membrane, and those labels left in the suspension medium, was quenched immediately after the addition of dithionite. Those fluorescent lipid labels located within cells decay very slowly, because of the slow entrance of dithionite into the cell. From the intensities  $F_0$ ,  $F_d$ , and  $F_b$  measured, respectively, before, immediately after, and long ( $>30$  min) after the dithionite addition to each sample, one may estimate the amount of labeled lipid protected within cells for that sample to be  $(F_d - F_b)/(F_0 - F_b)$ .

*N*-(5-Fluoresceinethiocarbamoyl)-1,2-dihexadecanoyl-PE (FPE) has been used to measure membrane surface pH, making use of the fluorescence sensitivity of fluorescein to pH (Langner et al., 1995). FPE (2 mole%) was added to make DNA:cationic liposome complexes containing DOPC or DOPE as helper lipids. After DNA:cationic liposome complexes were added, cells were washed and trypsinized at given treatment time points and transferred to fluorimeter cuvettes. The fluorescence intensity  $F$  of the pH probe FPE at 530 nm was measured at various time points.  $F$  represented the sum of fluorescence intensities of those probes in a lower pH environment, plus those remaining on the cell surface and elsewhere at the same pH as the buffering medium. At time 0, none of the probe was assumed to have been internalized, so that  $F_0$  represented all probes remaining on the cell surface after washing. The intensity change of those probes that experienced a change in pH at each time point was then given by  $F - F_0$ . The total amount of DNA:cationic liposome complexes associated with cells in each sample was measured after 0.1% Triton X-100 was added to the cuvettes to permeabilize cells. This fluorescent intensity,  $T$ , was measured at each time point. The increase in the total amount of probes associated (adsorbed and internalized) with cells was given by  $(T -$

$T_0$ ), whereas the net change in fluorescence due to change in pH environment was given by  $(T - T_0) - (F - F_0)$ . To compare results from repeating experiments, the measured intensities were normalized to the highest value of each set.

## Electron microscopy

DNA:cationic liposome complexes at different DNA/cationic liposome charge ratios were prepared as described above, except that the concentration was 10 times higher. Aliquots of 0.2  $\mu$ l of the suspension were sandwiched between thin copper sample holders for rapid freeze. The samples were rapidly frozen by plunging into liquid propane cooled at liquid nitrogen temperature, using a spring-loaded plunger (Fassel et al., 1991). Freeze fracture was done at  $-115^\circ\text{C}$  in a Polaron E-7700 unit, under a vacuum of  $10^{-7}$  torr. Replication by carbon/platinum and carbon was made using Cressington ion guns. Replicas were examined in a Hitachi-600 electron microscope at a magnification of 40,000 $\times$  to 100,000 $\times$ .

## Confocal microscopy

The fluorescent lipid label *N*-(lissamine rhodamine B sulfonate)-dihexadecanoyl PE (Molecular Probes), at 5 mol%, was mixed with other lipids in the preparation of cationic liposomes. A high-affinity DNA label YOYO-1 iodide (Molecular Probes) was used to label DNA. The dye was added to 1.38  $\mu$ g/ml plasmid DNA, at a ratio of 1 dye molecule to 50 bp DNA, in sterile deionized water. The mixture was dialyzed in a 12,000–14,000 dalton cutoff dialysis bag for 20 h at  $4^\circ\text{C}$ , to remove uncoupled dyes.

CHO cells were grown on sterilized coverslips for confocal microscopy (Hui and Zhao, 1995). Before cell treatment, 20  $\mu$ g/ml of labeled DNA was mixed with an equal volume of 0.133  $\mu$ mol/ml of labeled lipid suspension. The mixture was then added to cells in transfection medium, in a volume ratio of 3:7. After a given time period, coverslips carrying cells were washed twice with PBS and fixed in 2% formaldehyde for 20 min. After washing again twice in PBS and once in distilled water, the coverslips were mounted with glycerol on glass slides for confocal microscopy. A BioRad 600 confocal microscope was used to image these samples. Excitation at 512 nm was provided by an argon laser, and emissions at  $>540$  and  $<540$  nm were detected with respective dichroic mirrors, filters, and photomultipliers simultaneously. Serial images of rhodamine and YOYO fluorescence at 1- $\mu$ m z-intervals were recorded and combined. Stereo pairs were displayed with  $1^\circ$  stacking angle difference between the left and right images.

## Quasielastic light scattering

The sizes of liposomes, DNA:cationic liposome complexes, and granules were measured by quasielastic light scattering (QLS), using a particle sizer model 370 (Nicomp Particle Sizing Systems, Santa Barbara, CA). DNA:cationic liposome complexes were added to water, polyanion-free isotonic sucrose solution, phosphate-buffered saline solution (PBS), or serum-free transfection media in cuvettes immediately before measurement. The size change of aggregates was sampled for 1 min at each time point. The measurement was carried out at  $25^\circ\text{C}$ . Gaussian analysis of sizes was applied in most cases, except for the case of DOPE, which resulted in exceedingly high  $\chi^2$  values ( $>50$ ). A multimodal NICOMP analysis was applied to that case, and a two-mode distribution was found. The smaller size peak accounted for  $<20\%$  by volume and was not plotted. Most other size distributions were Gaussian, but the dispersion were wide at long time points, when the coefficients of variation might exceed 50.

