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N-Glycan Targeted Gene Delivery to the Dendritic Cell SIGN Receptor

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Abstract

A novel non-viral gene delivery vector composed of a high mannose N-glycan conjugated to a polyacridine peptide was prepared. The glycopeptide was designed to bind to plasmid DNA by a combination of polyintercalation and ionic binding, and to the DC-SIGN (Dendritic Cell-Specific Intracellular adhesion molecule-3 Grabbing Non-integrin) receptor expressed on CHO cells by recognition of the high mannose N-glycan. The glycopeptide conjugate was prepared by purification of a high mannose N-glycan from affinity fractionated soybean agglutinin (SBA). The SBA was proteolyzed to release the N-glycan which was then modified on its N-terminus with Tyr and a propionate maleimide. A DNA binding polyacridine peptide, Cys-(Acr-Lys)₄, was prepared by solid phase peptide synthesis using Fmoc-Lys(Acr), then conjugated to the maleimide on the N-glycan to produce a glycopeptide. The glycopeptide bound to DNA with high affinity as determined by fluorophore displacement assay and DNA band shift on agarose gel. When bound to Cy5 labeled DNA, the glycopeptide mediated specific uptake in DC-SIGN CHO (+) cells as determined by FACS analysis. In vitro gene transfer studies established that the glycopeptide increased the specificity of gene transfer in DC-SIGN CHO (+) cells 100-fold relative to CHO (–) cells. These studies suggest that a high-mannose N-glycan conjugated to a polyacridine peptide may also facilitate receptor mediated gene delivery in dendritic cells and thereby find utility in the delivery of DNA vaccines

Introduction

Dendritic cells (DC) are the primary antigen presenting cells in the immune system, and are therefore central to the development of vaccines (1). One of the challenges in developing DNA vaccines is the design of vectors that efficiently and selectively target DCs. The DC-SIGN (Dendritic Cell-Specific Intracellular adhesion molecule-3 Grabbing Non-integrin) receptor is a cell surface endocytosing lectin that is specifically expressed on DCs that binds to glycoprotein N-glycans of the high-mannose or complex type possessing terminal Le^X with a μM K_d (2–4). The receptor is therefore ideally located to facilitate receptor mediated gene delivery to DCs.

Several earlier studies have targeted either immunogenic proteins or plasmid DNA to internalize into DCs via DC-SIGN in an attempt to prime the immune system (5–18). These

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studies establish that either encapsulation of DNA into mannosylated liposomes or complexation of DNA with mannosylated-PEI, mannosylated-chitosan, mannan-polylysine, or mannosylated glycolipid, results in gene delivery with improved immune response when administered i.m. or i.p (10–18). Other studies have determined that high-mannose N-glycans on remodeled glycoproteins are able to target DC-SIGN resulting in uptake into DCs and enhanced immune response (5–8, 19). While gene delivery to DCs with mannose containing polymers and liposomes has been achieved, no study has yet demonstrated the delivery of DNA to DCs using a natural high mannose N-glycan.

Reversible binding of an N-glycan to DNA can be accomplished by covalently linking it to a polylysine peptide resulting in cationic polyplex (20). However, polylysine peptides of 18 residues or longer are necessary to achieve sufficient affinity to protect DNA, and the resulting electropositive polyplexes bind non-specifically to numerous proteins in vivo (21, 22). To overcome these limitations, we recently demonstrated that short cationic peptides possessing multiple acridines attached to the side chain of Lys residues bind DNA with much higher affinity and protect DNA from metabolism compared to much longer polylysine peptides (19, 23, 24).

Functionalized polyacridine peptides, modified with either PEG or a fusogenic peptide, have been shown to mediate gene transfer in vitro or in vivo (19, 23). Earlier studies have also demonstrated the efficacy of a polyacridine polymer functionalized with either a nuclear localization signal (NLS) or a neoglycopeptide (25, 26). These experiments demonstrate the importance of increasing the DNA binding affinity by increasing the number of acridines. An early report by Ueyama also demonstrated that polyacridine peptides possessing two to five Lys(Acr) residues results in progressively higher affinity for calf thymus DNA (27).

