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GENE DELIVERY

Development and in vitro validation of a targeted delivery vehicle for DNA vaccines

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Abstract

Usage of DNA vaccination has been limited by inefficient cellular expression of plasmid constructs used in DNA vaccines. We describe a novel system for enhancing delivery of DNA vaccine plasmids into cells and their nuclei. This delivery system uses recombinant reovirus type 3 σ1 attachment protein genetically modified with a nuclear localization sequence (σ1-NLS) as a targeting ligand. Purified σ1-NLS was covalently conjugated to the polycation polyethyleneimine (PEI) using a carboxyl-reactive cross-linking agent and complexed with plasmid DNA. The benefit of the NLS in enhancement of protein delivery into the nucleus was demonstrated by liposome-mediated loading of cells with σ1 or σ1-NLS. In L929 fibroblasts loaded with σ1-NLS, 69% of the internalized protein was recovered in the nuclear fraction after 6 h compared to just 10% when using unmodified σ1. Transfection of L929 cells with σ1-NLS-conjugated PEI complexed with a luciferase expression plasmid resulted in a mean 16-fold increase in luciferase activity over complexes made with unmodified PEI, compared to a mean 3-fold boost obtained using σ1-conjugated PEI. These results suggest that σ1-NLS is a useful bifunctional targeting ligand suitable for enhancing DNA delivery and subsequent gene expression for both DNA vaccine applications and nonviral gene therapy.
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1. Introduction

DNA vaccines represent an alternative to traditional vaccine approaches that employ live attenuated pathogens, inactivated pathogens, or purified pathogen antigens to induce a protective immune response. Parenteral administration of naked plasmid DNA leads to gene expression in the host and induction of a systemic immune response to the proteins encoded by the plasmid. DNA vaccines can stimulate immune responses to a variety of pathogenic microorganisms, are easy and safe to produce, and are more stable than several other types of vaccines [1].

Since the advent of DNA vaccination as an alternate method for eliciting a protective immune response, unmodified plasmid DNA has been used in most trials in experimental animals and humans. Immune responses to the antigen encoded by the DNA can be elicited by several methods of DNA administration including intramuscular injection, intradermal injection, and intranasal immunization. However, a constant challenge when using DNA vaccination is the relatively low level of gene expression attained following injection of naked plasmid DNA. This is particularly true when DNA is administered at mucosal surfaces (e.g. nasal or oral immunization) in an attempt to generate mucosal immunity. For DNA vaccination to realize its full potential in human vaccination programs, additional methods are needed that enhance expression of DNA vaccines by selective targeting of the plasmid DNA to cells that can express the DNA-encoded antigens and efficiently present these antigens to T cells.

In an effort to increase the potency of DNA vaccines, attempts have been made to give DNA that is either adsorbed to the surface of small particles or actually encapsulated within the

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particles (reviewed in [2]). Attachment of DNA to small particles offers a way of both protecting DNA from degradation before reaching cells involved in antigen presentation including specialized epithelial cells called M cells and dendritic cells (DCs) as well as taking advantage of their phagocytic activity [3]. Polyanionic DNA forms electrostatic bonds with polycationic polymer particles thus facilitating cellular uptake of DNA due to a decrease in net negative charge and also condensation of the DNA [4]. The polycationic polymer, polyethyleneimine (PEI), has been shown to condense DNA and is an effective transfection reagent [5–7].

Attachment of specific ligands to the surface of particles is a strategy for targeted delivery of DNA–polymer complexes to the cell types in which gene expression is most likely to elicit an immune response, especially DCs. An increased mucosal and systemic immune response has been demonstrated after combination of a DNA vaccine with the reovirus 3 attachment protein σ1 as a targeting ligand that allows DNA uptake by mucosal M cells [8,9]. M cells are responsible for sampling the gut lumen for antigens, thus, mediating antigen presentation to gut mucosal inductive tissue. Many enteric pathogens exploit M cells as means to gain entry into the host [10,11]. Reovirus is an enteric pathogen and infects the host following attachment to mucosal M cells [12,13]. Reovirus interactions with M cell surface receptors occur via the attachment protein σ1, which is expressed and located at the 12 vertices of the viral icosahedron representing the tips of the spike [12]. The σ1 attachment protein has been shown by crystallographic studies to consist of a C-terminal compact head domain that is conserved among different reovirus isolates and includes the binding site for junctional adhesion molecule 1 (JAM-1; also known as JAM-A) and an N-terminal fibrous tail that allows the natural σ1 protein to fold into a trimer and includes carbohydrate-binding domains that recognize sialic acid residues [14,15].

One of the major limitations in non-viral gene transfer efficiency is the entry of plasmid DNA from the cytoplasm into the nucleus to transfect the cells. Trafficking of nuclear proteins from the cytoplasm into the nucleus through nuclear pore complexes is mediated by nuclear localization sequences (NLS). The first NLS that was described is the simian cancer virus large T antigen derived sequence [16]. Several groups have demonstrated that the attachment of a SV40 nuclear localization sequence (SV40 NLS) either directly to DNA or to polymers that form complexes with DNA lead to an increased nuclear import of the DNA resulting in enhanced protein expression [17–19]. It is believed that the addition of nuclear localization sequences (NLS) allows larger DNA particles to be imported into the nucleus via specific interactions with and uptake through the nuclear import machinery in an importin-mediated manner [18]. Sequences flanking the minimal SV40 NLS sequence may also facilitate nuclear import [19].

