Synthetic Glycopeptide-Based Delivery Systems for Systemic Gene Targeting to Hepatocytes

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Received January 3, 2000; accepted January 24, 2000

Purpose. To design, synthesize, and test synthetic glycopeptide-based delivery systems for gene targeting to hepatocytes by systemic administration.

Methods. All peptides were synthesized by the solid phase method developed using Fmoc chemistry on a peptide synthesizer. The binding of galactosylated peptides to HepG2 cells and accessibility of the galactose residues on particle surface was demonstrated by a competition assay using ^{125}I-labeleld asialoorosomucoid and RCA lectin agglutination assay, respectively. DNA plasmid encoding chloramphenicol acetyl transferase (CAT) gene was complexed with a tri-galactosylated peptide (GM245.3) or tri-galactosylated lipopeptide (GM246.3) in the presence of an endosomolytic peptide (GM225.1) or endosomolytic lipopeptide (GM227.3) to obtain DNA particles of 100–150 nm in size. The plasmid/peptide complexes were added to HepG2 cell cultures or intravenously administered by tail vein injection into normal mice or rats. Plasmid uptake and expression was quantified by qPCR and ELISA, respectively.

Results. Multiple antennary glycopeptides that have the ability to condense and deliver DNA plasmid to hepatocytes were synthesized and complexed with DNA plasmid to obtain colloidally stable DNA/peptide complexes. Addition of DNA/GM245.3/GM225.1 peptide complexes (1:3:1 (−/+/−)) to HepG2 cell cultures yielded CAT expression in transfected cells. The transfection efficiency was significantly reduced in the absence of galactose ligand or removal of endosomolytic peptide. Intravenous administration of DNA/GM245.3 peptide complexes (1:0.5 (−/+)) into the tail vein of normal rats yielded DNA uptake in the liver. Substitution of GM245.3 by galactosylated lipopeptide GM246.3 resulted in more stable DNA particles, and a 10-fold enhancement in liver plasmid uptake. CAT expression was detectable in liver following intravenous administration of DNA/GM246.3 complexes. Addition of endosomolytic lipopeptide GM227.3 into the complexes (DNA/GM246.3/GM227.3 (1:0.5:1 (−/+/−))) yielded a 5-fold increase in CAT expression. Liver expression was 8-fold and 40-fold higher than lung and spleen, respectively, and localized in the hepatocytes only. The transfection efficiency in liver was enhanced by increasing DNA dose and injection volume. The plasmid uptake and expression in liver using DNA/GM246.3/GM227.3 complexes was 100-200-fold higher than DNA formulated in glucose. Tissue examination and serum biochemistry did not show any adverse effect of the DNA/GM246.3/GM227.3 (1:0.5:1 (−/+/−)) complexes after intravenous delivery.

Conclusions. Gene targeting to hepatocytes was achieved by systemic administration of a well-tolerated synthetic glycopeptide-based delivery system. The transfection efficiency of this glycopeptide delivery system was dependent on peptide structure, endosomolytic activity, colloidal particle stability, and injection volume.

KEY WORDS: gene delivery system; gene targeting; glycopeptide; hepatocyte; transfection efficiency.

INTRODUCTION

The liver is an attractive target for systemic gene therapy due to its large size, and blood flow (25% of the cardiac output) as well as the fenestrated endothelium that allows direct contact of blood components with the very large surface of the villous plasma membrane of hepatocytes (1–2). One of the key challenges for effective hepatocyte gene delivery using a hepatocyte-specific receptor ligand-based DNA delivery system is to produce colloidally stable DNA particles with a mean diameter between 100–150 nm (3). This size enables extravasation of plasmid complexes through the sinusoidal endothelial barrier of the liver, which has gaps of 100–200 nm and no basement membrane, and into the space of Disse. After extravasation through sinusoids into the space of Disse, DNA particles need to bind to specific hepatocyte receptors to induce receptor-mediated endocytosis. It has been proposed that after internalization by hepatocytes, the delivery system may need to contain a pH-sensitive lytic agent to facilitate the release of plasmid from the endosomes into the cytoplasm for efficient plasmid translocation to the nucleus (3).