## RESULTS

### Structure and granule formation of DNA:cationic liposome complexes using PC or PE as helper lipids, and their transfection efficiencies

Sonicated vesicles of mixtures of DOTAP:PC or DOTAP:PE (1:1 molar ratio) were typically 25–30 nm in diam-

eter, as measured by QLS and by EM. When these vesicles were mixed with the plasmid DNA containing the promoter and reporter gene pSV- $\beta$ -gal, which we used for transfection experiments, the resultant complexes had a broad size distribution up to 500 nm, depending on the ratio of DNA:cationic liposomes. Fig. 1, A and B, shows, respectively, PC-containing complexes and PE-containing complexes at a DNA:liposome charge ratio of 1:2. These complexes showed a common characteristic that vesicles or liposomes were aggregated, and the individual vesicles were larger than the sonicated vesicles shown beside the DNA:cationic liposome complexes (Fig. 1 B). Apparently, some sonicated vesicles have fused to become larger vesicles upon complex formation. In general, PE-containing complexes were slightly larger than PC-containing complexes. More interestingly, small pieces of string-like materials were seen around or on the surface of aggregated vesicles in DNA:cationic liposome complexes. From the dimension of these string-like materials, we believe that, if they were DNA-based, as suggested by their appearance in samples at higher DNA:lipid ratios, the DNA strands were likely to have a lipid coating on them (for detailed thickness analysis, see Hui et al., 1996). The electrostatic interaction between cationic lipids and DNA might have distorted some cationic liposomes to form at least a partial bilayer wrapping on DNA molecules during the complex formation. Other cationic liposomes adhered to the unwrapped parts of DNA to form aggregates. Observations of fusion of cationic liposomes, the change of zeta potential of the complexes, and the partial shielding of DNA from ethidium bromide staining upon DNA:cationic liposome complex formation (Xu et al., unpublished results) support this argument.

When DNA:DOTAP/DOPE complexes were added to the transfection medium containing bicarbonates and phosphates, granules were formed within the first few seconds. The rate of granule formation was monitored by QLS. No granules were formed when the complexes were added to polyanion-free sucrose solution. Fig. 2 shows the granule size as a function of time after the DNA:cationic liposome complexes were added to the F10 medium. The formation of

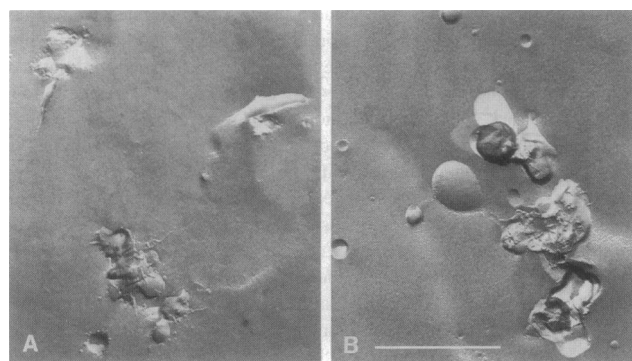


FIGURE 1 Freeze fracture electron micrographs of DNA:cationic liposome complexes. (A) DNA:DOTAP-DOPC complexes and (B) DNA:DOTAP-DOPE complexes, at charge ratios of 1:2. Bar = 1  $\mu$ m.

