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Utilization of modified surfactant-associated protein B for delivery of DNA to airway cells in culture

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ABSTRACT **Pulmonary surfactant lines the airway epithelium and creates a potential barrier to successful transfection of the epithelium *in vivo*. Based on the functional properties of pulmonary surfactant protein B (SP-B) and the fact that this protein is neither toxic nor immunogenic in the airway, we hypothesized that SP-B could be modified to deliver DNA to airway cells. We have modified native bovine SP-B by the covalent linkage of poly(lysine) (average molecular mass of 3.3 or 10 kDa) to the N terminus of SP-B and formed complexes between a test plasmid and the modified SP-B. Transfection efficiency was determined by transfection of pulmonary adenocarcinoma cells (H441) in culture with the test plasmid pCPA-RSV followed by measurement of activity of the reporter gene encoding chloramphenicol acetyltransferase (CAT). Transfections were performed with DNA·protein complexes using poly(lysine)$_{10kDa}$-SP-B ([Lys]$_{10kDa}$-SP-B) or poly(lysine)$_{3.3kDa}$-SP-B ([Lys]$_{3.3kDa}$-SP-B), and results were compared with transfections using unmodified poly(lysine)·DNA, unmodified SP-B·DNA, or DNA only. For [Lys]$_{10kDa}$-SP-B·pCPA-RSV preparations, CAT activity was readily detectable above the background of [Lys]$_{3.3kDa}$-SP-B or unmodified SP-B. The SP-B-poly(lysine) conjugates were effective over a broad range of protein-to-DNA molar ratios, although they were optimal at approximately 500:1–1000:1. Transfection efficiency varied with the tested cell line but was not specific to airway cells. Addition of replication-defective adenovirus to the [Lys]$_{10kDa}$-SP-B·pCPA-RSV complex enhanced CAT activity about 30-fold with respect to that produced by the [Lys]$_{10kDa}$-SP-B·pCPA-RSV complex alone. This increase suggests routing of the adenoviral·[Lys]$_{10kDa}$-SP-B·pCPA-RSV complex through an endosomal pathway. Effects of covalent modification on the secondary structure of SP-B were examined by Fourier transform infrared spectrometry (FTIR). Results of FTIR indicated that the conformation of [Lys]$_{10kDa}$-SP-B was comprised primarily of α-helical structure compared with a predominately aggregated structure of unmodified poly(lysine). We conclude that poly(lysine) conjugates of SP-B effectively deliver DNA *in vitro* and may have utility as DNA delivery vehicles to the airway *in vivo*.**

With the successful cloning of the cystic fibrosis transmembrane conductance regulator (CFTR) cDNA and correction of the cAMP-dependent chloride transport defect by transfection of cells, strategies to transfect pulmonary cells *in vitro* and *in vivo* are rapidly being developed with the goal of gene therapy of cystic fibrosis and other pulmonary diseases. Such corrective therapies are approachable in lung disease since noninvasive access to the epithelial surface of the organ is possible by conventional instillation or aerosolization technologies. Successful transfection of airway cells *in vitro* and *in vivo* reported in recent literature includes the use of viruses with specific tropism for airway epithelial cells but also other compounds not indigenous to the airway. To date these include replication-defective adenoviral vectors, adeno-associated viral vectors, cationic liposomes including Lipofectin and *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium, and protein·cationic peptide complexes (1–8). Recently, Curiel and associates (5) have applied the strategy of Wu and associates (4) to link poly(lysine) covalently to a cell surface receptor ligand to deliver DNA to lung cells via the receptor·endosomal pathway. Specifically, Curiel *et al.* (5) have shown that DNA complexed to poly(lysine) conjugates of the transferrin receptor was taken up and expressed by epithelial cells. The process was enhanced by coupling the ligand·DNA complex with adenovirus through an antibody or mixing the ligand·DNA with adenovirus (7). Recently, adenovirus complexed directly with poly(lysine) was shown to deliver DNA effectively *in vivo* (8).