The asialoglycoprotein receptor is a part of the surveillance system in the circulation that removes proteins as they age by the spontaneous loss of sialic acid (4). Systemic administration of DNA plasmid complexed with polylysine covalently coupled to asialoorosomucoid, a natural ligand for the asialoglycoprotein receptor, has been used to achieve gene transfer to the liver (5–7). While there are many advantages of the receptor-mediated gene delivery to the liver, such as high specificity and the availability of high affinity binding ligands, there are several formidable obstacles to the routine use of receptor-mediated gene delivery. The naturally occurring receptor ligands are either proteins or complex carbohydrates, which are extremely difficult to obtain consistently in high purity and in sufficient amount, and are potentially immunogenic when cross-linked to polylysine for DNA condensation (8). The polylysines are heterogeneous molecules giving rise to polydispersity in complexes (9) and variability in the rate of liver clearance (10). Polylysines are also toxic to living cells in nanomolar concentrations, which limits their general applicability (9).

Synthetic peptide-based DNA delivery systems consisting of carrier peptide analogs ($YKAK_nWK$) that condense DNA and a pH-sensitive endosomolytic peptide that facilitates release of the plasmid from endosomes after endocytosis of the DNA/peptide complexes, have been recently described for transfection of mammalian cells (11). With molecularly defined synthetic peptides it should be possible to obtain a DNA delivery system that is structurally well-characterized, non-toxic, biodegradable and non-immunogenic. In the present report, we

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ABBREVIATIONS: GM208.8, non-targeting carrier peptide $YKAK_8WK$; GM245.3, tri-galactosyl targeting carrier peptide; GM246.3, tri-galactosyl targeting carrier lipopeptide; GM212.8, non-targeted carrier lipopeptide; GM225.1, endosomolytic peptide; GM227.3, endosomolytic lipopeptide.

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describe the design, synthesis, and activity of a hepatocyte gene targeting system comprising of 1) a glycolipopeptide that condenses plasmids to colloidally stable particles that target the asialoglycoprotein receptor on hepatocytes, and 2) a pH-sensitive, endosomolytic peptide that enables the release of plasmids from the endosomes following receptor-mediated uptake by hepatocytes. The development of this glycopeptide/endosomolytic peptide-based delivery system for gene targeting to hepatocytes by systemic administration is discussed.

MATERIALS AND METHODS

Peptide Synthesis and Purification

All peptides were synthesized by the solid phase method developed by Merrifield (12) using Fmoc chemistry on an Applied Biosystems 433A peptide synthesizer. The synthesis of non-targeting carrier peptide GM208.8 ($YKAK_8WK$) and an endosomolytic peptide GM225.1 (GLFEALLELLESLWELLLEA-OH) was performed as described previously (9). The galactosylated targeting carrier peptide (GM245.3) was prepared in a similar manner to GM208.8 with the additional inclusion of an N-terminal covalently bound Gly-Ser flexible linker to provide the carrier backbone ($GSGSGSGSGSGYKAK_8WK$). Three sites for galactosylation were introduced by coupling Fmoc-Lys (Aloc) and Fmoc-Lys (Fmoc) to the backbone sequentially ($KKGSGSGSGSGSGYKAK_8WK$). Removal of the Fmoc protecting groups from this tri-antennary peptide was accomplished on the synthesizer using 20% piperidine, followed by manual Aloc removal employing tetrakistriphenylphosphine palladium (13) on the solid support to generate the desired tri-amino containing peptide. The simultaneous glycosylation of the three available amines was performed on solid support by treatment with a 12-fold excess of AcGalTP (14) using PyBop/HOBt activation. After cleavage with TFA/thioanisole (95:5), the crude acetylated glycopeptide was purified by reverse-phase HPLC and the acetyl groups removed from the galactose residues after incubation with 0.1N NaOH for 15 min at room temperature (15). The composition of the final product was confirmed by amino acid analysis and electrospray MS (M+ 3505.5).