To date, there have been no reports of the successful development and use of a polyacridine glycopeptide in gene transfer. Here, we report the purification of a high-mannose N-glycan from soybean agglutinin and its conjugation to a polyacridine peptide. The resulting glycopeptide binds to plasmid DNA with high affinity and mediates targeted uptake and gene expression in DC-SIGN expressing cells in vitro.

Materials and Methods

Sephacrose CL-4B, D-galactosamine HCl, 6-aminocaproic acid, Boc-tyrosine N-hydroxysuccinimide, 9-chloroacridine (97%), pronase, thiazole orange, phenol, super-DHB diisopropylethylamine, piperidine, triisopropylsilane (TIS), polyethylenimine (PEI), and acetic anhydride were from Sigma-Aldrich (St. Louis, MO). Sephadex G-50 resin was from Amersham Biosciences (Pittsburgh, PA). AG 50W-X2 cation exchange resin was from Bio-Rad (Hercules, CA). 1,1'-carbonyldiimidazole, and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide HCl were Fluka Biochemica products (St. Louis, MO). 3-maleimidopropionic acid N-hydroxysuccinimide ester was from ABD Bioquest, Inc (Sunnyvale, CA.). NuPAGE® Novex Bis-Tris Gels, Dulbecco's Modified Eagle Medium (DMEM), MEM non-essential amino acids, Dulbecco's phosphate buffered saline (DPBS) and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA). D₂O was from Cambridge Isotope Laboratories (Cambridge, MA). Untoasted soy flour was obtained from Archer Daniels Midland

(Decatur, Illinois). Sodium hydroxide was a Mallinckrodt product through Fisher Scientific (Pittsburg, PA). Fmoc-Lys-OH was from Novabiochem. Ethidium bromide was from Bio-Rad. Fmoc-Lys(Boc)-Wang resin, Fmoc-Lys(Boc)-OH, Fmoc-Cys(Trt)-OH, N-methyl-2-pyrrolidinone (NMP), O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluorophosphate (HBTU) and hydroxybenzotriazole hydrate (HOBt) were from Advanced ChemTech (Lexington, KY). N',N-dimethylformamide (DMF) was from Amresco (Solon, OH). Human DC-SIGN (+) and DC-SIGN (–) CHO cells were a gift from Drs. Chae Gyu Park and Ralph M. Steinman of the Laboratory of Cellular Immunology and Physiology at The Rockefeller University (28). Cy5TM *LabelIT* was from Mirus Biotechnology (Madison, WI). pGL3 control vector, a 5.3 kb luciferase plasmid containing an SV40 promoter and enhancer, was obtained from the Promega corporation (Madison, WI), amplified in DH5 α *E coli* from Invitrogen, and purified with a Giga kit from Qiagen (Valencia, CA). D-Luciferin was from Gold Biotechnology (St. Louis, MO), Adenosine 5'-triphosphate was from Roche (Indianapolis, IN), and BCA Protein Assay reagents were Pierce protein research products of Thermo Scientific (Rockford, IL). All reagents and solvents were certified ACS grade, molecular biology grade, or an equivalent.

Preparation of Sepharose N-caproylgalactosamine Affinity Resin

6-Aminocaproic acid was coupled to activated Sepharose CL-4B as described (29). Briefly, a moist cake (50 g) of Sepharose CL-4B was activated with 1,1'-carbonyldiimidazole (CDI) (1.2 g). After a 15 min reaction with CDI, the mixture was washed with 1 L of acetone, then reacted with 14 g of 6-aminocaproic acid in 90 ml of water. The pH of the slurry was adjusted to 10 with 1 M sodium hydroxide and stirred overnight at 4°C. Ligand coupling proceeded with the addition of 600 mg of galactosamine and 300 mg of CDI followed by reaction for 2 hrs (30). The affinity resin (30 ml) was washed with 90 ml each of 1 M sodium chloride, 1 M sodium chloride in 0.1 M Tris pH 8.6, 1 M sodium chloride in 50 mM sodium formate pH 3.0, 1 M sodium chloride in 0.1 M Tris pH 8.6, water, and PBS.