Pulmonary surfactant is a complex mixture comprised predominately of lipids with about 10% protein by weight. Pulmonary surfactant overlays the proximal and distal epithelium that forms the logical target cells for gene therapy of cystic fibrosis and potentially other pulmonary diseases. One of the critical protein components of surfactant is surfactant-associated protein B (SP-B). SP-B, which exhibits surface tension-lowering properties, is a component of various surfactant preparations used in the treatment of neonatal respiratory distress syndrome. Surfactant preparations have been shown to be nonimmunogenic and nontoxic when used in the therapy of respiratory distress syndrome (9). Due to its compatibility with surfactant and potential safety, SP-B may be useful in gene therapy strategies that require repetitive treatment of the airway necessary to achieve adequate or consistent levels of gene expression.

SP-B in its mature form is a nonglycosylated 79-amino acid polypeptide generated by translation of a 2.0-kb mRNA and subsequent proteolytic processing of an ≈39-kDa primary translation product as determined by sequence analysis with cDNA clones and direct amino acid sequence analysis (10–14). SP-B mRNA and protein are expressed at high levels in bronchiolar and alveolar epithelium.

Recently, Breslin and Weaver (15) showed that exogenous SP-B, in the absence or presence of isolated rat surfactant, is taken up by airway cells *in vitro* and found in endosomes, through a process that does not appear to be receptor mediated. Using competition assays with unlabeled and ^{125}I-labeled SP-B in the absence of surfactant lipids, Bates *et*

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Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; SP-B, pulmonary surfactant-associated protein B; CAT, chloramphenicol acetyltransferase; [Lys]$_n$, poly(L-lysine) of average molecular mass of *n* kDa; [Lys]$_n$-SP-B, poly(L-lysine) cross-linked to SP-B; FTIR, Fourier transform infrared spectrometry; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate.

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al. (16) demonstrated that uptake of SP-B by pulmonary type II cells was unsaturable. However, in the presence of surfactant phospholipids, the uptake of SP-B was saturable. These data led Bates et al. (16) to conclude that SP-B, in the absence of phospholipids, exists primarily as aggregates, and the mechanism of cellular uptake may be dependent on the physical state of the SP-B molecule. The intracellular fate of SP-B is not completely resolved, although it may be recycled to lamellar bodies. SP-B and several synthetic peptides of SP-B enhance phospholipid uptake and inhibit phospholipid secretion by type II epithelial cells *in vitro* and, therefore, may play an important role in recycling of surfactant in the alveolus (17). In addition, SP-B has been shown to promote fusion of membranes (18)—a property of SP-B that may play a role in its reuptake in type II cells.

In the present study, we provide evidence that poly(lysine) conjugates of SP-B are effective in delivering DNA to airway cells in culture. The modified forms of SP-B were preferentially linked at the N terminus with poly(lysine). The modified SP-B exhibited an increase in α-helical content compared to unmodified SP-B or unmodified poly(lysine). To determine the potential of these conjugates to deliver and enhance uptake of DNA in the airway epithelium for gene therapy, we tested the functional expression of test plasmid DNA delivered to cells in culture.

METHODS

Purification of Native SP-B. SP-B, 8.7 kDa (monomeric form), was isolated and purified as described (19–21).

SP-B·DNA and [Lys]$_n$-SP-B·DNA Complexes. *Synthesis of polycationic conjugates of SP-B.* Bovine SP-B was covalently labeled with poly(lysine) ([Lys]$_n$) at the amino group of the N terminus using the method of Jung *et al.* (22) with several modifications. Briefly, in the first step of the coupling procedure, the surfactant protein and linear poly(lysine), [Lys]$_n$ (3.3 or 10 kDa in size), were mixed with an ethanolic solution of *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) at 25°C in a 1:2 molar ratio, with constant stirring for 4 hr to form PDP-SP-B and PDP-[Lys]$_n$. While the [Lys]$_n$/SPDP reaction was carried out in 10 mM Tris buffer (pH 7.5), linkage of the SPDP to SP-B was performed in 2-propanol/H_2O, 3:1 (vol/vol), following adjustment of pH to 7.5 with 10 mM Tris buffer (pH 7.5). The PDP-[Lys]$_n$ was then treated with a 2-fold excess of dithiothreitol to thiolate the PDP moiety. The thiolated form of PDP-[Lys]$_n$ was mixed with PDP-SP-B at pH 7, 30°C (24 hr), to yield [Lys]$_n$-SP-B. Minimal linkage of the poly(lysine) to lysine and arginine residues of SP-B was controlled by pH. Purifications of the PDP intermediates and poly(lysine) conjugates of SP-B were performed by size exclusion chromatography using Sephadex G-75 and/or dialysis (cellulose tubing, molecular weight cut-off range of 12,000–14,000).