For the lipophillic series of glycopeptides the cationic oligopeptide "K9" (KKKKKKKKKWK) was used as the core condensing peptide in place of GM208.8 and the triethylene glycol spacer $HO_2CCH_2CH_2OCH_2CH_2OCH_2CH_2NH_2$ was replaced by the $(Gly\text{-}Ser)_5$-Gly spacer to provide better spacing of the sugars and less restricted mobility. The core "K9" peptide was synthesized on a Rink amide MBHA resin by standard solid-phase peptide synthesis methods with the following orthogonal strategy: the C-terminal lysine contained a Dde protecting group at the ε-amine, while the N-terminal lysine had Fmoc protecting groups in both the α-and ε-amines. The rest of the side chain amines were protected with t-Boc groups. The core peptide was dipalmitoylated by deprotecting the N-terminal lysine with 20% piperidine in NMP and coupling the resulting free amines with palmitoyl chloride (four molar excess over the amine) for several hours at 25°C in the presence of 2M DIEA. The targeting moiety was then built on the dipalmitoylated core peptide by removing the Dde protecting group from the side chain of the C-terminal lysine by manually treating the peptide resin with 2% hydrazine in NMP. The first triethylene glycol spacer was introduced by coupling $HO_2CCH_2CH_2OCH_2CH_2OCH_2CH_2NH$-Fmoc to the side chain of the C-terminal lysine by standard solid phase procedures. Two additional lysines were coupled to the first linker to provide, after deprotection, three amine functionalities, for coupling to galactose. After deprotection with 15% piperidine in NMP, four molar excess of AcGalTP was coupled to free amines in the presence of PyBOP/HOBt for 5 h at 25° C. The peptide was cleaved from the resin by treatment with TFA/EDT/H_2O (95:2.5:2.5) cleavage cocktail for 3 h at 25° C. After extraction and lyophilization, the crude peptide was deacetylated by treatment with 0.1N NaOH for 40 min at 25°C. Purification of the final tri-galactosylated lipopeptide was achieved by reverse-phase HPLC.

Initial attempts in our laboratory to hydrophobically modify the endosomolytic peptide GM225.1 (GLFEALLELLESLWELLLEA) (9) were unsuccessful due to insolubility of the resulting lipopeptide. As an alternative, a small lytic sequence with lytic properties similar to that of GM225.1 was identified and hydrophobically modified. The resulting peptide (Fig. 1), referred to as GM227.3, has been shown to enhance reporter expression *in vitro* to the same extent as GM225.1 (unpublished observation).

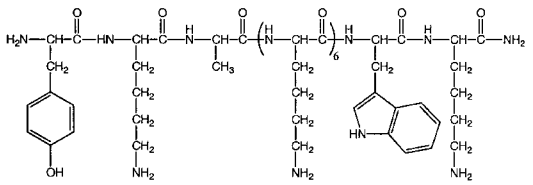
Plasmid Construction and Preparation of DNA/Glycolipopetide Complexes

The expression plasmid pCMV-CAT contained a CMV enhancer and promoter, an SV40 intron, a CAT coding region, and an SV40 late polyadenylation signal. The pDNA was isolated, purified, and characterized before *in vivo* administration as previously reported (16). The DNA plasmid was complexed with targeted tri-galactosylated peptide GM245.3, targeted galactosylated lipopetide GM246.3, or non-targeted lipopeptide GM212.8, at 1:0.5 (−/+) charge ratio under constant vortexing to get a 0.1 mg/ml final DNA concentration. The samples were allowed to stand for 30 min to ensure optimal complexation between the plasmid and peptide carrier. After 30 min, the endosomolytic peptide was added to the DNA/peptide colloidal suspension under vortexing. The mean particle size of DNA/peptide complexes in 5% glucose, 150 mM NaCl, or 5–20% mouse serum was determined by dynamic light scattering using an N4MD submicron particle sizer (Coulter, Miami, FL).