Purification of Soybean Agglutinin (SBA)

Soy flour (10 g) was dissolved in 200 ml of PBS and stirred overnight at 4°C. The slurry was centrifuged at $9,000 \times g$ for 15 min, and the supernatant was applied to the affinity column (2.5×10 cm). The column was washed with 90 ml PBS until the Abs_{280nm} of the effluent was below 0.02 units. The bound SBA was eluted with 90 ml of 0.2 M galactose in PBS (30). The SBA was pooled and dialyzed in 12–14 kDa MWCO tubing for 48 hrs against running water and freeze dried.

Purification of Man 9 from SBA

Affinity purified SBA was proteolytically digested to release Man 9 which was then purified following the method of Lis *et al* (31). Briefly, SBA (600 mg in 8 ml 1 mM hydrochloric acid) was heat denatured at 50°C for 15 min and digested with 12 mg of pronase for 48 hr at 37°C. The digest was centrifuged at $4,000 \times g$ and the supernatant applied to a Sephadex G-50 column (1.5×170 cm) eluted with 10 mM acetic acid. The fractions (8.5 ml) were monitored by Abs 280 nm and an aliquot of each fraction was analyzed for carbohydrate by the phenol sulfuric assay (32). Carbohydrate containing fractions were pooled, concentrated and re-purified by gel filtration on Sephadex G-50. Man 9 containing

fractionations were prepared in 1.5 ml of water then applied to a AG 50W-X2 cation exchange column (1.5×50 cm) eluted with 1.2 L of 1 v/v% pyridine/acetic acid, pH 3.2 while detecting carbohydrate by the phenol-sulfuric assay. The Man 9 containing fractions eluting a 880 ml were pooled and freeze dried. The recovery was estimated by phenol sulfuric assay to be 6.7 μ mol (45%) based on 18.5 nmol of Man₉GlcNAc₂Asn = 1 AU_{490nm}.

The purified Man 9 was analyzed by mass spectroscopy by preparing 2 nmol in 4 μ l of 10 mM sodium chloride containing 0.5 mM of super 2,5-DHB. The sample was spotted on the target and analyzed by MALDI-TOF (Biflex III, Bruker) in reflectron mode resulting in the detection of masses of 2019.72 and 2041.66 m/z corresponding to [M+Na] and [M + 2Na].

Man 9 was analyzed by high field NMR (600 MHz Varian) by preparing a 1 mM sample in D₂O with acetone as an internal standard. The chemical shift data corresponded with that published previously (33).

Synthesis of Cys-(Acr-Lys)₄

The nine amino acid peptide, Cys-(Acr-Lys)₄, was synthesized using standard Fmoc chemistry with HOBt and HBTU double coupling on a 30 μ mol scale with an APEX 396 solid phase peptide synthesizer using Fmoc-Lys(Acr) prepared as described previously (34). The peptide was cleaved from the resin and deprotected with 2 ml of TFA/TIS/H₂O (95:2.5:2.5 v/v/v) for 3 hr, followed by precipitation in 40 ml of cold ether. The precipitate was collected by centrifugation at $4,000 \times g$ for 10 min, and reconstituted in 0.1 v/v % TFA. The peptide was preparatively purified on RP-HPLC by injecting 2 μ mol onto a 2×25 cm Vydac C18 column eluted at 10 ml per min with 0.1% TFA and a 15–30% acetonitrile gradient over 45 min while detecting at Abs_{280nm}. The peptide was pooled, concentrated by rotary evaporation, reconstituted in 0.1 v/v % TFA, and then analyzed for purity by RP-HPLC, injecting 5 nmol onto a 0.47×25 cm Vydac C18 column eluted at 1 ml/min with 0.1% TFA and a gradient of 15–30% acetonitrile while detecting by positive mode ESI-MS. The doubly charged ion was detected with an m/z of 928.7, resulting in an observed mass of 1855.4 m/z (calc = 1855.5 m/z). The synthesis resulted in a 17% isolated yield ($\epsilon_{409\text{ nm}} = 37,064 \text{ M}^{-1} \text{ cm}^{-1}$) at >95% purity.

