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Calcium enhances the transfection potency of stabilized plasmid–lipid particles

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Abstract

Previous work from this laboratory has shown that plasmid DNA can be encapsulated in small (70-nm-diameter) stabilized plasmid–lipid particles (SPLP) that consist of a single plasmid encapsulated within a bilayer lipid vesicle. SPLP preferentially transfect tumor tissue following intravenous administration. Although the levels of transgene expression in vivo are greater for SPLP than can be achieved with naked DNA or complexes, they are lower than may be required for therapeutic benefit. In the present work we examine whether Ca^{2+} can enhance the transfection potency of SPLP. It is shown that Ca^{2+} can enhance SPLP transfection potency in bovine hamster kidney cells by 60- to 100-fold when treated in serum containing medium and an additional 60-fold when serum is absent for the initial 10 min of the transfection period. When cells are treated with SPLP in the presence of Ca^{2+}, there is a fivefold increase in intact plasmid in the cell. It is also shown that this Ca^{2+} effect involves the formation of calcium phosphate precipitates; however, these precipitates are not directly associated with the SPLP plasmid DNA. The ability of calcium phosphate to facilitate delivery of other macromolecules without direct association is also demonstrated by the release of large-molecular-weight dextrans from endosomal/lysosomal compartments in the presence of calcium phosphate. Finally, it is shown that, unlike naked DNA, SPLP transfection potency in the presence of calcium phosphate is not affected by nuclease activity.

Keywords: Liposomes; Cationic lipids; Calcium phosphate; Intracellular delivery; Gene therapy

For gene therapy to be clinically useful, an effective and safe gene delivery system is required. Viral vectors are relatively efficient gene delivery vehicles but suffer from a variety of limitations including the potential for reversion to the wild type and immune response concerns [1–4]. As a result, considerable efforts have been made to develop nonviral gene delivery systems [5–7]. Plasmid DNA–cationic liposome complexes (lipoplexes) are one of the most commonly employed nonviral gene delivery vehicles [8–15]. Lipoplexes can exhibit good transfection properties in vitro but have limitations in vivo. For example, lipoplexes are large, highly charged systems that are rapidly cleared from the circulation following systemic administration and that can elicit toxic side effects [16–19].

Work from this laboratory has shown that plasmid DNA can be encapsulated in small (~70 nm diameter) stabilized plasmid–lipid particles (SPLP)[1] that consist of a single plasmid encapsulated within a bilayer lipid vesi-

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[1] *Abbreviations used:* SPLP, stabilized plasmid–lipid particles; BHK, bovine hamster kidney; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; QELS, quasi-elastic light scattering.

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cle [20]. These SPLP contain the "fusogenic" lipid dioleoyl-phosphatidylethanolamine (DOPE) and low levels of cationic lipid and are stabilized in aqueous media by the presence of a poly(ethylene glycol) (PEG) coating. SPLP exhibit extended circulation lifetimes following intravenous (i.v.) injection, accumulate preferentially at distal tumor sites due to the enhanced vascular permeability in such regions, and can mediate relatively specific transgene expression at these tumor sites [21–23]. The levels of transgene expression in vivo are greater than those that can be achieved with naked DNA or complexes but are lower than those that may be required for therapeutic benefit [23–25]. One major factor hindering the transfection potency of the particle is inefficient release from endocytotic/lysosomal vesicles once taken up by the cell. Current efforts have therefore been focused on developing SPLP that have more effective intracellular delivery properties.

In previous work we have shown that Ca^{2+} can enhance the transfection potency of plasmid DNA–cationic lipid complexes by 20-fold or more [26]. This enhanced potency was ascribed to increased cell uptake of the complexes when Ca^{2+} is present. In the present work we examine whether Ca^{2+} can also enhance the transfection activity of SPLP. It is shown that the addition of Ca^{2+} can result in up to 60- to 100-fold enhancements in SPLP transfection potency. Under conditions where serum is absent during the initial incubation period, gene expression is increased by an additional 60-fold in BHK cells. It is shown that these effects are dependent on the presence of physiological levels of phosphate in the medium. The results are discussed with regard to the mechanism of calcium phosphate transfection and methods of improving the transfection properties of the SPLP system.