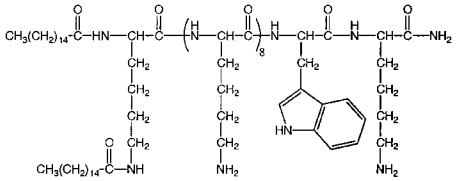
Binding Assay

The binding of targeted galactosylated peptide GM245.3 to asialoglycoprotein receptors on HepG2 cells (17) was assessed by a competition assay using ^{125}I-labeleld ASOR, a natural ligand for asialoglycoprotein receptors. The ability of GM245.3 to compete for ASOR binding to HepG2 cells was examined. HepG2 cells were grown to 70–80% confluency in a 24-well plate. The monolayers were washed 3 × with minimal essential medium and incubated at 4°C for 5 h with 11.8 nM ^{125}I-labeleld ASOR and 0–109 nM GM245.3. Cells were washed to remove unbound labeled ligand and incubated with 300 μl of 1N NaOH overnight at room temperature to obtain cell lysate. The amount of radioactivity associated with the cells was measured by counting an aliquot of the cell lysate in a gamma counter. Non-specific binding in the assay was determined by adding excess of unlabeled ASOR with the labeled ligand.

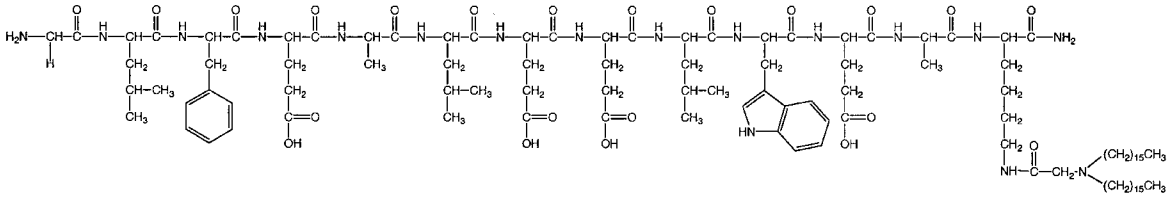
GM208.8 (Non-targeting carrier peptide)



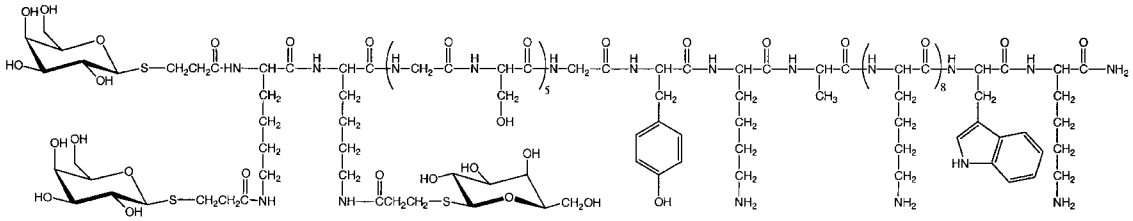
GM212.8 (Non-targeting carrier lipopeptide)



GM227.3 (Endosomolytic lipopeptide)



GM245.3 (Tri-galactosyl targeting carrier peptide)



GM246.3 (Tri-galactosyl targeting carrier lipopeptide)