Synthesis of a Man 9 Glycopeptide

Man 9 (500 nmol) was prepared in 500 μ l of 50 v/v % DMF and 100 mM sodium bicarbonate containing Boc-Tyr-NHS (5 μ mol) and reacted for 3 hr at RT. The reaction mixture was directly applied to a Sephadex G-25 column (0.5×50 cm) eluted with 10 mM acetic acid while detecting Abs_{280nm}. The Man 9 Tyr-Boc product peak eluting at 50 ml was concentrated by rotary evaporation and reconstituted in water. The product was analyzed for purity by analytical RP-HPLC eluting at 1 ml per min with 0.1% TFA and a 1–30% gradient of acetonitrile over 30 min while detecting by Abs_{280nm} and by negative mode ESI-MS. The doubly charged ion was detected with an m/z of 1129.4, resulting in a mass of 2260.8 m/z (calculated 2260.0 m/z). The Man 9 Tyr-Boc product was >95% pure and the yield ($\epsilon_{280\text{ nm}} = 1130 \text{ M}^{-1} \text{ cm}^{-1}$) was 95%.

To conjugate Man 9 Tyr-Boc to Cys-(Acr-Lys)₄, the Boc group was removed from the Tyr and the amine terminus was derivatized with propionate maleimide, prior to reaction with Cys-(Acr-Lys)₄. The Boc group was removed by treating Man 9 Tyr-Boc (500 nmol) with 50 μ l of 95% TFA for 5 min at RT, followed by freeze drying to remove TFA. The yield of Man 9 Tyr was quantitative and LC-MS analysis established a mass of 2160.3 m/z consistent with the anticipated mass (2160.9 m/z). Man 9 Tyr (500 nmol) was reacted for 1 hr with 3-maleimidopropionic acid NHS ester (5 μ mol) in 250 μ l of 50 v/v% DMF and 100 mM sodium bicarbonate. The reaction mixture was fractionated on a Sephadex G-25 column (1.5 $\times$ 50 cm), eluting with 0.1% acetic acid while detecting Abs_{280 nm}. The product peak (Man 9 Mal), eluting at 35 ml, was pooled and concentrated by rotary evaporation and purified to homogeneity on a semi-prep Vydac C18 RP-HPLC column (2 $\times$ 25 cm) eluted at 10 ml per min with 0.1% acetic acid and a 30 min acetonitrile gradient (5–15%) resulting in a 23% isolated yield. LC-MS analysis in the negative mode revealed that the Man 9 Mal product produced an ion of 1156.1 m/z, resulting in a mass of 2314.2 m/z, closely corresponding to the anticipated mass of 2312.1.

To complete the glycopeptide synthesis, Man 9 Mal (500 nmol) was reacted with Cys-(Acr-Lys)₄ peptide (600 nmol) in 500 μ l of 5 mM Hepes pH 7 for 2 hrs. The Man 9 glycopeptide product was purified from excess dimeric peptide on analytical RP-HPLC with a 30 min 15–30% acetonitrile gradient in 0.1% TFA. The purity and identity of the Man 9 glycopeptide were verified by analytical RP-HPLC detected by Abs_{280 nm} and positive mode ESI-MS. The product peak was >95% pure and produced a triply charged ion detected with an m/z of 1390.0 that deconvoluted to an observed mass of 4167.0 m/z (calculated 4167.4 m/z). The recovery was 102 nmol, resulting in a 23% yield.

Man 9 Glycopeptide DNA Band Shift Assay

pGL3 (1 μ g) was combined with 0.05, 0.2, 0.5, and 1 nmol of Man 9 glycopeptide. Each sample was incubated for 30 min before loading onto an agarose gel, that was electrophoresed at 80 V for 60 min (24). The gel was stained with 0.1 mg per ml of ethidium bromide at 4°C for 24 hrs, then photographed while illuminating on a transilluminator.