Materials and methods

Materials

N,*N*-Dioleyl-*N*,*N*-dimethylammonium chloride (DOD AC) was obtained from Dr. S. Ansell and 1-*O*-(2-(ω-methoxyetheneglycol)succinoyl)-2-*N*- arachidoylsphingosine (PEG-CerC_{20}) was synthesized by Dr. Z. Wang, at Inex Pharmaceuticals (Burnaby, BC). 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) was purchased from Northern Lipids (Vancouver, BC). 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rh-DOPE) was purchased from Avanti Polar Lipids (Alabaster, AL). DEAE Sepharose CL-6B anionic-exchange column, octylglucopyranoside (OGP), Triton X-100, sodium dodecyl sulfate (SDS), Hepes, Tris, $CaCl_2$, $MgCl_2$, sucrose, and NaCl were obtained from Sigma Chemical (St. Louis, MO); 12–14,000 molecular weight cutoff dialysis tubing was purchased from Spectrum Laboratories (Rancho Dominguez, CA) and 100,000 nominal molecular weight limit (NMWL) centrifugal filtration units were purchased from Millipore (Billerica, MA). The luciferase assay kit was purchased from Promega (Madison, WI). PicoGreen dsDNA detection reagent and Alexa Fluor 488 labeled 10,000 molecular weight dextran were purchased from Molecular Probes (Eugene, OR). Plasmid DNA (pCMVLuc) coding for the luciferase reporter gene under the control of the human CMV immediate early promoter–enhancer element was obtained from Protiva Biotherapeutics (Vancouver, BC). Bovine hamster kidney (BHK) cells were obtained from the American Tissue Culture Collection (ATCC CCL-10, Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplement with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin, purchased from Invitrogen (Carlsbad, CA).

Preparation of SPLP

SPLP were prepared as described by Wheeler et al. [20] with some modifications. Briefly, a total of 10 μmol of DODAC:DOPE:PEG-CerC_{20}:Rh-DOPE (8:81.5:10:0.5; molar ratio) was dissolved in chloroform and dried under a stream of nitrogen gas. Residual solvent was removed under high vacuum for 2 h. The resulting lipid film was hydrated in 1 ml of HBS buffer (20 mM Hepes and 150 mM NaCl, pH 7.5) containing 0.2 M OGP with continuous vortexing. Plasmid DNA (400 μg/ml) was added to the hydrated lipids and the mixtures were dialyzed against HBS buffer for 36–48 h with two buffer changes. Nonencapsulated plasmid was removed by DEAE anion exchange chromatography and empty lipid vesicles were removed by employing a sucrose density gradient as previously described [20]. SPLP were characterized with respect to plasmid entrapment using a previously described PicoGreen assay [25], and particle mean diameter was determined using a submicron quasi-elastic light scattering particle sizer (Nicomp, Santa Barbara, CA). SPLP formulations used in this study demonstrated a maximum of 5–10% untrapped plasmid following purification with particle sizes of 80–90 nm in diameter.

Reporter gene activity

Prior to transfection, BHK cells maintained as a monolayer at 37 °C in a humidified atmosphere containing 5.0% CO_2 were plated at a density of 8.75×10^3 cells/well in a 96-well plate overnight. A 500 mM $CaCl_2$ stock solution was prepared in dH_2O and sterilized using a 200-nm syringe filter. Plasmid DNA encapsulated in SPLP coding for the luciferase reporter gene was used at a concentration of 0.5–1.0 μg/well of transfection. SPLP were first added to appropriate concentrations of Ca^{2+}

as required by the experiment, after which culture medium was added to the mixture to obtain a final transfection volume of 125 μl/well (Ca^{2+} concentration was calculated with respect to the final volume of the transfection medium applied to cells). The final volume was composed of 20% Ca^{2+} and SPLP, and 80% culture medium. In nonserum studies, Ca^{2+} and SPLP were mixed with non-serum-containing DMEM and added to cells for the appropriate incubation period; then FBS was added to replenish the serum content to 10%(vol/vol). Experiments to investigate the effect of phosphates in the cell medium employed the same procedure as indicated; however, a phosphate-free formulation of DMEM was used (Invitrogen). Nuclease sensitivity experiments were conducted by assaying gene expression following cell transfections in DMEM containing FBS that was heated to 65 °C for 1 h (heat inactivation to deactivate serum nucleases) or FBS that was not heat inactivated prior to preparing the cell medium for transfection. In all experiments, cells were incubated with the transfection complexes for the appropriate time periods before assaying for luciferase expression as described previously [20]. Luciferase activity was normalized against cell protein concentration in the assayed lysate determined with the Micro BCA protein assay reagent kit (Pierce, Rockford, IL).

Determination of SPLP uptake into cells

BHK cells were plated at 8.75×10^3 cells/well in a 96-well plate overnight the day prior to the experiment. Cellular uptake determination was performed by incorporating 0.5 mol% Rh-DOPE into the lipid formulation: DODAC:DOPE:PEG-CerC_{20}:Rh-DOPE (8:81.5:10:0.5; molar ratio). SPLP were prepared using the detergent dialysis method as described above. Purified particles were mixed with increasing concentrations of Ca^{2+} (0–14 mM) and added to cells at a lipid dose of 12.6 nmol in complete medium (125 μl final volume). Cells were incubated at 37 °C for 24 h, washed 2× with Dulbecco's phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, CA), and lysed by the addition of Dulbecco's PBS containing 0.1% Triton X-100. Rhodamine fluorescence resulting from cell-associated SPLP was measured on a Molecular Dynamics Typhoon 8600 fluorescence imager (Piscataway, NJ) using λ_{ex} laser of 532 nm and λ_{em} of 580 nm. Lipid uptake was quantified by comparing cell lysate fluorescence to that of a Rh-DOPE lipid standard of known concentration and then normalized to the cell protein concentration in the assayed lysate.