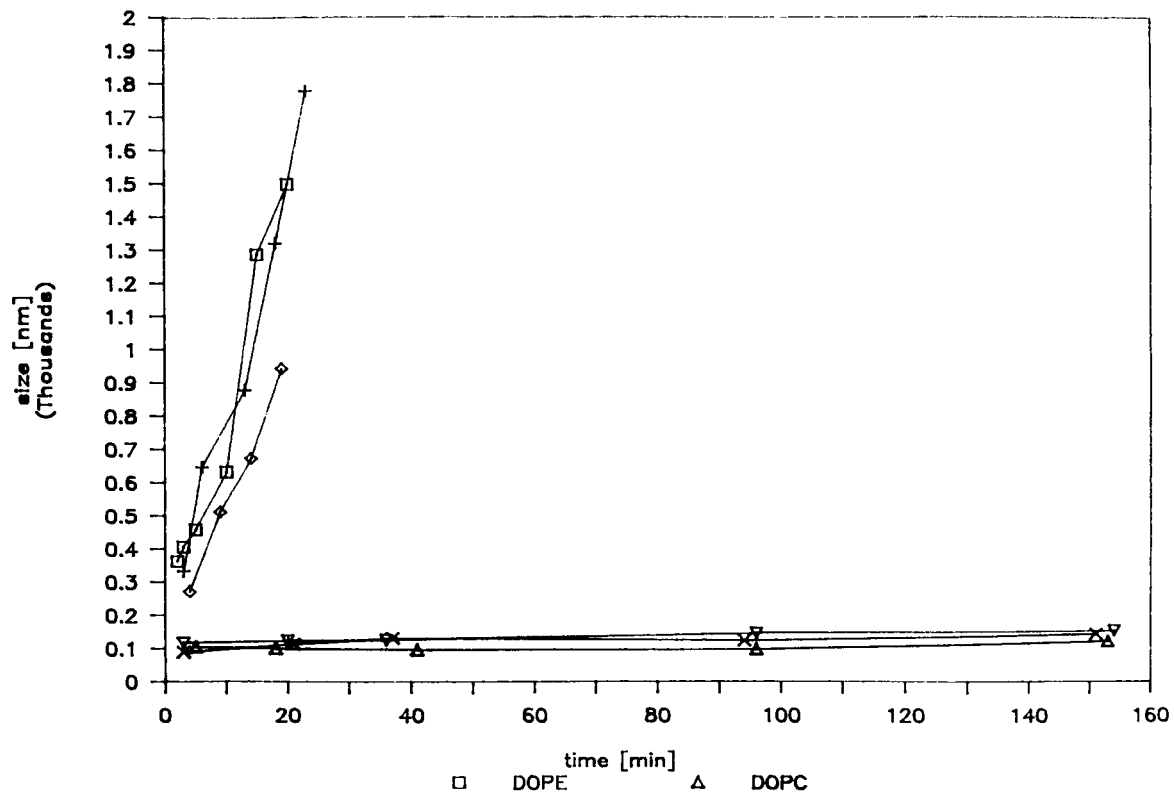


FIGURE 2 QLS measurement of aggregation of DNA:cationic liposome complexes containing DOPE (+, □, ◇) or DOPC (Δ, ▽, ×) with time, after they were added to F10 culture medium. Results represent three repeating experiments.

granules from the DNA:cationic liposome complexes was observable by microscopy, when final granules grew to several microns in diameter. However, when DNA:DOTAP/DOPC complexes were added to cell-free transfection medium, no granules were observed by QLS or by microscopy (Fig. 2).

To facilitate the observation of granule formation by fluorescence microscope, 1 mol% of the fluorescent lipid, rhodamine-PE (Rh-PE), were incorporated in cationic liposomes to monitor the association of lipids to cells and morphological changes of DNA:cationic liposome complexes. Granules of PE-containing complexes were observed within the first few seconds of addition to the cell-free, serum-free transfection medium containing bicarbonates and phosphates. No granules of PC-containing complexes were observed under the same conditions, in agreement with QLS results. When PE-containing complexes were added to cells in transfection media, several granules were seen on or in some cells immediately upon addition, and the number of granules did not change significantly over 1 h of treatment. On the contrary, when PC-containing complexes were added to cells, granules were seen to develop in/on cells within the first 10 min. Thereafter, more granules were seen on/in cells treated with PC-containing complexes than on/in cells treated with PE-containing complexes (Fig. 3 A). Furthermore, the number of cells with visible granules in/on them was higher in samples treated with PC-containing complexes than in samples treated with PE-containing complexes (Fig. 3 B).

The observation of more granule formation on cells and a higher percentage of granule-associated cells in PC-containing complex-treated samples than in PE-containing complex-treated samples also reflect their transfection efficiencies. Fig. 4 shows the number of transfected clones obtained in samples treated with PC-containing complexes and PE-containing complexes as functions of complex concentrations. In three repeating experiments, PC-containing complexes were consistently more efficient in producing transfected clones than were PE-containing complexes.

### The role of surface adhesion and endocytosis in DNA uptake

Because uncomplexed DNA, or DNA added with helper lipids only, has a very low transfection effect (results not shown), cationic liposomes do play a significant role in presenting DNA to cells. The obvious role of cationic liposomes is to mediate the adhesion of the complexes to negatively charged cell surfaces by electrostatic interaction. Removing negatively charged cell surface glycoproteins by pronase treatment, which generally increase cell electrofusion efficiency by reducing cell-cell electrostatic repulsion (Stenger et al.), reduced the transfection efficiency in a dose-dependent manner (Fig. 5 A). Pretreatment of cells with poly-L-lysine, which was supposed to shield the negative cell surface charge by the positively charged residues

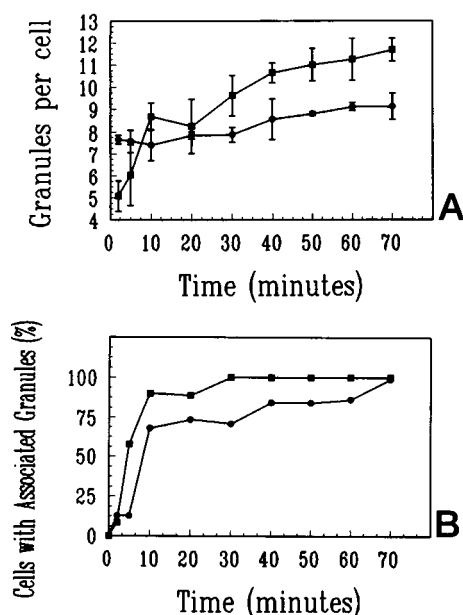


FIGURE 3 (A) The average number of fluorescent granules in/on cells treated with DNA:DOTAP-DOPC complexes (■) and DNA:DOTAP-DOPE complexes (●) as functions of treatment time. (B) The percentage of cells showing fluorescent granule association after treatment with DNA:DOTAP-DOPC complexes (■) and DNA:DOTAP-DOPE complexes (●).

of the peptide, resulted in an increase in the transfection efficiency at low concentrations (Fig. 5 B). The opposite trend is believed to be due to poly-L-lysine-mediated adhesion of DNA:cationic liposome complexes to the cell surface, as discussed later.