The degree to which SP-B and poly(lysine) were substituted with 2-pyridyldisulfide residues was determined by first thiolating the PDP intermediates with the addition of dithiothreitol (final concentration, 5 mM) followed by measurement of the absorbance at 343 nm. The amount of pyridine-2-thione released from the PDP-SP-B or PDP-[Lys]$_n$ intermediates was calculated using a molar extinction coefficient of 8.08×10^3 $M^{-1}\cdot cm^{-1}$. Concentrations of PDP-[Lys]$_n$ and PDP-SP-B were determined by the bicinchoninic acid protein assay (Sigma). Finally, N-terminal sequence analysis was performed on the final [Lys]$_n$-SP-B products (before and after thiolation with dithiothreitol) to ascertain cross-linkage between [Lys]$_n$ and SP-B.

Formulation of [Lys]$_n$-SP-B·DNA complexes, Lipofectin·DNA complexes, and transfection of H441 cells. Purified [Lys]$_n$-SP-B, suspended in 10 mM Hepes buffer (pH 7.5), was incubated with pCPA-RSV DNA in 10 mM Tris, pH 7.5/1 mM EDTA (TE) for 1/2–1 hr at 25°C. For experiments with Lipofectin and Lipofectin·DNA, samples were prepared in polystyrene tubes according to manufacturer's recommendations (GIBCO/BRL). The plasmid, pCPA-RSV, has been described elsewhere and is expressed well when transfected into the pulmonary adenocarcinoma cell line H441 (23). The [Lys]$_n$-SP-B·DNA or Lipofectin·DNA mixture was added to 60-mm culture plates containing cells at about 50% confluency in RPMI 1640 medium or Dulbecco's modified Eagle's medium (DMEM) without serum. After incubation overnight, medium was changed to RPMI medium plus 10% fetal bovine serum for H441 cells and DMEM plus 10% bovine serum for 3T3 or HeLa cells and incubated to ≈80–90% confluency. Cells were collected, lysed by three freeze–thaw cycles, and clarified by centrifugation, and volumes were normalized to the same cell lysate protein per assay for determination of chloramphenicol acetyltransferase (CAT) activity as described elsewhere (23). CAT assays were autoradiographed and then quantitated for determination of conversion of chloramphenicol to acetylated products using a PhosphorImager and ImageQuant software (Molecular Dynamics).

[Lys]$_n$-SP-B·DNA·lipid complexes. [Lys]$_n$-SP-B·DNA·lipid complexes were formed by first mixing (Lys)$_n$-SP-B (in saline) with pCPA-RSV plasmid DNA. After a 60-min incubation period (25°C), 6.5 μg per plate of surfactant lipids in the form of Survanta (an organic extract of bovine lung) was added to the SP-B·DNA complex and gently mixed. Suspensions of protein·DNA complexes were formulated in polystyrene tubes. The amount of Survanta added (from 25 mg of phospholipid per ml of 0.9% NaCl stock suspensions) was based on the dose used in treatment of a 1-kg infant with respiratory distress syndrome. Assuming that 1×10^9 cells yields 1 g of cells (see ref. 6) and a lung mass of 32 g/1-kg infant, an equivalent dose per 1×10^6 cells (in culture) would be 2.5 μg of surfactant. The amount of Survanta added per culture dish in the present experiments was ≈2.5-fold excess of the calculated dose.

Agarose gel analysis of protein·DNA complexes. One hundred femtomoles of DNA (about 250 ng) was mixed with picomolar amounts of [Lys]$_{10kDa}$-SP-B in polystyrene tubes and incubated for 1 hr. An aliquot of about 50 fmol (≈125 ng) of DNA in the presence of protein was electrophoresed in a 1% agarose gel to identify the presence of DNA not bound to protein.

Adenovirus enhancement. Defective adenovirus containing bacterial *lacZ* gene was generously provided by Bruce Trapnell (Genetic Therapy, Gaithersburg, MD). Virus was added to cells in serum-free medium immediately after addition of DNA·protein complexes or 2 days after transfection. Multiplicity of infection was calculated using viral titer in plaque-forming units/ml and cell counts of cells trypsinized from 50–60% confluent plates. At a multiplicity of infection of ≈1000:1, at least 60–70% of H441 cells are β-galactosidase positive (data not shown).