Intracellular processing of plasmid DNA

BHK cells were plated at 3×10^5 cells/well in six-well plates 18 h prior to the experiment. SPLP encapsulating 2.5 μg plasmid DNA were added to each well and incubated with cells for 2, 4, and 8 h, in the absence or presence of 8 mM Ca^{2+}. At the appropriate time points, cells were washed with Dulbecco's PBS and external SPLP removed by trypsinization. Trypsinized cells were pelleted by centrifugation and cells were resuspended and washed with isotonic buffer (250 mM sucrose, 3 mM $MgCl_2$, 50 mM Hepes, pH 7.2). Subsequently, pelleted cells were lysed by incubating with 250 μl of lysis buffer (10 mM Tris, pH 7.5, 0.5% SDS, 1 mM EDTA) containing Pronase E at 1 mg/ml (Sigma) overnight at 37 °C. DNA (genomic DNA and delivered plasmid DNA) was extracted from whole cell lysates as described previously [27]. DNA recovery was determined by measuring the absorbance at 260 nm. Total DNA (6 μg) from each sample was either dot blotted onto a nylon transfer membrane (Amersham, Piscataway, NJ) with a set of pCMVLuc standards (0–5 pg) or loaded into a 1% agarose gel and size fractionated at 60 V for 2 h for the Southern analysis. Both blots were hybridized overnight at 68 °C to a ^{32}P-labeled plasmid DNA probe, which was prepared with *Pst*I cut-pCMVLuc plasmid using the ^{T7}QuickPrime Kit (Amersham Pharmacia Biotech). Blots were washed three times with 2× SSC containing 0.1% SDS and were then exposed on a PhosphorImager screen that was subsequently scanned (PhosphorImager SI, Amersham).

Intracellular distribution of dextran

BHK cells were plated at 3.5×10^4 cells/well in Lab-Tek II chambered coverglass plates (Nuncbrand, Rochester, NY) 18 h prior to transfection. Alexa-Fluor-488-labeled 10,000 mol wt. dextran (70 nmol) was added to HBS with 0 or 50 mM Ca^{2+} and then diluted with non-serum-containing DMEM to yield 0 or 8 mM Ca^{2+} in the medium applied to the cells (total volume/well was 500 μl). Cells were incubated in this mixture for 30 min and then FBS was added to yield 10%(vol/vol) serum. After 2 h, the sample medium was removed, cells were washed 2× with Dulbecco's PBS, and fresh complete medium was added for an additional 10 h. At the 2- and 12-h time points, cells in live culture were observed for green fluorescence under 400× magnification using a Zeiss Axiovert S100 inverted microscope (Thornwood, NY).

Results

Ca^{2+} addition to the transfection medium induces a substantial increase in gene expression

Initial experiments characterized the influence of Ca^{2+} on gene expression in BHK cells following incubation with SPLP. Following transfection for 24 h the cells were washed 2× with Dulbecco's PBS and then assayed for luciferase reporter gene expression. The data