Cytochalasin-B (CB) affects actin polymerization and hence the formation of microfilaments that are instrumental in cytoskeleton-controlled membrane movement and endocytosis. Endocytic functions may be inhibited reversibly by pretreating cells with up to 20  $\mu\text{g/ml}$  of CB (Wagner et al., 1971). To determine the relative importance of cytoskeleton-directed lateral movement of membrane components and endocytosis in granule formation and in subsequent DNA entrance into cells, cells were pretreated with 20

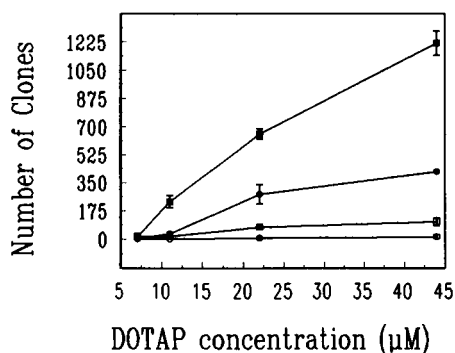


FIGURE 4 The average number of transfected clones per plate in cells treated respectively for about 4 h and 1 h with DNA:DOTAP-DOPC complexes (■, □) and DNA:DOTAP-DOPE complexes (●, ○).

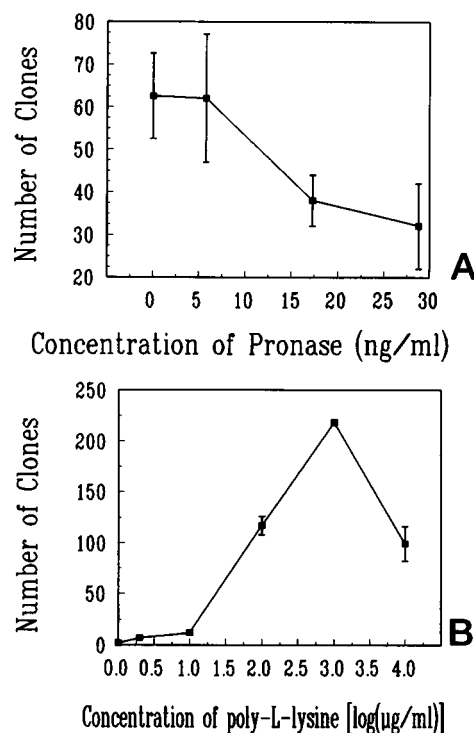


FIGURE 5 The effects of pronase or poly-L-lysine treatments on transfection efficiency. (A) The number of transfected clones per well as a function of pronase concentration. (B) The same as a function of the logarithm of poly-L-lysine concentration.

$\mu\text{g/ml}$  of CB before the addition of PC-containing complexes or PE-containing complexes. The resultant transfection efficiencies in PC-containing complex-treated and PE-containing complex-treated cells, with and without CB pretreatment, were also measured. The transfection efficiency decreased with increasing CB concentration; the effect was more pronounced for PC-containing complexes (Fig. 6). At 3 h after the addition of DNA:cationic liposome complexes, the number of granules in/on CB-treated cells and the percentage of fluorescent cells in a CB-treated population were less than those in the control, CB-free population (Hui et al., 1996). Again, the CB effect was more pronounced for PC-containing complex-treated cells than

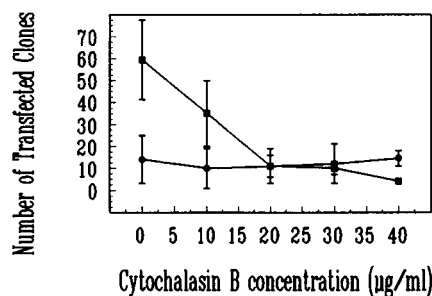


FIGURE 6 The number of clones in cells transfected with DNA:DOTAP-DOPC complex (■) and DNA:DOTAP-DOPE complex (●) as a function of cytochalasin B treatment.

for PE-containing complex-treated cells. Apparently, granule formation in PC-treated cells was more a biological event, whereas for those in PE-treated cells, it was more a chemical event unrelated to cytoskeleton-controlled motion of membrane components. Table 1 shows the ratio between PC-containing complex-treated and PE-containing complex-treated cells, in terms of the number of granules on cells, the percentage of fluorescent cells, and the number of transfected clones, at 24 h after 3 h of treatment in PC-containing complexes or PE-containing complexes. Few clones were observed in CB-pretreated cells. The results agree with the study of temperature effect on transfection, using the same cell line and plasmid (Hui et al., 1996).

The above results were not biased by the survival of cells due to PC-containing complex or PE-containing complex treatments. Although cells after PE-containing complex treatment were easier to detach, care was taken in the washing and replacement of culture media after the treatments. The cell density and viability after treatment, and after 24 h of posttreatment incubation, were virtually the same for PC-containing complex-treated and PE-containing complex-treated cells.