Fourier Transform Infrared Spectroscopy (FTIR) Analysis of Native Surfactant Protein and Modified Surfactant Protein Conformation. Analysis of the effects of modification of SP-B on its conformation, with respect to that of native SP-B, was performed by attenuated total reflection FTIR. The protein sample was dried directly to a germanium attenuated total reflection crystal and hydrated with minimal amounts of water. A total of 512 scans, recorded over the range of 2000 cm^{-1} to 1000 cm^{-1} and at 2 cm^{-1} resolution, was signal averaged and Fourier transformed with triangular apodization to obtain the FTIR spectra. Qualitative assessment of secondary structures, such as α-helical, β-sheet, β-turn, and random coil structures, was performed based on amide I band assignments as described by Byler and Susi (24).

RESULTS

Chemical Characterization of $[Lys]_n$-SP-B Conjugates. To determine the likely position and types of linkages obtained between poly(lysine) and SPDP or SP-B, the following analyses were performed. UV analysis of liberated pyridine-2-thione upon thiolation of all PDP-$[Lys]_n$ intermediates indicated that all PDP-$[Lys]_n$ preparations averaged 1.3 ± 0.4 mol of 2-pyridyldisulfide residues per mol of polypeptide, suggesting minimal reaction with the ε-amino groups of lysinyl side chains. Similar analyses indicated that SP-B was substituted to a degree of 1.1 ± 0.2 mol of 2-pyridyldisulfide residue per mol of SP-B monomer. N-terminal amino acid sequence analyses of the final $[Lys]_n$-SP-B product and purified thiolation products of the $[Lys]_n$-SP-B preparations yielded only N-terminally blocked peptides, a result that is consistent with successful cross-linking of $[Lys]_n$ with SP-B through SPDP.

Conformation of $[Lys]_{10kDa}$-SP-B as Determined by FTIR. To determine the conformation of the poly(lysine)-SP-B product, the FTIR spectrum of $[Lys]_{10kDa}$-SP-B was analyzed for secondary structural components using the characteristics of the amide I band. Fig. 1 shows a comparison of the amide I/amide II region of the FTIR spectra of $[Lys]_{10kDa}$-SP-B and $[Lys]_{10kDa}$. The amide I peak of $[Lys]_{10kDa}$ is very broad and has an apparent peak maxima at 1595 cm^{-1}. Since amide I maxima at <1625 cm^{-1} are indicative of peptide aggregation, it is likely that $[Lys]_{10kDa}$ exists in an aggregate state. In contrast, $[Lys]_{10kDa}$-SP-B exhibited a very narrow amide I band centered at 1655 cm^{-1}. Since α-helical structure has been assigned to maxima centered at 1655 cm^{-1}, $[Lys]_{10kDa}$-SP-B is comprised of primarily α-helical conformation, suggesting that covalent attachment of SP-B to $[Lys]_{10kDa}$ dramatically alters the conformation of poly(lysine).

Transfection of H441 Cells with $[Lys]_n$-SP-B Conjugates of Differing Poly(lysine) Chain Lengths. H441 cells are pulmonary bronchiolar adenocarcinoma cells of human origin that express SP-A and SP-B and serve as a model of distal airway epithelial cells. Our hypothesis that a poly(lysine)-SP-B conjugate would be effective in transfection of airway cells is based on the DNA binding properties of the poly(lysine) moiety and the fusigenic properties of SP-B allowing for internalization. To determine an effective poly(lysine) length, $[Lys]_n$-SP-B conjugates were synthesized using poly(lysine) preparations of average molecular masses of 3.3 kDa and 10.2 kDa (≈23 and ≈70 lysinyl residues, respectively). As illustrated in Fig. 2, CAT activity was readily detectable from preparations using $[Lys]_{10kDa}$-SP-B as the vehicle in the absence or presence of Survanta. Preparations using either $[Lys]_{3.3kDa}$-SP-B or unmodified $[Lys]_{10kDa}$ or uncomplexed DNA only did not result in detectable CAT activity. Purified unmodified native SP-B yielded only low-level CAT activity.