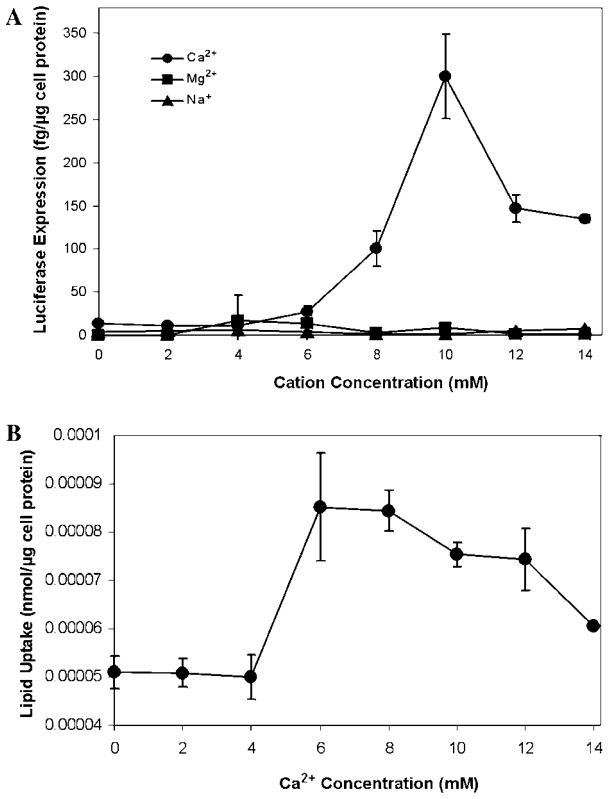

Fig. 1. Enhanced reporter gene activity relative to Ca^{2+} content in the cell culture medium during SPLP transfection, with a minor effect on cell uptake of SPLP. BHK cells were plated 18 h prior to transfection. Immediately before treatment, $CaCl_2$ was added to SPLP and then diluted with cell culture medium to yield a final Ca^{2+} concentration of 0–20 mM. The cells were treated at a dose of 12.6 nmol SPLP lipid/well (DODAC:DOPE:PEG-C_{20}:Rhod-DOPE, 8: 81.5:10:0.5; molar ratio) equal to 1 µg SPLP encapsulated pDNA/well. Following 24 h incubation, cells were washed 2× in Dulbecco's PBS and then lysed in a 0.1% Triton X-100 Dulbecco's PBS solution. To determine gene expression (A) the cell lysate was assayed for luciferase expression and SPLP uptake (B) was determined by assaying the lysate for Rh-DOPE-related fluorescence and correlated to fluorescence of dilutions of Rh-DOPE-labeled SPLP of known lipid concentrations. All data presented were normalized to cell protein content in the lysate assayed, and each data point represents an average of four samples ± SD.

presented in Fig. 1A demonstrate very low levels of expression until the Ca^{2+} concentration was at least 6 mM. Gene expression peaks at approximately 10 mM Ca^{2+}, yielding a 30-fold increase in expression, and then declines at Ca^{2+} levels of 12 mM and above. Replacing Ca^{2+} with other cations including magnesium and sodium at similar concentrations did not affect gene expression (Fig. 1A). Also, there was no evidence for changes in cell viability resulting from the Ca^{2+} addition in this concentration range, determined both by visual examination and by quantifying the cell protein content per well (data not shown).

A potential cause of enhanced gene expression is increased uptake of SPLP in the presence of Ca^{2+}. Cells were treated with the same range of Ca^{2+} concentrations as described above for experiments conducted in Fig. 1A. Following a 24-h incubation, cells were washed 2× with Dulbecco's PBS and then assayed for uptake of SPLP. Uptake was measured by quantifying a rhodamine-labeled PE phospholipid included in the SPLP membrane. The maximum increase in uptake in the presence of Ca^{2+} was in the range of 60% (Fig. 1B) and cannot therefore account for the 30-fold increase in gene expression levels.

Ca^{2+} increases the level of intracellular delivery of intact plasmid

If SPLP plasmid can escape from the endosome more readily in the presence of Ca^{2+}, it should avoid breakdown in the lysosomal pathway, and more intact intracellular plasmid DNA should be available to migrate to the nucleus. A dot blot assay was employed to measure intracellular delivery of plasmid DNA, and the integrity of the plasmid was examined by using Southern blot analysis. Cells were incubated with SPLP in the absence or presence of 8 mM Ca^{2+} for 2, 4, and 8 h. The levels of intact, intracellular plasmid DNA for the different systems were compared after isolation of DNA from the cells as described under Materials and methods, and the results are shown in Fig. 2. As shown in Fig. 2A, when cells were transfected with SPLP in the presence of Ca^{2+}, the amount of intact plasmid in the BHK cells was increased by approximately fivefold after an 8-h incubation period. This is also reflected by the Southern analysis, which showed that more intact plasmid DNA was present in cells transfected with SPLP in the presence of Ca^{2+} (Fig. 2B). Such enhanced levels of intact plasmid DNA were not observed when Mg^{2+} was substituted for Ca^{2+}, demonstrating the specificity of Ca^{2+} (Fig. 2B).

The Ca^{2+} effect is amplified in the absence of serum

It has been previously established that the presence of serum can have a significant influence on the efficiency of nonviral gene transfer [28–30]. To determine the effect of serum on gene expression in the presence of Ca^{2+}, cells were transfected with SPLP in the presence and absence of 8 mM Ca^{2+}, in cell culture medium containing serum levels ranging from 0 to 10%(vol/vol). After a 30-min incubation period, additional serum was added where required to yield 10% serum in all of the sample wells. At 24 h, cells were washed 2× in Dulbecco's PBS and then assayed for luciferase expression. In Fig. 3A it is demonstrated that a complete absence of serum in the presence of 8 mM Ca^{2+} for 30 min enhances gene expression by an additional 60-fold as compared to cells treated in the presence of 10% serum and 8 mM Ca^{2+}. The presence of as little as 2% serum for the initial 30-min incubation yields expression levels similar to that observed for 10% serum treatments (Fig. 3A). Cell survival and division were reduced when cells were treated with 0% serum in