In the present study, a combined targeting ligand DNA delivery system was developed consisting of recombinant reovirus type 3 σ1 attachment protein modified with a NLS (σ1-NLS), covalently conjugated to the polycation PEI, and complexed with a plasmid encoding for luciferase DNA, as a model DNA vaccine. This system was validated by in vitro studies demonstrating that PEI conjugated to σ1-NLS performed substantially better as a transfection agent than either PEI alone or PEI conjugated to σ1. This combined targeting ligand DNA delivery system has the potential to improve the in vivo performance of DNA vaccines.

2. Materials and methods

2.1. Expression of fusion proteins encoding σ1 and σ1-NLS

A plasmid (D4/6) containing a 1416 bp full length cDNA encoding the σ1 protein encoded by serotype 3 Dearing reovirus was kindly provided by Dr. Terence Dermody (Vanderbilt University, Nashville, TN). PCR amplification of D4/6 was used to clone the σ1 cDNA flanked by *Eco*RI on the 5′ end and *Hin*dIII on the 3′ end into pGEM-T Easy (Promega, Madison, WI). A modified σ1 cDNA with an in-frame insertion of a nuclear localization sequence derived from SV40 large T antigen (PKKKRKV) just N-terminal to the initial methionine of the σ1 cDNA coding sequence was also prepared by PCR. The inserts were sequenced in their entirety. The cloned PCR products were subcloned into the pET30a bacterial expression vector (EMD Biosciences, San Diego, CA) to create pET30a-σ1 and pET30a-σ1-NLS. The σ1 and σ1-NLS fusion proteins with a His-tag were expressed in the BL21 strain of *Escherichia coli* (Stratagene, La Jolla, CA). The fusion proteins were purified by nickel affinity chromatography using denaturing conditions (8 M urea) and His·Bind columns (EMD Biosciences, San Diego, CA), followed by refolding by dialysis into PBS. Protein concentration for the fusion protein preparations was determined by BCA assay (Pierce, Rockford, IL).

2.2. Expression of a mutant σ1-NLS fusion protein

Substitution of an asparagine for one of the lysines in the SV40 NLS has been shown previously to prevent this sequence from promoting nuclear localization [20]. A mutant form of the σ1-NLS in which the PKKKRKV sequence was changed to PKKNRKV was prepared by site directed mutagenesis of pET30-σ1-NLS using the QuikChange II kit (Stratagene). σ1-NLS-mutant protein was expressed in BL21 cells and purified as described for σ1 and σ1-NLS protein.

2.3. Antibodies

Hybridoma cells producing the neutralizing mouse monoclonal antibody 9BG5 specific for σ1 protein from serotype 3 reovirus were purchased from ATCC (Manassas, VA). The 9BG5 antibody binds to the C-terminal head region of σ1 and blocks interaction of σ1 with the JAM-1 cell surface receptor [15,21]. The antibody was used in the form of hybridoma supernatant or purified IgG prepared by Protein G Sepharose affinity chromatography. A rat IgG antibody of irrelevant specificity (2.4G2) was used as a negative control for experiments testing binding of 9BG5 to σ1 fusion protein. A mouse monoclonal antibody recognizing the His-tag epitope tag (BD PharMingen, San Diego, CA) was used for immunological

detection of the His-tagged fusion proteins. Polyclonal antisera to the pET30a-σ1-encoded fusion protein were prepared in groups of BALB/c mice (Jackson Laboratories, Bar Habor, ME) and Wistar rats (Charles River Laboratories, Wilmington, MA) by intradermal immunization with 5 μg of fusion protein emulsified in TiterMax Gold adjuvant (Sigma). Secondary antibodies used included goat anti-mouse IgG labeled with horseradish peroxidase (HRP) or FITC (Southern Biotechnology, Birmingham, AL), and goat anti-rat IgG labeled with HRP (Santa Cruz Biotechnology, Santa Cruz, CA).

2.4. Immunoassays for detection of antibody or JAM-1 binding to σ1/σ1-NLS fusion proteins and PEI-σ1/PEI-σ1-NLS fusion proteins

For detection of antibody binding to σ1 and σ1-NLS by ELISA, 96 well Maxisorb microplates (Nunc, Rochester, NY) were coated overnight at 4 °C with 1 μg of recombinant σ1 or σ1-NLS or with 10 μg of PEI-σ1 or PEI-σ1-NLS, respectively, in 100 μl of 0.015 M carbonate buffer (pH 9.6). Plates were blocked with 200 μl of 1% (w/v) bovine serum albumin (BSA) (Sigma) in phosphate-buffered saline (PBS, Cellgro, Herndon, VA) for 1 h at 37 °C. After three washes with 0.05% (v/v) Tween 20 (Fisher, Fair Lawn, NJ) in PBS (wash buffer), 100 μl of diluted primary antibody was added in 0.5% BSA in PBS (sample dilution buffer) and incubated for 60–90 min at 37 °C. After washing three times with wash buffer, 50 μl of the appropriate secondary reagent (diluted 1 : 1000 in sample dilution buffer) was added and incubated for 60 min at 37 °C. Following three more washes, 100 μl of 3,3′,5,5′ tetramethylbenzidine (TMB) substrate solution (BD PharMingen) was added to each well. After color development, the absorbances were read at 650 nm on a Spectramax plate reader (Molecular Devices, Sunnydale, CA). To study the interaction of recombinant σ1 or σ1-NLS with human JAM-1, a binding assay was developed using biotinylated recombinant human JAM-1 kindly provided by Dr. Charles Parkos (Emory University, Atlanta). The recombinant JAM-1 was prepared by thrombin cleavage of a GST-JAM-1 fusion protein expressed in bacteria to release the extracellular portion of JAM-1 [22]. The protocol for this binding assay was the same as described above for the antibody binding ELISA, except that 100 μl of recombinant JAM-1 (0.5 μg/ml) was used as the primary detection agent and 50 μl of 1 : 1000 diluted HRP-conjugated streptavidin (Caltag, Burlingame, CA) was used as the secondary reagent.