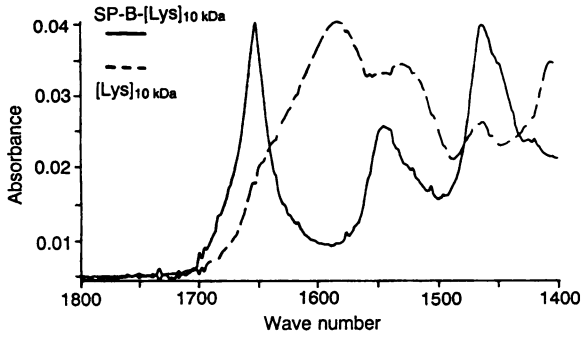

FIG. 1. Amide I/amide II region of the FTIR spectra of hydrated $[Lys]_{10kDa}$ and $[Lys]_{10kDa}$-SP-B on a ZnSe attenuated total reflection crystal (23°C). Acquisition parameters are described in the text.

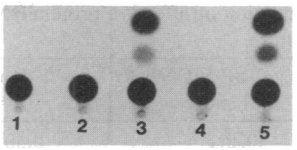

FIG. 2. Transfection of H441 cells. CAT assays were performed for 6 hr with 48 μg of cell extract protein per assay. Fifteen picomoles of pCPA-RSV DNA and ≈2 nmol of protein conjugate were used for each transfection. Lane 1, CAT assay of cells transfected with DNA only; lane 2, with $[Lys]_{10kDa}$; lane 3, with $[Lys]_{10kDa}$-SP-B in the presence of Survanta (see text); lane 4, with $[Lys]_{3.3kDa}$-SP-B; lane 5, with $[Lys]_{10kDa}$-SP-B.

Transfected cells were visually examined daily before, during, and after transfection, and no apparent cellular toxicity was observed at the DNA·protein concentrations examined.

Determination of Effective $[Lys]_{10kDa}$-SP-B-to-DNA Molar Ratios for Cell Transfection. Initially, concentrations of 0.25–8 nmol of $[Lys]_{10kDa}$-SP-B conjugate to 15 pmol of pCPA-RSV plasmid DNA were examined for optimal transfection of H441 cells in culture. Visual examination of the autoradiogram revealed effective DNA delivery with protein amounts of 1.0–2.0 nmol or greater. We subsequently demonstrated that as little as 1 pmol of pCPA-RSV (2.7 μg) was effectively delivered (data not shown). To determine the molar ratio of $[Lys]_{10kDa}$-SP-B·DNA at which DNA is completely bound, we examined the retardation of DNA·protein complexes in agarose gels by agarose gel electrophoresis. Protein-to-DNA molar ratios of 250:1 or greater resulted in complete retardation of detectable DNA (Fig. 3A).

To analyze efficiency of transfection, protein·DNA molar ratios were varied and used to transfect H441 cells with complexes formed by varying DNA concentration (Table 1) or protein concentration (Fig. 3B). The most effective protein·DNA molar ratios yielding maximal CAT conversion in culture were 625:1 (Table 1) or 1000:1 (Fig. 3).

Cellular Specificity of $[Lys]_{10kDa}$-SP-B Complexes. In a phospholipid-poor environment uptake of SP-B is not receptor dependent (15, 16). Transfections in serum-free medium mimic these conditions. Lung epithelial cells and non-lung