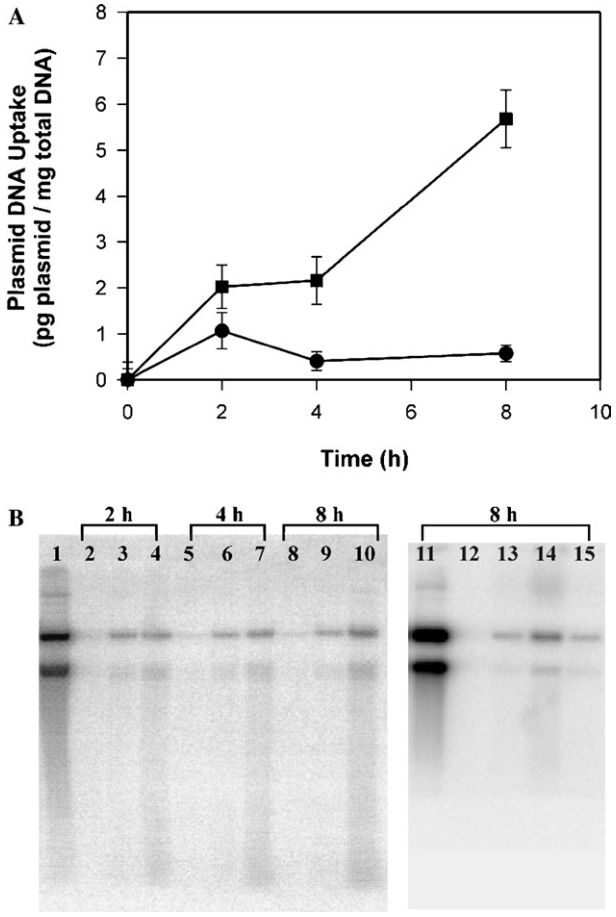

Fig. 2. Influence of Ca^{2+} on the integrity of SPLP plasmid following uptake of SPLP into BHK cells. SPLP (DODAC:DOPE:PEG-C_{20}, 7:83:10; molar ratio) containing 2.5 μg plasmid DNA was used to transfect BHK cells as described under Materials and methods. At appropriate time points (2, 4, and 8 h), DNA was extracted from the cells and intracellular plasmid DNA was detected by hybridization to a specific ^{32}P-labeled plasmid DNA probe. (A) Levels of plasmid DNA uptake in the absence of (●) and in the presence of (■) 8 mM Ca^{2+} as determined by dot blot analysis described under Materials and methods. (B) Integrity of intracellular plasmid DNA determined using the Southern blot analysis. Lanes 1 and 11, pCMVLuc control; lanes 2, 5, 8, and 12, nontransfected control; lanes 3, 6, 9, and 13, cells transfected with SPLP; lanes 4, 7, 10, and 14, cells transfected with SPLP and 8 mM Ca^{2+}; lane 15, cells transfected with SPLP and 8 mM Mg^{2+}. Each data point represents an average of three samples ± SD.

the presence of Ca^{2+}. There was no evidence of changes in cell morphology or growth rate in the absence of Ca^{2+} when serum content was in the range of 0–10% (data not shown).

To minimize the reduction in cell growth rates observed in the presence of 0% serum and 8 mM Ca^{2+}, we attempted to establish the shortest incubation period required to induce increased expression. Fig. 3B relates the time in non-serum-containing medium to the observed Ca^{2+}-induced increase in gene expression after 24 h. A lack of serum in the cell medium for 10 min or longer resulted in the same level of enhancement in transfection levels. However, if serum was added within