2.5. Flow cytometry

Flow cytometry was used to analyze the interaction of the σ1 and σ1-NLS fusion proteins or PEI-σ1-NLS complex with murine L929 fibroblast cells (ATCC) that constitutively express receptors that can bind σ1 from type 3 reovirus [23]. L929 cells grown in complete Dulbecco's Modification of Eagle's Medium (DMEM) (Cellgro) were detached from tissue culture flasks by the addition of 10 mM EDTA for 30 min. The cells were left untreated or treated with 1 μg of σ1 or σ1-NLS fusion protein, or 10 μg of PEI-conjugated σ1-NLS, respectively, in 150 μl of sample dilution buffer or 1 μg of σ1 that was first pre-incubated with 50 μl of 9BG5 hybridoma supernatant for 15 min. After a 20–30 min incubation at 4 °C, the cells were washed with FACS buffer (PBS with 0.2% BSA and 0.02% sodium azide), and 100 μl of the monoclonal anti His-tag antibody was added (1 : 1000 dilution) and incubated for 20–30 min at 4 °C. After washing with FACS buffer, 50 μl of 1 : 1000 diluted goat anti mouse IgG-FITC was added and incubated at 4 °C for 15 min. Cells were washed and analyzed using a FACS Calibur flow cytometer (BDBiosciences, San Jose, CA).

2.6. Cell loading with recombinant protein and analysis of nuclear vs. cytoplasmic partitioning

L929 cells and CHO-K1 cells (also from ATCC) maintained in complete DMEM were loaded with recombinant proteins using ProteoJuice transfection reagent (EMD Bioscience). Cells at approximately 80% confluency in 100 mm dishes were loaded by incubating 165 μl of serum free DMEM with 15 μl of ProteoJuice and 150 μl of σ1, σ1-NLS, or σ1-mutant NLS fusion protein (15 μg of each) on a rotator for 20 min to form complexes. This mixture was added dropwise to cells washed four times with serum-free DMEM before addition of 2.7 ml of serum-free DMEM. Following a 4 h incubation, 7 ml of complete DMEM was added to each plate and the incubation continued for 2 more hours followed immediately by isolation of the nuclei. In some experiments, cells were pretreated with 2 μg/ml of the cell cycle inhibitor aphidicolin (Sigma) for 4 h to growth arrest the cells. Cell nuclei were isolated using the Nuclei EZ Prep nuclear isolation kit (Sigma) according to the manufacturer's recommendations. Ice-cold Nuclei EZ lysis buffer was added to plates of washed cells and the cells were harvested with a cell scraper. Nuclei were separated from the cytosolic fraction by centrifugation at 500 ×*g* for 5 min at 4 °C. Nuclear lysates were prepared from nuclei (centrifuged at 16,000 ×*g* at 4 °C for 5 min) resuspended in nuclear lysis buffer (20 mM HEPES–KOH, pH 7.9, 1.5 mM $MgCl_2$, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 1 : 100 dilution of protease inhibitor cocktail, Sigma) and incubated on ice for 30 min. The mixture was cleared of debris by centrifugation, dialyzed against PBS, and analyzed for the concentration of σ1, σ1-NLS, or σ1-mutant NLS using a quantitative ELISA assay.

For the quantitative ELISA assay, wells of 96-well microplates were coated with 0.2 μg of the monoclonal anti-His-tag antibody in 100 μl of carbonate buffer overnight at 37 °C. After blocking with 200 μl of 1% BSA in PBS and 3 washes with wash buffer, 100 μl of σ1, σ1-NLS, or σ1-mutant NLS-containing nuclear lysates or cytosolic fraction samples were added and incubated at 37 °C for 60–90 min. After 3 washes, 100 μl of a 1 : 500 dilution of polyclonal anti-rat σ1 serum was added and incubated at 37 °C for 60–90 min. After 3 washes, 50 μl of 1 : 1000 goat anti-rat IgG-HRP was added and incubated for another 60 min at 37 °C. Following 3 wash steps, secondary antibody binding was detected by adding 100 μL of TMB substrate solution and reading absorbance at 650 nm after 10 min of development. A standard curve was prepared using known concentrations of purified recombinant σ1 protein.

2.7. Conjugation of σ1 fusion proteins to polyethyleneimine

The recombinant proteins σ1, σ1-NLS, or σ1-mutant NLS were covalently conjugated to the linear polycationic polymer polyethyleneimine (PEI, 50% (w/v) solution with MW of approximately 60 kDa, Sigma) via carboxyl groups on the fusion proteins and free amines on the polymer molecule using the carboxyl-reactive cross linking reagent 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride (EDC, Pierce). The two reactants, PEI and EDC, were diluted to 1 mg/ml each in 0.1 M 2-[N-morpholino]ethane sulfonic acid (MES) containing 0.9% NaCl, at pH 4.7 (BupH MES Buffered Saline Pack, Pierce). In a microfuge tube, 400 µl of protein solution (σ1, σ1-NLS, or σ1-mutant NLS at 100 µg/ml in MES) was added to 200 µl of polymer at a polymer to protein ratio of 5 : 1 (unless specified differently in the text) with gentle vortexing. Next, 100 µl EDC was added with gentle vortexing and the reaction mixture incubated at room temperature on a rotator for 2 h. Unreacted reagents were removed by dialysis against 50 ml PBS buffer overnight at 4 °C with 2–3 buffer exchanges. The molar ratio of PEI to protein in the resulting conjugates was determined using a combination of A280 measurements to detect aromatic amino acids (tryptophan) in the fusion protein and the ninhydrin reaction to detect primary amines in the polymer.