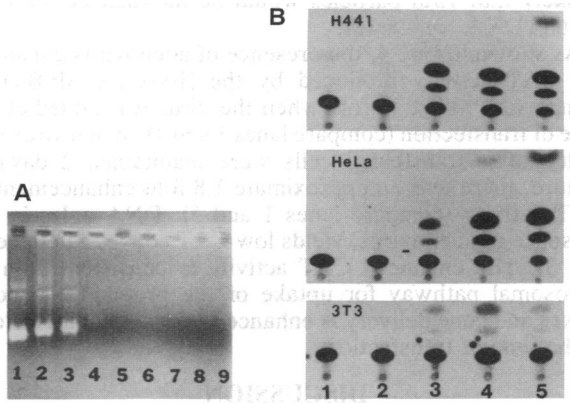

FIG. 3. (A) Analysis of $[Lys]_{10kDa}$-SP-B·DNA complexes by agarose gel electrophoresis. Protein·DNA (pCPA-RSV) complexes were prepared in polystyrene tubes and incubated for 1 hr (25°C) at the following protein·DNA molar ratios: lane 1, DNA only; lane 2, 31.2:1; lane 3, 62.5:1; lane 4, 125:1; lane 5, 250:1; lane 6, 500:1; lane 7, 1000:1; lane 8, 2000:1; lane 9, 100 pmol of SP-B only. About 50 fmol (≈125 ng) of DNA was analyzed on a 1% agarose gel. (B) Comparative transfection of H441, HeLa, and 3T3 cells. $[Lys]_{10kDa}$-SP-B·DNA complexes were formed with 1 pmol of DNA, varying the quantity of $[Lys]_{10kDa}$-SP-B to achieve protein·DNA molar ratios as follows: lane 1, 62.5:1; lane 2, 125:1; lane 3, 250:1; lane 4, 500:1; lane 5, 1000:1. CAT assays from transfected H441, HeLa, and 3T3 cells are designated. Cell lysate proteins (≈130 μg) were normalized to compare CAT activity; assays were 4 hr.

Table 1. Determination of effective protein·DNA molar ratio for transfection in culture

$[Lys]_{10kDa}$-SP-B·DNA,* mol/mol	% conversion (n = 3)†
5000:1	0.30 ± 0.06
2500:1	1.4 ± 0.3
1250:1	15.0 ± 3.3
625:1	80.2 ± 3.0

*Each transfection contained 1.0 nmol of $[Lys]_{10kDa}$-SP-B.
†Five-hour CAT assays were performed in triplicate. Data are reported as mean ± SEM.

3T3 and HeLa cells were capable of being transfected with the $[Lys]_{10kDa}$-SP-B conjugates (Fig. 3*B*). The level of CAT conversion varied with the cell type and the protein·DNA molar ratio used. At a protein·DNA ratio of 250:1, H441 cells showed the highest activity (Fig. 3*B*, lane 3), but at 1000:1 HeLa cells showed the greatest activity (Fig. 3*B*, lane 5). All cell types showed detectable CAT activity at a 250:1 protein·DNA molar ratio.

Comparison of Transfection Efficiency of $[Lys]_{10kDa}$-SP-B with Cationic Lipids. To determine the efficiency of transfection of airway cells with $[Lys]_{10kDa}$-SP-B compared with cationic lipids, CAT activity from H441 cells was determined by comparing transfections with equimolar amounts of protein conjugates or Lipofectin with the protein·DNA molar ratio at 1000:1. With 1 nmol of $[Lys]_{10kDa}$-SP-B, CAT conversion was 15.2% ± 2.9%, and Lipofectin at 1 nmol yielded CAT conversion of 10.8% ± 1.4%. In contrast, 2 nmol of $[Lys]_{10kDa}$-SP-B showed ≈3-fold greater CAT activity than 2 nmol of Lipofectin (43% ± 3.4% compared to 12.9% ± 0.5%).

Enhancement of Gene Expression in the Presence of Adenovirus. An accepted method for enhancing DNA uptake from endosomal pathways is to cotransfect DNA·protein conjugates with adenovirus (7). To test whether $[Lys]_{10kDa}$-SP-B·DNA conjugates are routed through the endosome, we utilized a defective β-galactosidase-expressing adenovirus to test the efficiency of transfection in the presence of adenovirus. A multiplicity of infection of ≈1000:1 was chosen to ensure transfection of 60–70% of the cells in culture and make it likely that viral particles would be internalized with the protein·DNA conjugates.

As shown in Fig. 4, the presence of adenovirus enhanced the CAT activity produced by the $[Lys]_{10kDa}$-SP-B·DNA complex by about 30-fold when the virus was added at the time of transfection (compare lanes 1 and 4). When virus was added after transfected cells were maintained 2 days in culture, there was an approximate 1.8-fold enhancement of CAT activity (compare lanes 1 and 5). DNA only, in the presence of adenovirus, yields low CAT conversion (lanes 2 and 3). The enhanced CAT activity is consistent with an endosomal pathway for uptake of the protein·DNA complexes and gene delivery is enhanced with adenovirus added at the time of transfection.