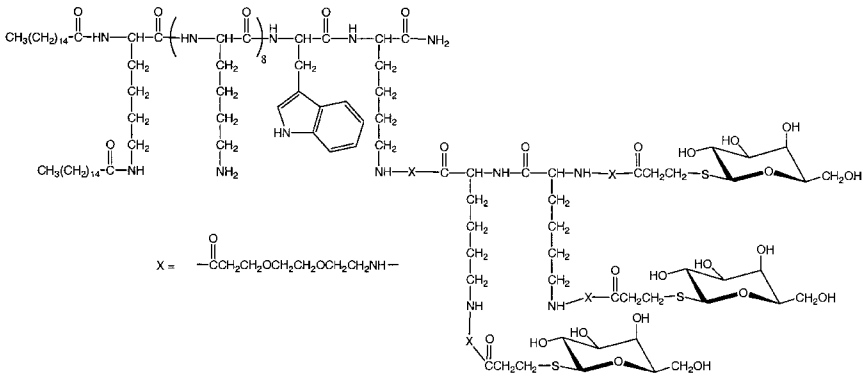


Fig. 1. Structure of synthetic glycopeptides and endosomolytic peptides.

Agglutination Assay

An agglutination assay with RCA lectin (a galactose binding protein from *Ricinus cummunis*) was used to confirm the availability of galactose on the surface of DNA/glycopeptide complexes. The DNA plasmid was complexed with targeted galactosylated lipopeptide GM246.3 or non-targeted lipopeptide GM212.8, at 1:0.5 (−/+) charge ratio, as described above. A 100 μl aliquot of the complexes containing 5–10 nM of the galactosylated peptide was mixed with equal volume of 1 mg/ml RCA lectin (Sigma Chemicals, St. Louis, MO) and the time dependent absorbance was measured at 460 nm using Du 640 Spectrophotometer (Beckman Coulter Inc., Fullerton, CA).

In Vitro Gene Transfer

HepG2 cells were purchased from the American Type Culture Collection (Rockville, MD). Cells were plated at an

initial density (80,000 cells/well) in 24-well tissue culture plates. 4 μg of DNA/GM245.3 or DNA/GM208.8 complexes were added to each well and allowed to incubate 4 h at 37° C. Afterward, 1 ml of the cell culture medium in 10% FBS was added to each well. Cells were harvested 1 day after transfection, washed 3 times with PBS, and cell lysate was prepared by addition of 0.25 ml lysate buffer, pelleted by centrifugation and supernatant was assayed for CAT using an ELISA (Boehringer Mannheim, Indianapolis, IN).

In Vivo Gene Transfer

Normal CD1 mice (22–25 g) or Sprague Dawley rats (250–300 g) were obtained from Charles River Laboratories (Raleigh, NC) and housed in the Laboratory Animal Resources Vivarium at VALENTIS, INC. and maintained on *ad libitum* water (temp 23° C, humidity 50%; light/dark cycle 12 h/12 h). Animal use conformed to NIH guidelines.

Mice were anesthetized by intraperitoneal administration of a mixture of ketamine (42.8 mg/ml), xylazine (8.6 mg/ml) and acepromazine (1.4 mg/ml) at a dose of 0.5–0.7 ml/kg. Transfection complexes containing 120 μg plasmid in 1.2 ml were administered intravenously into the tail vein. Liver, lung, and spleen were collected at appropriate time intervals after DNA administration, immediately frozen in liquid N_2, and stored at −80° C.

Hepatocyte Isolation

To determine hepatocyte localization of plasmid delivery, rat liver was enzymatically dispersed into single cells and hepatocytes were isolated and purified as described below. Animals were anesthetized prior to liver perfusion with intraperitoneal administration of acepromazine (0.73 mg), Ketamine (73.96 mg), and xylazine (3.74 mg) at a dose of 1.8—2.0 ml/kg. After flushing the liver with Earle's solution (1.8—2 ml/minutes, 37° C) for 5 minutes, the liver was perfused with collagenase solution (1.8—2 ml/min, 37° C) for 10 minutes. The liver was harvested and disrupted into single cells with a pipette tip in 5–10 ml DMEM with 10% FBS, filtered through nylon mesh, and centrifuged at 50 × g for 2 minutes. The pellet was washed three times, and purified on a percoll gradient. The gradient purified cell fraction was 95% hepatocyte rich as determined by light microscopy and electron microscopy (data not shown).