Thiazole Orange Displacement Assay

A control sample of pGL3 (1 μ g) was combined with 40 nM thiazole orange in a total volume of 500 μ l of 5 mM Hepes buffer pH 7.4. The Man 9 glycopeptide (0.2, 0.3, 0.4, 1 and 2 nmol) or Cys-(Acr-Lys)₄ peptide was added to pGL3 (1 μ g) samples containing thiazole orange, and incubated for 30 min, followed by measuring the fluorescence intensity at excitation 500 nm and emission 530 nm (21). The percent fluorescence intensity relative to control was plotted versus the stoichiometry of glycopeptide or peptide added to DNA to determine the equivalence point.

Glycopeptide DNA Polyplex Binding to DC-SIGN (+) and (–) CHO Cells

pGL3 plasmid (5 μ g) was labeled with *LabelIT* according to the manufacturer's instructions to prepare Cy5-DNA. The Man 9 glycopeptide (0.5 nmol) was combined with 1 μ g Cy5-DNA in 60 μ l of water and incubated for 30 min to form a polyplex. DC-SIGN (+) and (–) CHO cells (1×10^6) (28) were each plated in triplicate in 35 mm wells and grown in DMEM

supplemented with 7% FBS and 1% nonessential amino acids for 24 hrs. CHO (+) and (–) cells were transfected for 24 hrs with 1 µg of Cy5-DNA polyplex. The CHO cells were lifted by treatment with 5 ml of 1 mM EDTA in PBS and concentrated by centrifugation by 2,000 × g, before diluting to a final volume of 1 ml in PBS. The transfected CHO (+) and (–) cells were analyzed by FACS on a Becton Dickson LSRII with excitation 488 nm and emission 575 nm, and compared to untransfected CHO cells.

In Vitro Transfection with Glycopeptide DNA Polyplexes

DC-SIGN (+) CHO cells (1×10^5) were plated in 6-well plates in triplicate in 1 ml DMEM high glucose supplemented with 7% FBS and 1% MEM non-essential amino acids. After incubation for 24 hrs at 37°C in 5% CO₂, the media was removed, the cells were washed with 1 ml DPBS, replaced with 1 ml DMEM high glucose media supplemented with 2% FBS and 0.3% MEM non-essential amino acids.

Cells were transfected in triplicate for 24 hrs with pGL3 polyplexes (1 µg DNA and 0.6 nmol of glycopeptide in a total volume of 30 µl). The efficiency of glycopeptide DNA polyplex transfection was compared with PEI-DNA transfection performed by combining 1 µg DNA with 1.2 µg of PEI (N:P of 9) in 100 µl of 5 mM Hepes, 270 mM mannitol, pH 7.4. After 24 hrs, cells were washed with 2 ml of DPBS and lysed with 0.5 ml of lysis buffer (25 mM tris chloride, 1 mM EDTA, 8 mM magnesium chloride, 1% Triton X-100, pH 7.8) for 10 min at 4°C. Cell lysates were scraped, transferred to 1.5 ml microcentrifuge tubes, and centrifuged for 10 min at 13,000 g (at 4°C) to pellet cell debris. Lysis buffer (400 µl) and ATP (4.3 µl of a 165 mM solution at pH 7) were combined in a test tube, mixed briefly, and placed in the luminometer. The relative light units were determined with 10 sec integration after automatic injection of 100 µl of 0.43 mM D-luciferin. Protein concentrations were measured by a BCA assay with bovine serum albumin as a standard (35). The amount of luciferase recovered in each sample was normalized to mg of protein and reported as the mean with standard deviation obtained from triplicate transfections.

Results

A strategy was developed to reversibly bind a high mannose N-glycan to plasmid DNA for the purpose of targeted uptake and expression of a reporter gene in cells expressing the DC-SIGN receptor. While in the present study, glycopeptide mediated gene transfer was only tested in a model cell system, the ultimate purpose of this gene delivery system would be to selectively transfect dendritic cells in humans to improve the efficacy of DNA vaccines (36).

The polyacridine peptide was designed to reversibly bind to plasmid DNA by a combination of intercalation and ionic interaction as we and others have previously reported (19, 23, 25, 26, 37). The solid phase synthesis of Cys-(Acr-Lys)₄ necessitated the large scale synthesis of Fmoc-Lys(Acr) as an unnatural amino acid. Using this approach, a high affinity polyacridine peptide containing an N-terminal Cys was obtained in good yield.