2.8. Gel retardation assays

Varying amounts of conjugates of PEI to σ1-NLS were added to 100 µl of serum-free DMEM in a microfuge tube. Then 0.5 µg of pGL3 Control plasmid DNA (Promega) encoding for firefly luciferase was added and incubated for 20 min at room temperature on a rotator. Sample aliquots were loaded into wells of 0.5 µg/ml ethidium bromide-containing 0.8% agarose gel and were electrophoresed at 100 V for 50 min. Bands corresponding to plasmid DNA were detected under UV light. Different amounts of PEI–protein conjugate were added to a

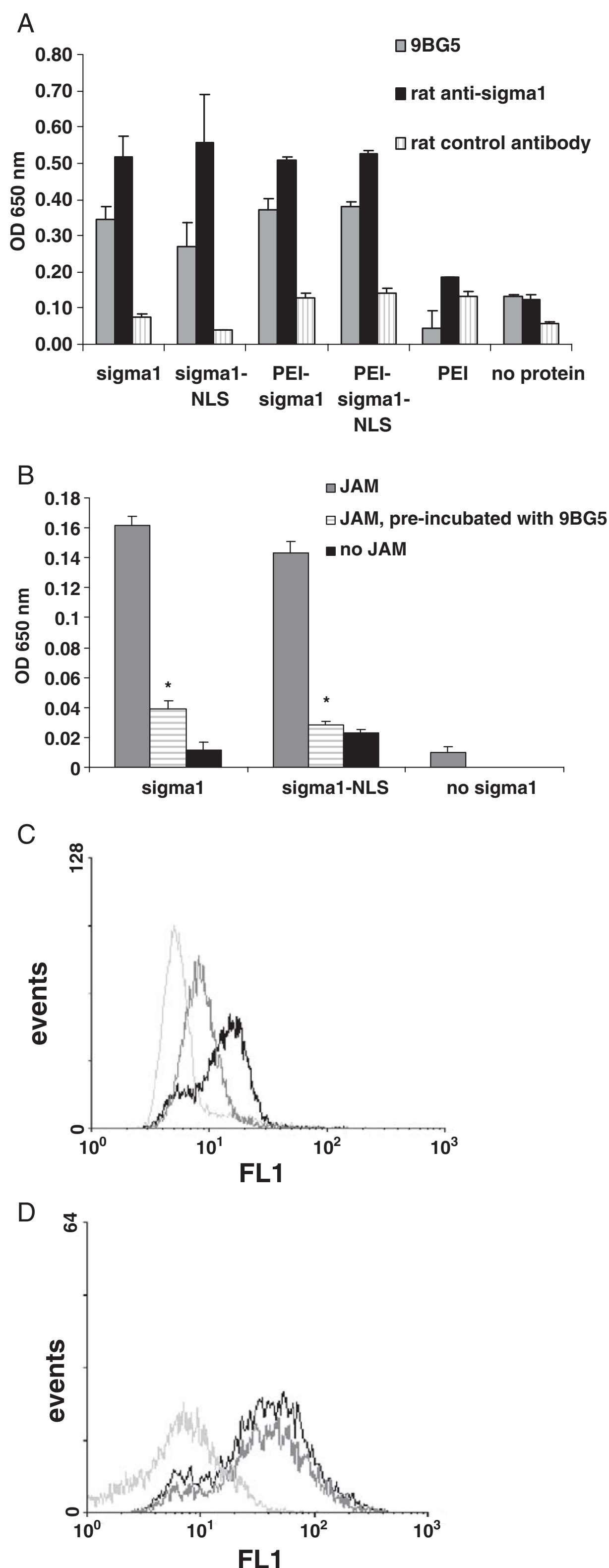

Fig. 1. Structural integrity of the recombinant proteins σ1 and σ1-NLS. (A) Antibody recognition of the recombinant proteins σ1 and σ1-NLS as well as σ1 and σ1-NLS conjugated to PEI in ELISA assays as compared to the controls of PEI and no protein. Plates were coated with 1 µg σ1 or σ1-NLS or 10 µg PEI-σ1 or PEI-σ1-NLS, respectively, blocked with 1% BSA, and σ1-specific monoclonal antibody 9BG5 or polyclonal rat anti-reovirus antibody were added. Detection was performed with goat anti-mouse IgG-HRP or goat anti-rat IgG-HRP using TMB as a substrate with absorbances read at 650 nm. Data are mean ± S.D. for $n = 3$ replicates. (B) Binding of σ1 and σ1-NLS to JAM-1. Plates were coated with 1 µg σ1, blocked with 1% BSA and incubated with biotinylated recombinant human JAM-1. For detection, streptavidin-HRP was added and TMB was used as substrate with absorbances read at 650 nm. Binding was blocked by pre-incubating σ1 with the σ1-specific antibody, 9BG5. Data are mean ± S.D. for $n = 3$ replicates. *Significantly different from JAM group. (C) Flow cytometric histogram of recombinant σ1 binding to L929 cells. L929 cells were incubated with σ1 or σ1 pretreated with 9BG5 monoclonal antibody or without σ1 (negative control), respectively, and detected with the monoclonal anti His-tag antibody and FITC-goat anti-mouse IgG. Data shown is representative of three separate experiments. Line of lightest color intensity corresponds to no σ1 negative control, line of darkest color intensity corresponds to σ1, line of medium color intensity corresponds to σ1 pretreated with 9BG5. (D) Flow cytometric histogram of recombinant σ1-NLS as well as PEI-σ1-NLS binding to L929 cells. L929 cells were incubated with σ1–NLS, or PEI-σ1-NLS, or without σ1-NLS (negative control), respectively, and detected with the monoclonal anti His-tag antibody and FITC-goat anti-mouse IgG. Line of lightest color intensity corresponds to no σ1-NLS negative control, line of medium color intensity corresponds to σ1-NLS, line of darkest color intensity corresponds to PEI-σ1-NLS.