Plasmid Isolation and Quantification

Plasmid DNA was isolated from liver tissue homogenate or isolated hepatocytes by incubation with digestion buffer (100 mM NaCl, 10 mM Tris-HCl, [pH 8.0], 25 mM EDTA [pH 8.0], 0.5% SDS, and proteinase K [0.1 mg/ml]) at 50° C. The samples were extracted with an equal volume of Tris-buffered phenol (pH 8.0), followed by extraction with chloroform:isoamyl alcohol (24:1, v/v) and ethanol precipitation. The DNA precipitates were dissolved in TE buffer (10 mM Tris [pH 7.5], 1 mM EDTA), and DNA concentration was measured by UV absorption at 260 nm.

A quantitative polymerase chain reaction (PCR) assay using the Taqman methodology and the ABI Sequence Detection System (Perkin-Elmer, Foster City, CA) was used to quantify the amount of DNA plasmid associated with the tissue or cell extract. The primers used in the reaction were a forward primer, 5′-GCC GTA ATA TCC AGC TGA ACG-3′ (Genosys Biotechnologies, The Woodlands, TX), which primes in the CMV 5′ untranslated region (UTR), and a reverse primer, 5′ GCA AGT CGA CCT ATA ATG CCG-3′, which primes in the CAT reporter gene coding region. The probe sequence was 5′-(FAM)-CCA GCC TCC GGA CTC TAG AGG A-(TAMRA)-3′. The initial copy numbers of unknown samples were determined by using the Applied Biosystem 7700 sequence detector to compare them with a standard curve generated from purified pCMV-CAT of known initial copy numbers.

Assay for CAT Expression in Mouse Liver

Tissue samples were homogenized in 5 volumes of TENT (Tris 10 mM, EDTA 1 mM, NaCl 0.1 M, Triton X-100 0.5%) buffer. The tissue homogenate was centrifuged at 10,000 × g for 15 min and supernatant was assayed for CAT using an ELISA (Boehringer Mannheim, Indianapolis, IN).

CAT Immunohistochemistry

5 μm paraffin liver sections (Leica RM2035) were first incubated overnight with sheep anti-CAT antibody (Boehringer-Mannheim) (1:100,000), washed three times in phosphate buffered-saline (PBS) and then incubated with biotinylated rabbit anti-sheep IgG (Vector Laboratories, Burlingame, CA) (1:400) for 1 h. The specimens were rinsed 3 × in PBS and then incubated with Vector *elite* ABC system (Vector Laboratories) (1:800) for 1 h. The antigen-antibody complex was visualized with DAB (Vector Laboratories) and counterstained with hematoxylin.

RESULTS AND DISCUSSION

Chemistry and Synthesis of Glyocopeptide Systems

In order to achieve the selective DNA delivery to hepatocytes with a peptide-based system we developed a convenient solid phase method to prepare multiple antennary glycopeptides that have the ability to condense and deliver DNA plasmid to select cells via receptor-mediated endocytosis. Galactosylated DNA carrier peptides were designed and synthesized to target the asialoglycoprotein receptor of hepatocytes (Figs. 1). These peptides were based on core cationic sequences that have been previously shown to promote transfection in a variety of cell lines (11). Both the number of galactose residues per peptide and the spacing of the galactose subunits were varied and subsequently evaluated for *in vitro* and *in vivo* activity (data not shown). The tri-antennary galactosylated peptide (GM245.3) was chosen for further optimization and subsequently formulated with an endosomolytic peptide (GM225.1) for *in vitro* evaluation. The evidence of GM245.3 binding to asialoglycoprotein receptor in HepG2 cells is demonstrated by a competition assay with ^{125}I-ASOR (Fig. 2). GM245.3 inhibited the ^{125}I-ASOR binding in a concentration dependent manner with an IC_{50} of 35.6 nM and Kd of 19 nM.