The high-mannose N-glycan is a known ligand for DC-SIGN receptor and is the only N-glycan present on SBA (3, 38). Because SBA is a galactose specific lectin, it is readily purified in a single affinity chromatography step. The affinity purification of SBA is

essentially the same as first reported by Sharon (38), with the exception that the affinity resin was prepared by attaching galactosamine to 6-aminohexanoic acid functionalized Sepharose resin prepared using CDI as reported by Bethel and Ayers (29). This approach afforded the scalable preparation of the column resin without the use of cyanogen bromide.

The high-mannose N-glycan on SBA is not easily released by direct treatment with N-glycosidase F. Likewise, affinity purified SBA is very resistant to digestion with trypsin. Consequently, as first described by Sharon, SBA is most efficiently converted to peptides by prolonged pronase digestion which produces the Man 9 N-glycan possessing a single Asn (38). Starting with 10 g of soy flour, the resulting Man 9 N-glycan was purified in good yield (6.7 μ mol, 45%) using a combination of gel filtration and cation exchange chromatography as described (38, 39). High field ^1H -NMR analysis of the purified Man 9 N-glycan established the presence of 9 mannose residues based on the resonance frequency of the anomeric proton for each (33). MALDI-TOF analysis was also consistent with the isolation of Man 9 N-glycan containing a single Asn residue.

The conjugation of the Man 9 N-glycan to Cys-(Acr-Lys)₄ required functionalization through the Asn residue (Fig. 1). To facilitate reaction monitoring by RP-HPLC, the Man 9 N-glycan was first modified by attaching Boc-Tyr to the N-terminus. Analysis of the Man 9 Tyr-Boc obtained from gel filtration chromatography revealed a single peak on analytical RP-HPLC with retention time of 19 min when detected by Abs_{280nm} (Fig. 2A). Removal of the Boc group by treatment with TFA resulted in a shift to an earlier retention time, confirming complete removal of Boc (Fig. 2B). The resulting amine terminus on Tyr was then modified with a propionate maleimide to provide a functional group that reacts with the Cys residue on Cys-(Acr-Lys)₄. The resulting Man 9 Mal N-glycan product was also purified by gel filtration, and produced a single product when analyzed on RP-HPLC (Fig. 2C). Each N-glycan product described produced ESI-MS consistent with the reported structure.

To complete the synthesis of the Man 9 glycopeptide, the Man 9 Mal was reacted with nearly a stoichiometric amount of Cys-(Acr-Lys)₄ (Fig. 1). In addition to the desired glycopeptide product, a dimeric peptide product formed. The RP-HPLC analysis of the purified Man 9 glycopeptide established its high purity and produced an ESI-MS consistent with the reported structure (Fig. 2D, inset).

To examine the binding of the Man 9 glycopeptide to DNA, an agarose gel electrophoresis band shift assay was used. The electrophoretic migration of DNA was significantly retarded at increasing glycopeptide to DNA stoichiometry such that at 0.4 nmol of glycopeptide per μ g of DNA or higher the band shift was complete (Fig. 3). In addition, the DNA bands were not visible by incorporating ethidium bromide into the gel prior to electrophoresis. Only post-staining with ethidium bromide following electrophoresis resulted in displacement of the polyacridine containing glycopeptides and the detection of DNA.

An additional analysis of glycopeptide DNA binding affinity utilized a fluorophore displacement assay. Increasing the stoichiometry of Man 9 glycopeptide to DNA resulted in the displacement of thiazole orange from DNA as determined by a decrease in fluorescence

intensity. An asymptote in the fluorescence intensity at 0.4 nmol Man 9 glycopeptide per 1 μ g DNA established this as the equivalence point for polyplex formation (Fig. 4). By comparison, Cys-(Acr-Lys)₄ appears to have slightly greater DNA binding affinity. Based on this and the results in figure 3, a stoichiometry of 0.5 nmol per μ g of DNA was used to prepare polyplexes and evaluate cell uptake and in vitro gene transfer.