constant amount of plasmid DNA to provide a range of PEI–nitrogen to DNA–phosphate (N / P) ratios from 0.25 to 4.0. The N / P ratio was calculated based on 330 and 43 Da per charge for DNA and PEI, respectively [24].

2.9. Transfection of L929 cells with DNA encoding for luciferase

One day before transfection, 1×10^5 L929 cells were plated in wells of a 24 well plate containing 0.5 ml of media. PEI conjugated to σ1, σ1-NLS, or σ1-mutant NLS (0.55 μg) or 20 μl of FuGENE 6 (Roche Diagnostics, Indianapolis, IN) as a positive control were added to 500 μl of serum-free DMEM in a microfuge tube. Then 0.5 μg of pGL3 Control plasmid DNA (Promega) encoding for firefly luciferase was added, gently vortexed, and incubated for 20 min at room temperature on a rotator. Prior to transfection, the cells were washed several times with PBS and serum-free DMEM and the transfection mixtures added to the cells and incubated for 4 h at 37 °C. After 4 h, the supernatant was aspirated and 500 μl of complete DMEM was added. Luciferase expression was measured after 48 hours of further incubation using the Luciferase Assay System (Promega) and luciferase activity measured in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

2.10. Data analysis

Statistical analysis was performed using ANOVA with the general linear model routine in Minitab software (Release 13, Minitab Inc., State College, PA). p-values of ≤ 0.02 were considered to be statistically significant.

3. Results

3.1. σ1 and σ1-NLS fusion proteins are recognized by antibodies to σ1

ELISA assays were performed to determine if the recombinant σ1 and σ1-NLS fusion proteins could be detected by a σ1-specific monoclonal antibody (9BG5) directed against an epitope in the head region critical for viral binding to JAM-1 (Fig. 1A). The 9BG5 antibody bound to the recombinant σ1 and σ1-NLS fusion proteins. The fusion proteins also reacted with a polyclonal rat anti-σ1 antibody raised against the σ1 fusion protein. No significant differences in antibody binding were observed between σ1 and σ1-NLS. Conjugation of recombinant σ1 and σ1-NLS to PEI did not significantly alter their ability to bind to 9BG5 or the polyclonal rat anti-σ1 antibody (Fig. 1A).

3.2. Binding of σ1 and σ1-NLS to soluble JAM-1 and to L929 cells

Native reovirus type 3 σ1 protein binds to several M cell surface receptors, one of which is JAM-1 [25,26]. To investigate whether the recombinant σ1 and σ1-NLS fusion proteins retained the ability to bind to JAM-1, wells coated with σ1 or σ1-NLS were incubated with the soluble extracellular portion of human JAM-1 labeled with biotin (Fig. 1B). Biotinylated JAM-1 bound to both σ1 and σ1-NLS, and the binding was inhibited by the 9BG5 monoclonal antibody which binds to the same head region of the σ1 protein containing the JAM-1-binding domain.

The binding of recombinant σ1 to mouse L929 cells was analyzed by flow cytometry since L929 cells is a cell line known to express multiple plasma membrane proteins capable of binding native reovirus σ1 protein [23]. Binding of recombinant σ1 or σ1-NLS to NLS cells was detected with a His-tag specific monoclonal antibody and FITC-conjugated goat anti-mouse IgG (Fig. 1C and D, respectively). Pretreatment of the σ1 with the blocking mouse monoclonal antibody 9BG5, resulted in a slight decrease in σ1 binding to L929 cells. PEI-conjugated σ1-NLS