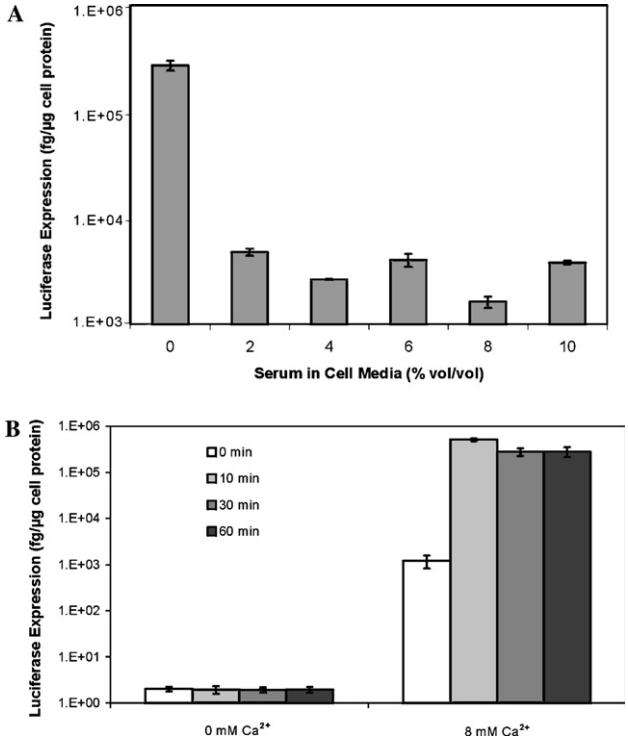

Fig. 3. Serum affects the Ca^{2+}-related increase in gene expression of SPLP-delivered pDNA. BHK cells were plated 18 h prior to transfection. Immediately before treatment, $CaCl_2$ was added to SPLP and then diluted with non-serum-containing medium to yield a final Ca^{2+} concentration of 8 mM. The cells were treated with this mixture at a dose of 1 μg SPLP (DODAC:DOPE:PEG-C_{20}, 8:82:10; molar ratio) encapsulated pDNA/well for 30 min in 0, 2, 4, 6, 8, and 10%(vol/vol) serum (A) and for 0, 10, 30, and 60 min in 0%(vol/vol) serum (B). Following the indicated treatment period, serum was added to yield 10%(vol/vol) in the medium. Cells were transfected for a total of 24 h and then washed 2× in Dulbecco's PBS followed by lysis in 0.1% Triton X-100 Dulbecco's PBS solution. Aliquots of cell lysate were then assayed for luciferase expression. Gene expression data presented were normalized to cell protein content in the assayed lysate, and each data point represents an average of three samples ± SD.

30 s to the cell medium, the expression was similar to that observed in the presence of 10% serum. In the non-Ca^{2+}-containing controls, there was no increase in expression resulting from exposure to nonserum conditions.

Ca^{2+}-induced increases in transfection require the presence of phosphate

It is well known that calcium phosphate can stimulate transfection using naked plasmid [31–33]. To determine whether calcium phosphate would be playing a role in the Ca^{2+}-dependent increases in expression observed for SPLP, the transfection levels of SPLP and Ca^{2+} mixtures were assayed in both phosphate-containing and phosphate-free DMEM cell culture medium. Cells were transfected with SPLP and 8 mM Ca^{2+} for 24 h. Ca^{2+}-free controls were included to ensure that the expression levels were not affected by the lack of phosphate in the medium.

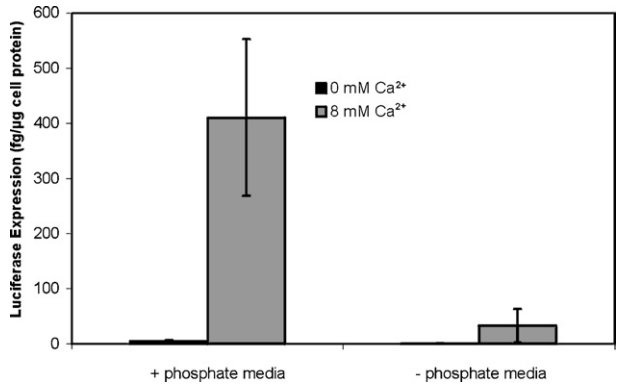

Fig. 4. Ca^{2+}-related increases in gene expression of SPLP-delivered pDNA are dependent on phosphates in the cell medium. BHK cells were treated as described in the legend to Fig. 3 with SPLP at a dose of 1 µg SPLP (DODAC:DOPE:PEG-C_{20}, 8:82:10; molar ratio) encapsulated pDNA/well in 0 and 8 mM Ca^{2+} for 24 h in phosphate-free and phosphate-containing DMEM medium. Following a 24-h transfection, cells were washed 2× in Dulbecco's PBS followed by lysis in a 0.1% Triton X-100 Dulbecco's PBS solution. The lysate was assayed for luciferase expression. Gene expression data presented were normalized to cell protein content in the lysate assayed, and each data point represents an average of three samples ± SD.

In Fig. 4 it is demonstrated that transfections conducted in phosphate-free medium reduced expression to levels observed in wells containing phosphate but no Ca^{2+}. Also, there was no significant difference in gene expression of the Ca^{2+}-free controls, treated in the presence as compared to the absence of phosphate (both values are within 1 SD of each other). The cell morphology or growth rates were not affected by the absence of phosphate in the medium for the duration of this 24-h experiment.

Ca^{2+} does not destabilize SPLP structure

SPLP with the PEG-CerC_{20} coating are highly stable systems that exhibit extended circulation times in vivo, protect encapsulated plasmid from external nucleases, and do not interact readily with cells [23,25,34]. However, it is important to demonstrate that the enhanced transfection properties of SPLP in the presence of Ca^{2+} do not arise due to destabilization of the SPLP leading release of free plasmid, for which calcium phosphate is well known to enhance transfection. The stability of the SPLP in the presence of Ca^{2+} was examined by employing quasi-elastic light scattering (QELS) to detect changes in size and the Picogreen fluorophore assay to detect DNA leakage. For the QELS experiments, $CaCl_2$ was added to the SPLP suspension to achieve concentrations as high as 50 mM. No change in the SPLP size or size distribution was observed (results not shown). For the plasmid release experiments, SPLP were incubated at 37 °C in HBS buffer containing 10% FBS in the presence or absence of 8 mM Ca^{2+}. Plasmid release was assayed over 24 h by employing the Picogreen assay. No plasmid release was observed (results not shown).