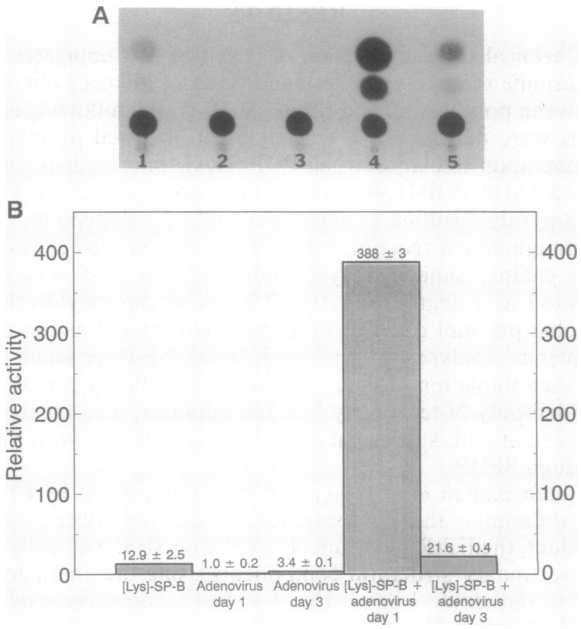

FIG. 4. (*A*) Enhancement of CAT activity by transfection with adenovirus. CAT assays were performed on cell lysates normalized to extract protein. Transfections were with 1.0 nmol of $[Lys]_{10kDa}$-SP-B and 1.0 pmol of pCPA-RSV. Lane 1, $[Lys]_{10kDa}$-SP-B·pCPA-RSV; lane 2, pCPA-RSV, adenovirus was added at time of transfection; lane 3, pCPA-RSV, adenovirus was added after cells were maintained for 2 days in culture; lane 4, $[Lys]_{10kDa}$-SP-B·pCPA-RSV, adenovirus was added at time of transfection; lane 5, $[Lys]_{10kDa}$-SP-B·pCPA-RSV, adenovirus was added after cells were maintained for 2 days in culture. (*B*) Relative CAT activities (mean ± SEM) resulting from preparations similar to those described in *A* were calculated in separate experiments (n = 3). To ensure linearity of the CAT assays, 0.5 nmol of $[Lys]_{10kDa}$-SP-B and 1.0 pmol of pCPA-RSV were used to form the complexes.

DISCUSSION

In this study, we have used the general approach described by Wu *et al.* (4) of cross-linking cationic peptides to other peptides taken up by cells to deliver DNA to cells. A similar strategy has been applied by Curiel *et al.* (5, 7) with human transferrin to deliver DNA to pulmonary cells. Our strategy was to use natural airway peptides known to be recycled in the airway, but not necessarily requiring a receptor for uptake. We covalently modified pulmonary surfactant protein SP-B with poly(lysine) to yield a molecule that is effective in transfection of DNA into airway cells. The chemical analysis of the degree of cross-linking of $[Lys]_{10kDa}$-SP-B samples used in these studies indicated linkage of approximately two poly(lysine) chains per SP-B molecule (dimer), with modification at the N terminus of SP-B. Information obtained from the amide I bands of the infrared spectra of $[Lys]_{10kDa}$ and $[Lys]_{10kDa}$-SP-B suggests that covalent attachment of SP-B to $[Lys]_{10kDa}$ dramatically alters the conformation of poly(lysine), yielding an SP-B-lysine conjugate composed of nearly 100% α-helical components. This conformation is also in contrast to that of native (unmodified) SP-B, which is comprised of 50% α-helix and 20% β-structure (25). The increase in α-helical content upon formation of $[Lys]_{10kDa}$-SP-B may enhance binding of DNA to the poly(lysine) moiety of the conjugate, thereby enhancing effective DNA delivery.

We chose SP-B since, unlike transferrin, it is taken up by airway cells in a non-receptor-mediated process through an endosomal pathway (15), and in an aggregated state (as would occur with $[Lys]_{10kDa}$-SP-B·DNA complexes) uptake of SP-B is not saturable (see ref. 16). Although transfection efficiency varied with cell cultures, $[Lys]_{10kDa}$-SP-B nonspecifically transfects the lung cell line H441 and the non-lung cell lines 3T3 and HeLa (Fig. 3*B*). The latter two cell lines would be unlikely to contain a putative SP-B receptor. Thus, $[Lys]_{10kDa}$-SP-B may deliver DNA to the cells through the fusigenic properties of SP-B (18) and through the poly(lysine) moiety, and not exclusively through a specific SP-B receptor.