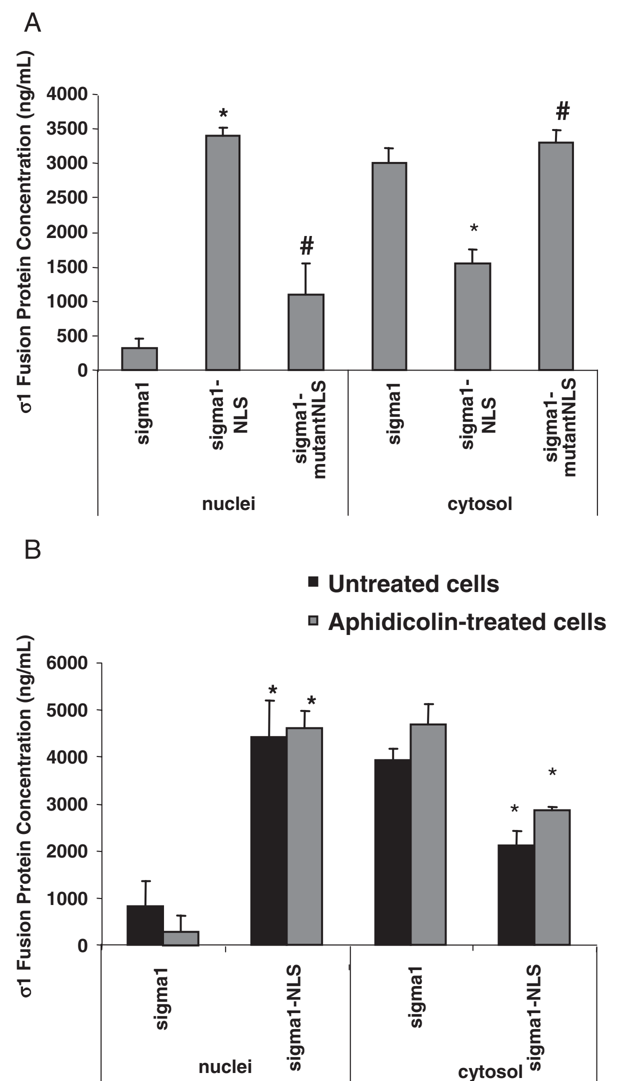

Fig. 2. Selective partitioning of σ1-NLS to nuclear fractions. L929 (A) or CHO-K1 (B) cells were loaded with σ1 or σ1-NLS by protein transfection. The L929 cells were alternatively loaded with σ1-mutant NLS as a fusion protein control (A). After 4 h of transfection and 2 h of subsequent incubation, nuclear lysates and cytosolic fractions were collected and analyzed for the concentration of σ1, σ1-NLS, or σ1-mutant NLS by a quantitative ELISA assay. In (B), CHO K1 cells were preincubated with 2 μg/ml aphidicolin to induce growth arrest. Data represents mean ± S.D. for $n = 3$ replicates. *: Significantly different from σ1; #: significantly different from σ1-NLS.

Table 1

Sample	Molar ratio*	Normalized luciferase activity
PEI		2
FuGENE6		249
PEI-σ1	1 : 1	1
	2.5 : 1	10
	5 : 1	69
	10 : 1	10
PEI-σ1-NLS	1 : 1	1
	2.5 : 1	3
	5 : 1	160
	10 : 1	97

*Molar ratio=Polymer : Fusion protein ratio.
Gene expression in cells transfected by complexes of pGL3 Control DNA with PEI, Fugene, or PEI conjugated with σ1 or σ1-NLS at different PEI to protein molar ratios. PEI and σ1 or σ1-NLS were mixed at different ratios in a microfuge tube. One hundred μl EDC was added with gentle vortexing and the reaction mixture incubated at room temperature on a rotator for 2 h. Unreacted reagents were removed by dialysis against 50 ml PBS buffer overnight at 4 °C with 2–3 buffer exchanges. Transfection agents were used to transfect L929 cells and luciferase expression levels were normalized to the luciferase expression level obtained using naked plasmid DNA.

bound to L929 cells in a similar extent as the unconjugated recombinant protein (Fig. 1D).

3.3. Improved partitioning of recombinant σ1-NLS to cell nucleus compared to unmodified σ1

The partitioning of σ1 and σ1-NLS to the nuclear and cytosolic factions was compared in cells transfected using a liposome reagent (ProteoJuice) optimized for protein transfection. A sandwich ELISA assay specific for σ1 was developed and standard curves with purified σ1 and σ1-NLS showed that this assay detected σ1 protein concentrations between 0.2 and 20 μg/ml (data not shown). Following transfection of L929 cells (Fig. 2A) or CHO-K1 cells (Fig. 2B) with the two proteins, the σ1 concentration in the nuclear lysates was significantly higher after loading cells with σ1-NLS as compared to loading cells with σ1. Transfer of the σ1-mutant NLS fusion protein to the nucleus was far less than for σ1-NLS (Fig. 2A), confirming that the single mutation in the NLS was sufficient to block most of the nuclear localization effect. Pretreatment of CHO-K1 cells with aphidicolin to trigger growth arrest did not significantly change the partitioning of the recombinant proteins (Fig. 2B). For cells loaded with σ1-NLS, the σ1 concentration in the nuclear lysates was 5.3 (CHO-K1) or 10.5 (L929) times higher than the concentration in cells loaded with unmodified σ1. Of the σ1-NLS protein that was recovered from CHO-K1 cells (Fig. 2B), 68% (62% in growth arrested cells) was found in the nucleus compared to only 20% (6% in growth arrested cells) in cells loaded with σ1. This trend was similar in L929 cells (Fig. 2A), where 69% of the σ1-NLS protein was detected in the nucleus and only 10% of σ1 was found in the nucleus.

3.4. Optimal ratio of PEI to recombinant protein in conjugates for cell transfection

The recombinant proteins were conjugated to PEI via protein carboxyl groups using the carboxyl reactive cross-linking reagent 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC). Different ratios of PEI to σ1 and σ1-NLS were used for conjugation and luciferase expression was compared after transfection of L929 cells with those different constructs (Table 1). For both PEI-σ1 and PEI-σ1-NLS, optimal transfection efficiency was observed when a polymer to protein molar ratio of 5 : 1 was used. Based on charge ratios, we estimate that a single DNA plasmid is on average complexing with about ten molecules of PEI. At a polymer to protein ratio of 5 : 1, an average of two σ1-derivatized PEI molecules are predicted to be associated with each copy of plasmid included in the polyplex.