### Subcellular location of PC and PE from DNA:cationic liposome complexes

The accessibility of NBD-lipid probe to added dithionite was employed to determine the subcellular location of PC and PE after their respective DNA:cationic liposome complexes were added to cells. Fig. 7 shows the fluorescence intensity that was not immediately quenchable when dithionites were added to cells treated for various times by PC-containing complexes or by PE-containing complexes. Most fluorescence from PE-containing complex-associated probes was immediately quenchable in cells treated with PE-containing complexes, at all time points. For cells treated with PC-containing complexes, the amount of fluorescence from probes that was protected from immediate quenching increased with treatment time, indicating increasing amount of lipids being taken into the cell interior.

The cellular pH environment of added PC-containing complexes or PE-containing complexes, at given time points of treatment, were measured using pH-sensitive fluorescein-conjugated phospholipids. The pH-sensitive changes in fluorescence intensity,  $F$ , were measured before the Triton X-100-induced cell lysis. The total amount of lipids associated (adsorbed and internalized) with cells

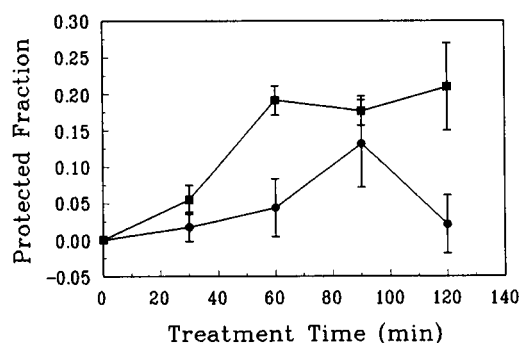


FIGURE 7 The fraction of protected fluorescence intensities of NBD-labeled lipids in DNA:DOTAP-DOPC complexes (■) and DNA:DOTAP-DOPE complexes (●) from instantaneous quenching by the addition of dithionite ions, as functions of DNA:liposome treatment time.

treated with PC-containing complexes or PE-containing complexes were also measured after Triton X-100 was added to these cells. The amount of lipids associated with cells treated with PC-containing complexes were much larger (Fig. 8 A). The pH-sensitive portion of the fluorescence intensity,  $(T - T_0) - (F - F_0)$ , was also higher in cells treated with PC-containing complexes than those with PE-containing complexes, and increased with treatment time (Fig. 8 B). The results indicate that 1) more PC-containing complexes were adsorbed on cell surfaces at any

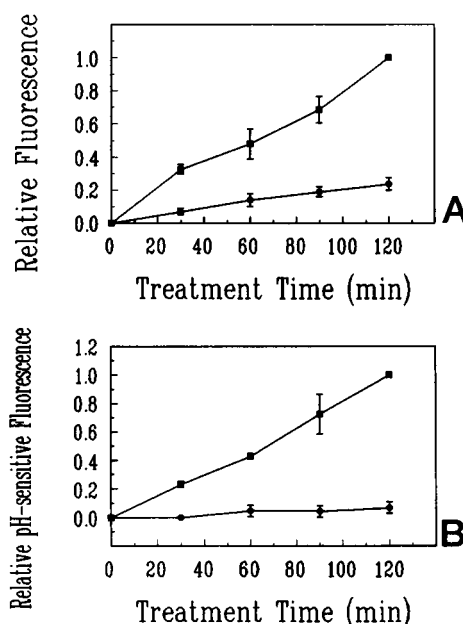


FIGURE 8 (A) The total fluorescence intensities of fluorescein-PE in/on cells treated with labeled DNA:DOTAP-DOPC complexes (■) or DNA:DOTAP-DOPE complexes (●), for different lengths of time and lysed in Triton X-100. The intensities represent the additional amount of lipids associated (adsorbed and internalized) with CHO cells. (B) The change in fluorescence intensities before and after Triton X-100 treatment, representing the fluorescence of fluorescein-PE in cells in a different pH environment before cell lysis. Results are from treatments by labeled DNA:DOTAP-DOPC complexes (■) or DNA:DOTAP-DOPE complexes (●).

**TABLE 1** Relative effects of helper lipids (DOPC/DOPE) in terms of the number of fluorescent granules per cell, percentages of fluorescent cells, and the number of transfected clones

Relative effect of PC/PE	CB <sup>-</sup>	CB <sup>+</sup>
No. of granules	3.8	1.7
Fluorescent cells (%)	2.6	1.6
No. of clones	2.4	—

time point of treatment, and 2) they were more likely to be internalized into a lower pH environment, possibly endosomes, whereas lipids in PE-containing complexes remained more or less in the same pH environment throughout 2 h of treatment.

### Confocal microscopic observation of the uptake of granules of DNA and lipid

To determine the role of granules in the uptake of DNA and lipids, we labeled the cationic liposomes with 1 mol% of rhodamine-PE and followed their uptake process by confocal microscopy (Hui and Zhao, 1995). If the major uptake of the lipids is by endocytosis of granules, the lipids in the granules are likely to appear granular immediately after uptake. These granules should be visible within cells. On the other hand, if fusion between cationic liposomes and plasma membranes is the major route of uptake, the plasma membrane should become fluorescent early in the treatment time, and the number of fluorescent lipid granules within cells should be much reduced.