$[Lys]_n$-SP-B conjugates with poly(lysine) chains of an average molecular mass of 10 kDa were more effective than 3.3-kDa poly(lysine) moieties. We hypothesize that a shorter poly(lysine) chain may not allow accessibility of the cationic sites due to steric effects of the protein.

In vitro protein·DNA molar ratios of 500:1 to 1000:1 were most effective for DNA transfection. When 0.5–1.0 nmol of $[Lys]_{10kDa}$-SP-B was used, a protein·DNA molar ratio of

5000:1 or 2500:1 resulted in reduced effectiveness (Table 1) as did ratios <250:1 (Fig. 3). Agarose gel retardation of protein·DNA migration revealed that a protein·DNA ratio of at least 250:1 was necessary to completely bind detectable DNA. Since the amount of protein necessary to completely retard DNA is two to four times less than the amount needed for the most efficient CAT activity (Fig. 3 and Table 1), it is likely that the stoichiometry required for uptake at the cell surface is different from that needed to simply retard DNA in the agarose gel. At higher protein·DNA ratios, the stoichiometry of the DNA·protein complexes may be inefficient for internalization. At lower protein·DNA ratios, free DNA is not likely to be effectively internalized, thereby less DNA would be delivered to the cell. To determine effective molar ratios with other reporter gene plasmids or other peptide conjugates, these variables will need to be examined for each combination.

Complexes that are likely to work *in vivo* will have to be compatible with surfactant lipids and should not be in competition with airway proteins. To simulate this environment, Survanta was added to the $[Lys]_{10kDa}$-SP-B·DNA complexes. Survanta consists primarily of phospholipids but also contains about 0.4% (wt/wt) SP-B and 1.6% (wt/wt) SP-C (J.E.B., unpublished data). Thus, the amount of Survanta used in the experiments presented would contain ≈125 ng of SP-B. Since the $[Lys]_{10kDa}$-SP-B used in the experiments was in a 52-fold excess (6.5 μg) of unlabeled SP-B of Survanta, no competition was expected or resulted from its presence. These *in vitro* results in the present study are consistent with the concept that $[Lys]_n$-SP-B is compatible with airway lipids.

Since the $[Lys]_{10kDa}$-SP-B is not specific for transfecting lung cells, other strategies are necessary to specifically target airway epithelial cells. The surfactant protein B, Clara cell 10-kDa protein, and surfactant protein C gene promoter sequences are expressed exclusively in lung epithelial cells (26–28). Plasmids containing these promoters should be expressed only in epithelial cells.

Poly(lysine) alone is not known to be immunogenic (29) and the presence of circulating antibodies specific for SP-B was not detected in infants treated with preparations containing bovine SP-B (9). Since SP-B is a component of pulmonary surfactant, it is unlikely that surface immunity to this protein will arise. Also, since SP-B is recycled in the airway, it is unlikely that toxic amounts of SP-B will accumulate following repetitive treatment of the airway. Therefore, the [Lys]-SP-B conjugates may not be toxic or immunogenic when applied *in vivo*.

Adenovirus disrupts endosomal membranes through the action of the protein capsid (30, 31). Enhanced uptake through the endocytotic pathway has been shown for ligand–toxin complexes and DNA·protein conjugates in the presence of adenovirus (5, 30–32). In the transfections shown in Fig. 4, adenovirus markedly enhanced CAT activity of the transfected $[Lys]_{10kDa}$-SP-B·DNA complexes. These results are consistent with the hypothesis that the $[Lys]_{10kDa}$-SP-B is being transfected through an endosomal pathway. Enhancement of CAT activity was maximal when virus was added at the time of transfection, suggesting that complexes are trapped and degraded in the endosomal pathway. The slight enhancement (1.8-fold) was observed even when adenovirus was added after the cells had been in culture for 2 days. It is possible that routing of the $[Lys]_{10kDa}$-SP-B complexes is slow such that a small amount of transfected DNA remains within the endosomal compartment. Lysomotrophic agents (such as chloroquine) may be useful for enhancing delivery of DNA.

In summary, we have modified surfactant-associated protein B by covalent linkage to poly(lysine) of average molecular mass of 10 kDa to form a conjugate useful in gene delivery to airway cells in culture. With successful application of this strategy to SP-B and with potential modification of other surfactant-associated proteins, it may be possible to develop a surfactant-based method for gene delivery *in vivo*.

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