3.5. Conjugation of σ1 fusion proteins to PEI

Complex formation between DNA and the polymer–protein conjugates was assessed by gel retardation assays. The effect of increasing concentrations of PEI within the PEI-σ1-NLS complex on the electrophoretic migration of pGL3 plasmid was investigated in a 0.8% agarose gel (Fig. 3). At an N / P ratio of 2.0 (lane 5, Fig. 3) the plasmid's mobility was retarded, indicating that it was retained within the polymer complexes preventing it from migrating into the agarose gel. This ratio was considered optimal and was subsequently used in transfection experiments. The viability of L929 cells was not affected by the PEI-σ1-NLS conjugate at the concentration of 1.1 μg/ml used for transfections as demonstrated by cell viability assays measuring trypan blue exclusion after 48 h of co-culture with the conjugates (data not shown).

3.6. Transfection of L929 cells with PEI conjugated with σ1-NLS

The PEI-σ1-NLS–DNA complexes were assessed for in vitro transfection efficiency in L929 cells and compared to the controls of PEI-σ1-mutant NLS–DNA, PEI-σ1-DNA, PEI–DNA, naked plasmid DNA, and a commercially available

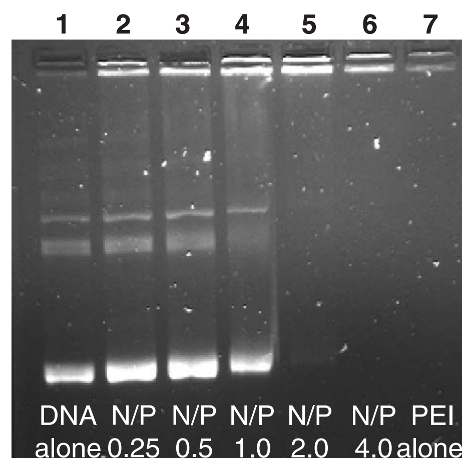

Fig. 3. Gel retardation assay. Agarose gel electrophoresis of PEI-σ1–NLS–DNA complexes at varying N / P (DNA / polymer) ratios compared to the controls of DNA alone and PEI alone. Increasing amounts of PEI were added to 0.5 μg of DNA. Detection was performed by ethidium bromide staining.

transfection reagent (FuGENE 6). In each experiment performed, the luciferase signals obtained were normalized to the luciferase signal obtained using naked plasmid DNA. As shown in Fig. 4A, transfection of cells with PEI-σ1–DNA complexes resulted in a luciferase expression level 3-fold higher than that observed for cells transfected with PEI–DNA. Cells transfected with PEI-σ1-NLS–DNA complexes demonstrated an average 16-fold increase in luciferase activity compared to cells transfected with PEI–DNA. The addition of the NLS to the σ1 protein increased the resultant cell transfection rates significantly over the PEI-σ1-DNA complex. Pretreatment of L929 cells with aphidicolin to growth arrest the cells did not significantly change the transfection rates (Fig. 4A). Transfection with PEI-σ1-mutant NLS–DNA complexes resulted in a significantly lower luciferase expression than transfection with PEI-σ1-NLS–DNA complexes (Fig. 4B).

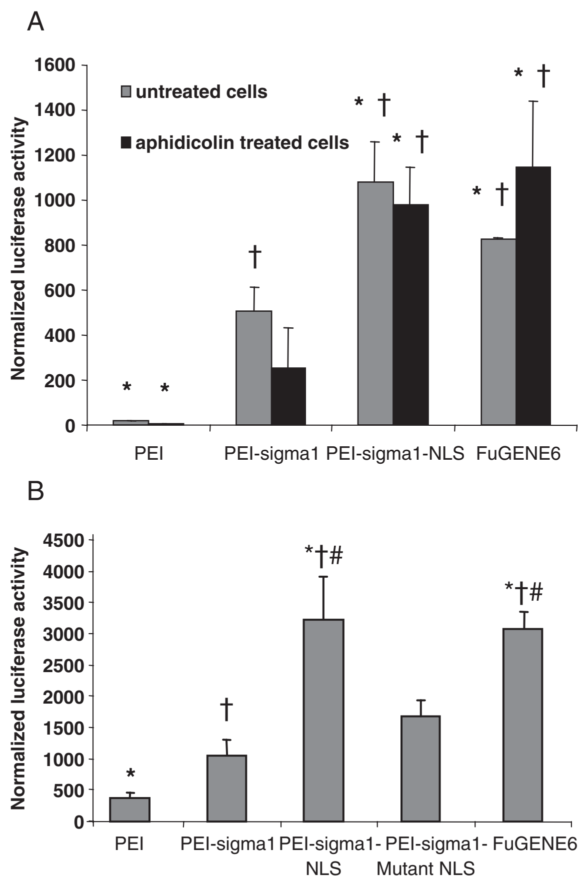

Fig. 4. Gene expression in cells transfected by complexes of DNA with PEI-conjugated σ1 or σ1-NLS showing effect of growth arrest (A) and transfection with σ1-mutant NLS as a fusion protein control (B). Complexes of plasmid DNA with PEI, PEI-σ1, PEI-σ1-NLS, FuGENE 6 (A, B) or PEI-σ1-mutant NLS (B) were prepared using plasmid DNA from a luciferase expression vector (pGL3 Control). In (A), L929 cells were preincubated with 2 μg/ml aphidicolin to induce growth arrest. L929 cells were transfected with the various reagents for 4 h and luciferase expression was assayed after 48 h of incubation at 37 °C. For each individual experiment, the luminometric data were normalized to the luciferase expression level obtained using naked plasmid DNA. The mean ± S.D. is presented for the data from a total of 3 experiments; *: significantly different from PEI-σ1 group, #: significantly different from PEI-σ1-mutant NLS group; †: significantly different from PEI-group.