Immediately after exposure to DNA:DOTAP/DOPE complexes, cells were seen to have a few bright granules on the surface, but the cell bodies were not fluorescent, and no granules were observed within the cells (Fig. 9 A). At 30 min of exposure, more granules were seen on cell surfaces, but none within cells. At 1 h, granules were seen within cells as well as on cell surfaces (Figs. 9, B–E). More

granules were visible on the supporting substrate when viewed at that level (Fig. 9 D), but several granules were definitely within cells, especially when viewed at 4  $\mu\text{m}$  above the substrate level (Fig. 9 C), and in an  $xz$ -plane display (Fig. 9 E). Cell membranes were weakly fluorescent. By 3–4 h of exposure, cell membranes became more fluorescent, and more granules were seen within cells, although a large number of granules remained on the cell surface (results not shown). Because endocytic granules were common and were visible generally before cell membranes became fluorescent, we may deduce that lipid uptake was mainly through endocytosis of granules, and fusion with plasma membrane was only a minor pathway.

To distinguish the pathways of lipid and DNA uptake, we labeled the lipids with Rh-PE, and plasmid DNA with a high-affinity dye for DNA, YOYO. The red emission of Rh-PE and green emission of YOYO were distinctly visible in fluorescent confocal micrographs (Fig. 10). At 0 h, green (DNA) granules were visible on the cell surface in the stereo projection (Fig. 10 A), indicating that some lipids were already fused with plasma membranes, leaving DNA-rich granules near the cell surface. However, the amount of lipid fused with plasma membrane was not sufficient to be visible. Granules outside cells and on substrates were yellow in color, indicating that both lipid and DNA were present in the same granules. At 1 h of exposure, red granules within cells, as well as green and yellow granules near the cell surfaces, were seen (Fig. 10 B). In those optical section confocal micrographs where areas outside and inside the cell could be clearly distinguished, green-labeled DNA-rich granules resided predominantly outside the cell boundary (Fig. 10 B). The process advanced further at 4 h of exposure. Red granules within cells might be endocytic vesicles mostly devoid of DNA, which might have escaped when endosomal membranes fused with cationic liposomes of the internalized DNA:cationic liposome complexes. The escaped DNA no longer aggregated, and the dispersed molecules were difficult to detect by fluorescence microscopy. Cell membranes also became red and visible. Invariably, green granules were seen only near cell surfaces, indicating a considerable amount of aggregated DNA was left on or adjacent to the cell surface when the lipids of granules formed by DNA:cationic liposome complexes fused with plasma membranes. Although fusion between cationic liposomes and plasma membranes took place at an early time point, this event did not result in the delivery of DNA deep into cells.

When DOPC was used in place of DOPE as the helper lipid, granules did not form in transfection medium, but instead on cell surfaces. There were generally more granules on PC-treated cells than on PE-treated cells. Fig. 10 C shows a cell after 4 h of exposure to DNA:cationic liposome complexes containing DOPC as the helper lipid. In this particular cell, membranes did not become fluorescent with Rh-PE, unlike some other cells in the same culture well. Both green and yellow granules were observed near the cell surfaces, with several yellow granules within the cell, indi-

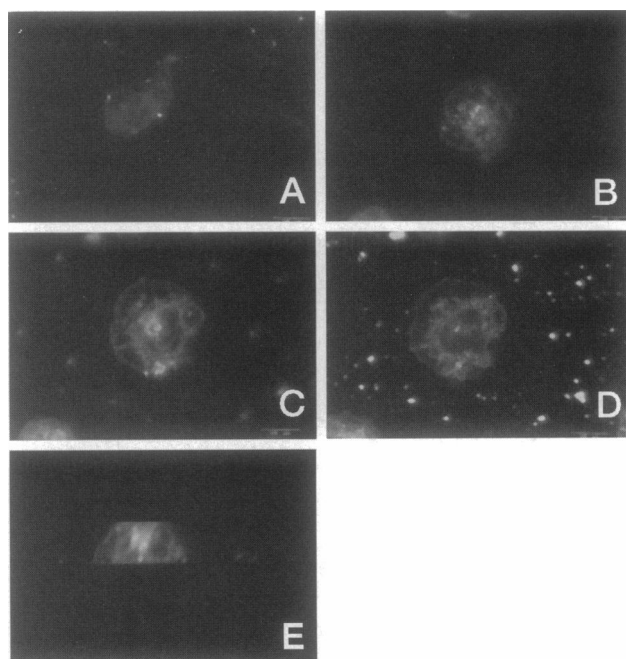
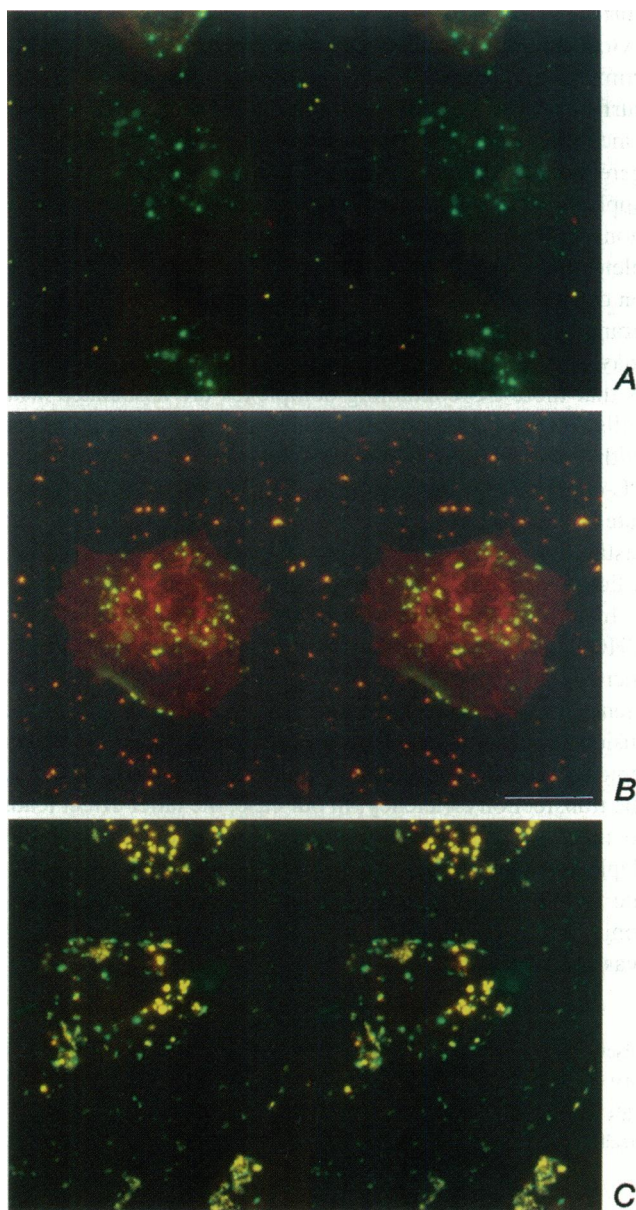