Calcium phosphate-mediated enhancements in gene expression of SPLP are not affected by serum nucleases

A calcium-phosphate-dependent enhancement in transfection by naked plasmid requires that the plasmid be protected from serum nucleases ubiquitous in the blood. To explore the effect of nucleases on calcium phosphate transfection of plasmid DNA, we conducted calcium phosphate transfections of free and SPLP-encapsulated plasmid DNA in cell medium containing active serum nucleases or reduced serum nuclease activity (see Materials and methods). At 24 h, cells were washed 2× in Dulbecco's PBS and then assayed for luciferase expression. Transfection with free plasmid in medium containing active serum nucleases resulted in a 120-fold reduction in gene expression (Fig. 5). In contrast, the presence/absence of nuclease activity in the medium did not affect gene expression of the encapsulated system (Fig. 5).

Calcium phosphate facilitates the transfer of large molecules trapped in endosomes/lysosomes into the cytoplasmic component of the cell

To investigate the underlying mechanism behind calcium phosphate transfection, experiments were conducted to identify differences in cell distribution of SPLP following uptake. Cells were transfected with Rh-DOPE-labeled SPLP and Ca^{2+} and then observed using

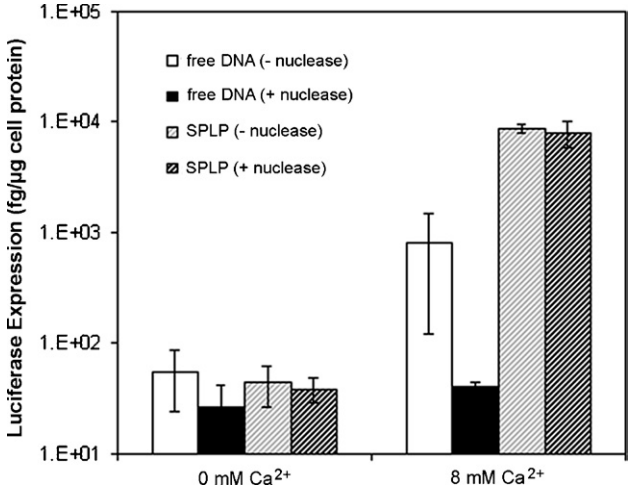

Fig. 5. Calcium-phosphate-enhanced transfection of SPLP is not affected by serum nucleases. BHK cells were treated as described in the legend to Fig. 3 with SPLP at a dose of 1 µg SPLP (DODAC:DOPE:PEG-C_{20}, 8:82:10; molar ratio) encapsulated pDNA/well with 0 and 8 mM Ca^{2+} for 24 h in nuclease-containing or nuclease-deficient DMEM. After 24 h, cells were washed 2× in Dulbecco's PBS followed by lysis in a 0.1% Triton X-100 Dulbecco's PBS solution. The lysate was assayed for luciferase expression. Gene expression data presented were normalized to cell protein content in the lysate assayed, and each data point represents an average of three samples ± SD.

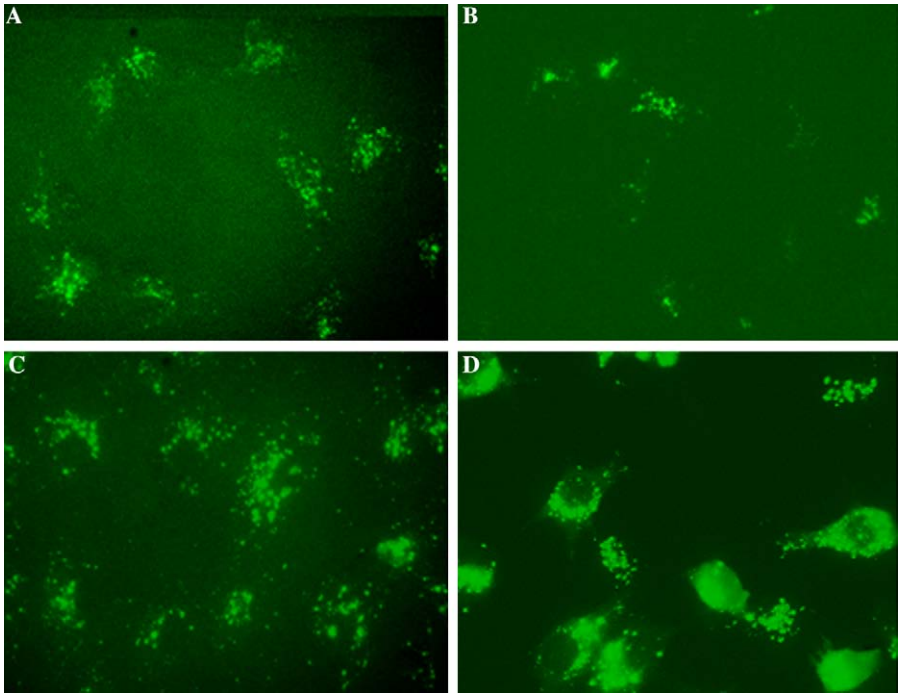

Fig. 6. Calcium-phosphate-mediated intracellular release of dextrans following cell uptake. BHK cells were treated with Alexa-Fluor-488-labeled 10,000 mol wt. dextran as described under Materials and methods. Treatments were conducted in the presence and absence of 8 mM Ca^{2+}. Serum was absent for the first 30 min in all wells, after which additional FBS was added to yield 10%(vol/vol) in the medium. Cells were incubated for an additional 1.5 h and then washed 2× in Dulbecco's PBS and incubated in serum-containing medium for an additional 10 h. Micrographs were taken of live cells at 2 h (A,B) and 12 h (C,D) to compare dextran distribution in the presence (C,D) and absence (A,B) of Ca^{2+} under 400× magnification using an inverted fluorescence microscope.