4. Discussion

The present study demonstrates that a bifunctional targeting ligand consisting of recombinant reovirus type 3 σ1 attachment protein modified with a SV40-derived NLS can be covalently attached to the polycation PEI to create an efficient DNA delivery vehicle. This targeting ligand DNA delivery system can achieve significantly higher gene expression levels in vitro compared to using either PEI alone or PEI attached to σ1 without the NLS for transfection of the same DNA expression vector. The addition to DNA of proteins or small molecules that interact with cell surface receptors on specific cells has been previously used as a targeted gene delivery strategy to facilitate cellular uptake of DNA. The first example of such a targeted gene delivery system was the cross linking of asialoglycoprotein to poly-L-lysine for targeting of galactose receptors on hepatocytes [27]. Another laboratory has previously described a DNA vaccine delivery system based on use of a fusion protein between maltose binding protein and reovirus σ1 as a targeting ligand and using polylysine as a polycation carrier [28]. This DNA delivery system was shown to increase gene expression levels in vitro and boost antibody and CD8 T cell responses in vivo following intranasal immunization with a DNA vaccine [8,9].

Our studies confirm that a nonglycosylated form of reovirus σ1 expressed in bacteria provides an effective targeting ligand for DNA delivery into cells. We have also shown that insertion of a short NLS into a σ1 fusion protein confers enhanced partitioning to the cell nucleus and does not interfere with binding of σ1 to recombinant JAM-1 or cells that bind unmodified σ1. Nuclear localization sequences, covalently or non-covalently conjugated to DNA or to DNA complexing polymers, have been shown to increase the DNA delivery into the nucleus [6,17,18] by using the nuclear import machinery [29]. Our protein transfection data indicate that the enhanced nuclear delivery is responsible for the increased activity of our σ1-NLS targeting ligand when used as a delivery vehicle for in vitro gene expression studies. To our knowledge, this is the first example of inserting a short NLS peptide into the sequence of a viral attachment protein to create a novel bifunctional targeting ligand that will specifically bind to plasma membrane cell surface receptors when located outside of the cell and engage the nuclear import machinery for enhanced nuclear translocation after uptake into cells.

Our σ1-based DNA delivery vehicle used PEI as the polycation for DNA condensation rather than polylysine, which was used by Wu et al. for their σ1-based system of DNA vaccine delivery [28]. A variety of polycationic polymers or modifications thereof [30–32], cyclodextrins [33], and dendrimers [34] have been used to increase gene delivery by forming complexes and condensing DNA, thus protecting it from degradation and facilitating cellular uptake. In our experiments with the σ1-NLS targeting ligand, PEI was a better attachment matrix for DNA delivery than two other polycations that we tested (polylysine and polyornithine; data not shown). We and others [5] demonstrated that PEI forms complexes with DNA that condense and protect DNA and can also be used as a scaffold for the covalent attachment of targeting ligands. In vitro transcription assays

have demonstrated that PEI–DNA complexes are transcribed as effectively as naked DNA [35]. Morphological studies of cells that have internalized PEI–DNA complexes have shown that most of these complexes end up in an endocytic compartment rather than the nucleus where PEI aggregates are difficult to detect [35]. These studies indicate that the rate limiting step in cellular transfection by PEI–DNA complexes is transfer of the complexes from the endosomal compartment into the nucleus. Our σ1-NLS targeting ligand is equipped to increase the efficiency of this rate-limiting step. Another benefit of using PEI is that it functions as a "proton sponge" [5]. After uptake of PEI–DNA complexes into the endosomal compartment, PEI will buffer the acidic pH, protecting the DNA from degradation and causing a swelling of the vesicles and release of the PEI–DNA complexes into the cytoplasm. After reaching the cytoplasm, the NLS on our σ1-NLS targeting ligand can engage the nuclear import machinery located at the nuclear membrane. This nuclear localization was shown to be NLS specific, since insertion of a point mutation that impairs the NLS function showed decreased transfection efficiency. This result demonstrates that the effect observed on adding of the NLS moiety to the σ1 fusion protein is not merely a result of adding positively charged amino acids to the molecule when adding the NLS, but is the result of specific transport via the nuclear import machinery.

One issue to be examined in future studies on this DNA vaccine delivery system is the intracellular trafficking of complexes between plasmid DNA and PEI or targeting ligand modified-PEI. By using fluorescently labeled forms of targeting ligand and DNA, the fate of PEI-σ1 NLS–DNA complexes within individual cells can be analyzed using fluorescence video microscopy and confocal microscopy. These results should provide further insights into how incorporation of the NLS in the targeting ligand affects the cellular trafficking of the various components of the combined targeted DNA delivery system. The next step to demonstrate the utility of this DNA vaccine delivery system will be in vivo murine studies in which the immune responses elicited by intradermal, intramuscular, or mucosal routes of administration of plasmid DNA complexed with PEI-σ1-NLS or PEI-σ1 will be compared to the immune responses elicited by naked DNA and DNA condensed by PEI without a targeting ligand.

5. Conclusions

A bifunctional fusion protein consisting of recombinant reovirus type 3 σ1 attachment protein incorporating a 7 amino acid NLS is an efficient targeting ligand when covalently attached to PEI and used for delivery of a model DNA vaccine. Nuclear uptake of σ1-NLS was enhanced compared to unmodified σ1 when the purified fusion proteins were transfected into cells. Plasmid DNA complexed with the PEI-σ1-NLS delivery vehicle resulted in substantially greater levels of gene expression in vitro using a luciferase expression system. The PEI-σ1-NLS delivery vehicle is an improvement on DNA vaccine delivery strategies based on use of viral attachment proteins as targeting ligands and is predicted to elicit enhanced immune responses in vivo to antigens encoded by DNA vaccines.

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