FIGURE 9 Fluorescence confocal micrographs showing selected optical sections of cells treated with rhodamine-PE-labeled DNA:DOTAP-DOPE complexes. (A) At 8–10  $\mu\text{m}$  from the supporting substrate at 0 h of treatment. (B) At 9  $\mu\text{m}$  from the supporting substrate at 1 h of treatment. (C) Same cell at 2–5  $\mu\text{m}$  from the supporting substrate. (D) Same cell at 1  $\mu\text{m}$  from the supporting substrate. (E)  $xz$ -z section of the same cell. Bar = 10  $\mu\text{m}$ .





**FIGURE 10** Stereo pairs of fluorescence confocal micrographs showing doubly labeled DNA (YOYO, green) and lipid (rhodamine-PE, red) components of DNA:DOTAP-DOPE complexes (*A, B*) and DNA:DOTAP-DOPC complexes (*C*) in/on cells. (*A*) A cell immediately after the addition of DNA:DOTAP-DOPE complexes, showing green granules on cell surfaces, and yellow granules on supporting substrate. (*B*) A cell after 1 h of treatment, showing green granules on the surface and yellow and red granules within the cell. The nuclear membrane and other cell membranes are also labeled red. (*C*) A cell without fluorescent membranes after 4 h of treatment with DNA:DOTAP-DOPC complexes, showing green and yellow granules on the surface and inside. Bar = 10  $\mu\text{m}$ .

cating granules containing both DNA and lipids were internalized as a whole.

## DISCUSSION

Cationic liposomes have been successfully used in transfection experiments. In most applications, helper lipids are

used, but their roles in the gene transfer process are not well defined. The current belief is that these helper lipids function as fusogens in the breaking out of endosomes when DNA escapes to the cytosol. The more fusogenic the helper lipid, the more effective it is in gene transfer (Caplen et al., 1995). Our experiments are designed to answer 1) What is the most significant factor, or rate-limiting step, controlling the DNA transfer process? and 2) What role does the helper lipid play in this process? To answer these questions, we must understand the fundamentals of DNA delivery mechanism.

From our study and those reported by others, we know that successful gene transfer by cationic liposomes involves 1) the packaging (condensation and protection) of DNA, 2) adhesion of packaged DNA to cell surfaces, 3) internalization of DNA, 4) escape of DNA from endosomes if endocytosis is involved, and 5) DNA entrance and expression in cell nuclei.

Because the transfection efficiency of CHO cells by naked (uncomplexed) plasmid is near zero (results not shown), the need for a delivery vehicle is obvious. Cationic lipids accomplish two goals as a vehicle; the first is to package DNA by forming a sheath around the DNA strands, thereby partially protecting it from DNAase degradation, and the second is to mediate their adhesion to cells, overcoming the generally negative polarity of most cell surfaces. The first role is helpful but not necessary, because electroporation and other methods of introducing naked DNA into cells do result in significant transfection. The second role is seen by the charge ratio dependency of transfection efficiency, with the best transfection results in the 1:1 to 1:2 ratios, when the negative DNA charges are largely shielded by the positive charges of cationic liposomes (Xu et al., unpublished results; Caplen et al., 1995). The residual positive charges of the complexes facilitate the adhesion to cell surfaces. The cationic bridging effect is the common driving mechanism for DOTAP as well as poly-L-lysine-mediated transfection (Fig. 5 *B*) (Midoux et al., 1993). Excess cationic liposomes or poly-L-lysine shields the negatively charged cell surfaces and prevents further adhesion of DNA:cationic liposome complexes (Fig. 5 *B*). Pronase treatment reduces the electrostatic attraction between DNA:cationic liposome complexes and cell surfaces, leading to a decrease in transfection (Fig. 5 *A*). Therefore, the presentation of DNA to the cell surface, more so than the ensheathing of DNA, is a primary contribution of cationic liposomes to the gene transfer process.