The uptake of Man 9 glycopeptide Cy5-DNA polyplexes into CHO cells, either with (+) or without (–) stably transfected DC-SIGN receptor, was assayed by FACS analysis (Fig. 5). A 24 hr 37°C incubation of Man 9 glycopeptide Cy5-DNA polyplexes with CHO (+) and (–) cells was used to simulate conditions used for in vitro transfection. FACS analysis revealed uptake of Man 9 glycopeptide Cy5-DNA polyplexes in both CHO (+) and (–) cells relative to untreated cells. CHO (–) cells were able to bind Man 9 glycopeptide Cy5-DNA polyplexes non-specifically through ionic interaction. However, the cell uptake in CHO (+) cells was 10–50 fold higher than CHO (–) cells based on the intensity of fluorescence (Fig 5). The results suggest that DC-SIGN on CHO (+) cells increases the uptake of Man 9 glycopeptide Cy5-DNA polyplexes by receptor mediated endocytosis.

To determine if the increased uptake of Man 9 glycopeptide DNA polyplexes by CHO (+) cells results in increased gene expression, a series of gene transfer experiments were performed in CHO (+) and (–) cells. The results established that PEI-DNA polyplexes were the most efficient in gene transfer but were not selective for (+) or (–) cells (Fig. 6). The Cys-(Acr-Lys)₄ peptide mediated low level gene expression in CHO cells, but did not demonstrate selectivity. In contrast Man 9 glycopeptide DNA polyplexes mediated approximately 100-fold greater gene expression in CHO (+) cells relative to an identical experiment conducted in CHO (–) cells (Fig. 6). These results are consistent with the cell uptake studies and support the hypothesis that the DC-SIGN receptor can facilitate receptor mediated endocytosis of DNA polyplexes.

Discussion

The clinical application of DNA vaccines for the treatment of disease is currently limited by the low efficiency of nonviral delivery systems (36, 40). While it will be possible to optimize the DNA vector, it is also necessary to improve the delivery system. A large body of growing evidence suggest that targeting of DNA, as well as adjuvant, to enter dendritic cells leads to an enhanced immune response (9, 36, 40). It has been demonstrated that DC-SIGN is an endocytosing, cell surface C-type lectin that can be used to target proteins and DNA into dendritic cells (9). Given that the specificity of DC-SIGN is for mannose or Le^X on N-glycans, we explored an approach to reversibly attach a high-mannose N-glycan ligand to plasmid DNA to establish DNA uptake and gene expression in cells expressing DC-SIGN.

To achieve this, a well-known purification of a high-mannose N-glycan from soybean agglutinin was used (38). This required the development of a large-scale galactose affinity column by applying a carbodiimide coupling strategy. The purified N-glycan was then systematically modified on the Asn N-terminus to allow coupling to a DNA binding peptide. The conjugation of Boc-Tyr resulted in introduction of a hydrophobic chromophore that could be iodinated. Removal of Boc proceeded in neat TFA to afford the Tyr amine.

Modification of the amine with a propionate possessing a maleimide group resulted in a Man 9 Mal that reacted with a thiol on the peptide.

The polyacridine peptide was modeled after similar peptides first reported by Ueyama (27). However, a Cys residue was included on the N-terminus to allow specific coupling to Man 9 Mal. This conjugation led to a Man 9 glycopeptide that possessed affinity for DC-SIGN and DNA. The DNA binding affinity of a polyacridine peptide is related to the number of acridines and the identity of the spacing amino acid (19). Two different experiments established that the glycopeptide binds to DNA. A band shift assay and a fluorophore displacement assay both confirmed that the Man 9 glycopeptide binds maximally to DNA at 0.4 nmol per μ g of DNA. The binding affinity for the unglycosylated peptide was slightly greater than that of the glycopeptide.

The ability of DC-SIGN to bind to Man 9 glycopeptide DNA polyplexes was established using a CHO cell line stably transfected with the human DC-SIGN receptor. The results of FACS analysis using Cy5-DNA glycopeptide polyplexes confirmed preferential binding to CHO (+) cells compared to CHO (–) cells. It should be noted that Cy5-DNA glycopeptide polyplexes bind weakly to CHO (–) cells perhaps due to the cationic nature of the polyplexes.