fluorescence microscopy over a 24-h time period. However, visual evidence for differences in the distribution of SPLP in the cell in the presence or absence of Ca^{2+} was subtle, with indication of a Ca^{2+}-related increase in lipid content in the cells and much larger punctate structures where most of the particle was accumulated (data not shown). For more definitive visual evidence, a 10,000 molecular weight fluorescently labeled dextran was employed to investigate differences in cellular distribution following uptake when Ca^{2+} is present. Dextran was selected since it resists biological degradation and is known to enter the cell primarily via phagocytotic and endocytic pathways similar to the route of entry for nonviral systems [35]. Cells were incubated with Alexa-Fluor-488-labeled dextran and 8 mM Ca^{2+} in non-serum-containing medium for 30 min, and then serum was added to yield 10%(vol/vol) in the well. Following 2 h, the cells were washed, replenished with fresh medium, and then left to incubate for a further 10-h period. At the 2- and 12-h time points, cells were observed under 400× magnification for cellular distribution of the fluorescently labeled dextran. It is demonstrated in Figs. 6A and C that after 2 h, the cells treated with and without Ca^{2+} show that dextran is limited to punctate structures distributed in the cytoplasmic region of the cell. After 12 h, the cells treated with Ca^{2+} show distribution of dextran in a more diffuse pattern throughout the cytoplasm of the cell and in very large punctate structures (Fig. 6D). Conversely, in the absence of Ca^{2+}, very low levels of dextran remained in the cell, still evident in small punctate structures in the cytoplasmic region (Fig. 6B).

Discussion

The results presented in this paper demonstrate that Ca^{2+} can dramatically stimulate the transfection potency of the SPLP system in vitro, that this effect appears to depend on the presence of calcium phosphate, and that this effect is not related to calcium-phosphate-dependent destabilization of the SPLP structure. We discuss these areas in turn.

The addition of Ca^{2+} enhances SPLP potency by 60- to 100-fold under conditions where serum is present and by up to 6000-fold when serum is absent from the medium for the initial 10-min period of the transfection procedure. This Ca^{2+} effect occurs only in the presence of phosphates, providing strong evidence to support that the increase is related to the presence of calcium phosphate precipitates. The Ca^{2+} and phosphate concentrations required are similar to other methods utilizing calcium phosphate transfection protocols [33,36]. Interestingly, the Ca^{2+} concentrations required to achieve this

increase in gene expression fall over a narrow range. The reduction in expression at levels above the optimal peak concentration of Ca^{2+} is commonly attributed to the formation of calcium phosphate precipitates that become too large for effective delivery [33,37].

The observation that Ca^{2+} does not cause detectable destabilization of the SPLP system suggests that the calcium-phosphate-dependent enhancement in transfection levels does not result from direct interaction with SPLP plasmid DNA. It is possible that small calcium phosphate precipitates could adsorb to the SPLP surface. In this regard, SPLP purification yields particles with approximately 5% of the plasmid exposed to the extracellular environment that cannot be removed by further purification on a DEAE column (data not shown). Alternatively, it is possible that calcium phosphate directly causes destabilization of the endosomal membrane without direct association with the SPLP particle. This possibility is supported by the ability of calcium phosphate to directly enhance the intracellular delivery of fluorescently labeled dextran. Dextran, which lacks a negative charge, would not be expected to coprecipitate with calcium phosphate. This observation correlates with the hypothesis that the precipitates are acting primarily as lysosomotropic agents which, at lower pH, encourage destabilization of endosomal/lysosomal membranes [38].

The potential utility of this calcium phosphate effect to enhance transfection properties of SPLP in vivo is questionable since methods to locally generate calcium phosphate crystals to enhance transfection at disease sites such as a tumor site are not available. However, the development of methods to associate calcium phosphate with SPLP systems may prove beneficial. Also, the ability of the SPLP system to protect encapsulated plasmid from degradation by serum nucleases, while maintaining sensitivity to calcium phosphate, clearly extends the utility of the calcium phosphate method.

In conclusion, the data presented facilitate further understanding of the mechanism behind calcium phosphate transfection, indicating that direct association of calcium phosphate with plasmid is not required and that calcium phosphate alone facilitates intracellular delivery. The observation that calcium phosphate can dramatically enhance the transfection potency of SPLP also suggests interesting new directions to take to improve the in vivo potency of the SPLP system.

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