Two possible membrane fusion steps are involved in the cationic liposome-mediated gene transfer process. The first is the fusion of cationic liposomes of the complexes with plasma membranes, resulting in the possible transfer of some DNA into cells. Because DNA is not encapsulated within cationic liposomes as shown by EM morphology (Fig. 1), this mode of content delivery is topographically inefficient. Our confocal observation of cells treated with dual-labeled complexes indicates that this type of fusion results in most aggregated DNA of the DNA:cationic lipo-



some complexes being left at the cell periphery (Fig. 10). Inhibition of endocytosis prevents transfection (Fig. 6), although fusion of cationic liposomes to plasma membrane is not entirely suppressed (Hui et al., 1996). The evidence points to the conclusion that, for CHO cells, endocytosis rather than plasma membrane fusion is the major pathway of DNA uptake during the gene transfer process. The second step is the fusion between internalized cationic liposomes of the complexes with endosomal membranes, thereby allowing plasmid DNA to escape into the cytoplasm. From the significant number of granules seen within cells (Figs. 9 and 10), and the proportion of transfection-competent PC-containing lipids being protected in a low-pH environment (Figs. 7 and 8), we expect fusion between liposomes and endosomal membranes to be an important step.

What is the contribution of helper lipids? Indeed, helper lipids are not necessary in the transfection of certain cell lines (such as CS1 cells; Xu et al., unpublished results). However, in the case of CHO cells, the addition at an equal molar ratio of DOPE improved the transfection efficiency of using DOTAP alone by a factor of 5–10. In most other reports (Legendre and Szoka, 1993; Farhood et al., 1995; Felgner et al., 1994), PE also enhances the transfection efficiency, whereas PC reduces it. One tends to assume the function of PE to be that of a membrane fusion promoter, by virtue of its curvature stress-related bilayer instability (Epand, 1996; Hui and Sen, 1989). Fusogenicity of the helper lipid may influence the transfection efficiency in at least two ways. 1) The bilayer instability affects the rate of preaggregation of complexes upon adding to polyanion-containing media (Fig. 2). However, presenting preaggregated granules to cells may not enhance endocytosis, but instead may deplete smaller complexes from the medium and prevent them from reaching the cell surface. 2) The contribution of fusogenic helper lipids at the escape step as well as at the plasma membrane fusion step may enhance DNA delivery. In our case, either fusion step is secondary to endocytosis in determining the transfection efficiency. This is indicated by the finding that the nonfusogenic PC is much more effective in transfection in our case (Table 1 and Fig. 4).

Our finding that more PC-containing complexes are taken up by CHO cells, leading to higher transfection efficiency, may be reasoned as follows. PE-containing complexes aggregate into granules more readily in transfection media (Fig. 2). Some of the granules fall on cell surfaces. Those of a size recognizable by the cell may be endocytized, whereas the majority of granules remain on the cell surface or on the surrounding substrate. After the cationic liposomes in the DNA:cationic liposome complexes fuse with the plasma membranes, the DNA aggregates are left at the cell periphery and possibly remain inactive (Fig. 10). On the other hand, PC-containing complexes form granules very slowly in transfection medium. The excess positive charges on these PC-containing complexes bring them to the cell surfaces. For certain cells such as CHO cells, the accumulation and adsorption of smaller PC-containing complexes on their

surfaces may imitate receptor cross-linking and initiate a cytoskeleton-associated lateral movement of cell surface components, leading to the formation of granules on cell surfaces, similar to the capping process in lymphocytes. Once the granules reach a certain size, endocytosis is triggered, and the granules are internalized. This process also applies to PE-containing complexes; however, the aggregation of PE-containing complexes in the liquid media depletes PE-containing complexes available for accumulation on cell surfaces, reducing the extent of cell-directed surface granule formation. Readily formed aggregated granules may be too large to be recognized by the cell. The end result is that more PC-containing complexes are associated with cells (Fig. 8 A), and more granules are seen on cells treated with PC-containing complexes than for those treated with PE-containing complexes (Fig. 3). For cells whose uptake rate is limited by endocytosis, such as CHO cells, the instability (propensity to fusion) of the helper lipid could be a liability rather than an asset for DNA delivery.

In conclusion, the rate-limiting step in the transfection of CHO cells is endocytosis. Any measures that can lead to an increase in endocytosis also increase the transfection efficiency. Other factors such as cationic liposome-membrane fusion, being enhanced by the curvature stress of helper lipids such as PE, play only a secondary role in determining the transfection efficiency. In our case, the latter effect leads to premature granule formation in treatment media, thus depleting unaggregated complexes from cell-directed granule formation/endocytosis. The role of helper lipids in cationic liposome-induced gene transfer is more complex than was once believed.

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