The results of FACS analysis compare favorably with those of gene transfer studies. A 100-fold increase in gene transfer was determined when transfecting CHO (+) cells with glycopeptide DNA polyplexes compared to the same transfection of CHO (–) cells. Unglycosylated peptide formed cationic polyplexes that mediated non-specific gene transfer. While the levels of gene transfer are much lower than that mediated by PEI DNA, the selectivity of Man 9 glycopeptide mediated gene transfer support the hypothesis that DC-SIGN binds the ligand and transports the DNA polyplex into the cell.

Taken together, this study demonstrates the first application of a high-mannose N-glycan to mediated gene transfer via DC-SIGN, and demonstrates the application of a polyacridine anchor peptide to reversibly bind Man 9 to DNA. These results may be expanded to eventually improve the efficacy of DNA vaccines for use in humans.

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Figure 1. Synthesis of a Man 9 Glycopeptide

A high-mannose N-glycan containing a single Asn residue was purified from soybean agglutinin and then functionalized to possess an N-terminal Tyr and propionate maleimide. The resulting Man 9 Mal was reacted with Cys-(Acr-Lys)₄, which was prepared by solid phase peptide synthesis using Fmoc-Lys(Acr). The resulting Man 9 glycopeptide possesses affinity for DC-SIGN and for binding to plasmid DNA through polyintercalation.

Figure 2. RP-HPLC Analysis of Man 9 Derivatives

Man 9 Asn obtained from SBA was N-terminally modified with Boc-Tyr then analyzed for purity by RP-HPLC (panel A). The removal of Boc from Tyr with TFA results in an earlier retention time (panel B). Conjugation of an N-terminal propionate maleimide increased the retention time (panel C) and conjugation of Man 9 Mal with Cys-(Acr-Lys)₄ resulted in the formation of Man 9 glycopeptide that rechromatographed as a single peak on RP-HPLC (panel D). The inset in panel D illustrates the ESI-MS analysis of the purified glycopeptide. See figure 1 for structures.

Figure 3. Glycopeptide DNA Binding By Agarose Gel Band Shift

A DNA band shift assay was performed by adding increasing amounts of glycopeptide to plasmid DNA. Lane 1 represents control plasmid DNA (1 μg) whereas lanes 2–5 are the result of adding 0.2, 0.4, 0.6 and 1 nmol of Man 9 glycopeptide to 1 μg of DNA.

Figure 4. Glycopeptide DNA Binding by Fluorophore Displacement Assay

The displacement of a thiazole orange intercalator dye by increasing amounts of Man 9 glycopeptide or Cys-(Acr-Lys)₄ results in an asymptote in the fluorescence intensity at 0.2–0.4 nmol per µg of DNA.

Figure 5. Cy5-DNA Glycopeptide Polyplex Binding to DC-SIGN Expressing CHO (+) Cells
The results of FACS analysis of Cy5-DNA Man 9 glycopeptide polyplex mediated uptake in DC-SIGN CHO (+) and (–) cells are illustrated. The fluorescence intensity in CHO (+) was nearly 10-fold higher than CHO (–) cells whereas, untreated CHO (+) or (–) cells demonstrated negligible fluorescence intensity.

Figure 6. In Vitro Transfection of CHO (+) and (-) DC-SIGN Cells

The result of in vitro transfection of CHO (+) (open bar) and (-) (closed bar) with different gene transfer agents is illustrated. Transfection of CHO (+) and (-) cells with PEI DNA polyplexes (N:P of 9) leads to high level of luciferase expression which is non selective. Likewise, transfection with Cys-(Acr-Lys)₄ peptide polyplexes results in lower levels of non-selective gene transfer. Alternatively, transfection with Man 9 glycopeptide (GP) polyplexes results in a 100-fold selective gene transfer in CHO (+) cells over CHO (-) cells. Each result is the mean and standard deviation of six independent transfections.