This DNA/GM245.3/GM225.1 delivery platform relies upon electrostatic interactions to facilitate the association of the carrier and endosomolytic peptides with the DNA. While this approach provides a modular means of incorporating multiple functionalities, there is the likelihood of complex disassociation in the presence of ionic conditions found in a biologic

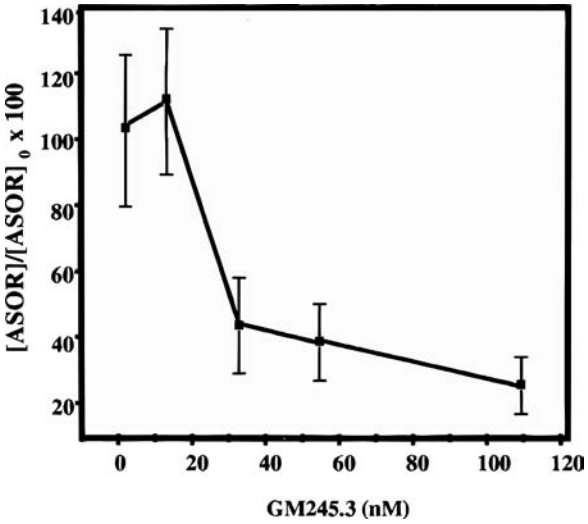


Fig. 2. Competition of tri-galactosyl peptide GM245.3 and ^{125}I-ASOR for binding to HepG2 cells in culture. The effect of various concentrations of GM245.3 on ^{125}I-ASOR binding to HepG2 cells was determined as described in the *Methods*. The values represent mean +/− S.D. (n = 3).

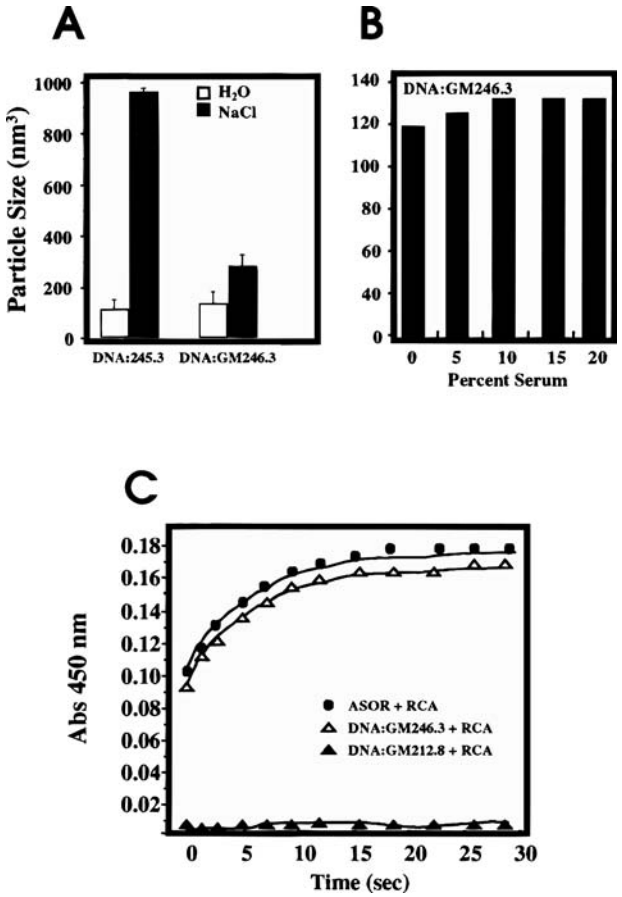


Fig. 3. Increased colloidal stability of DNA/glycopeptide complexes by hydrophobic modification of the glycopeptide. pCMV-CAT was complexed with tri-galactosylated peptide GM245.3 (1:0.5 (−/+)) or tri-galactosylated lipopeptide GM246.3 (1:0.5 (−/+)) at a final DNA concentration of 0.1 mg/ml. The particle size of the DNA/peptide complexes was determined 2 min after the addition of 150 mM NaCl or equivolume of H_2O (A) or mouse serum (B). Agglutination of RCA lectin by DNA/glycopeptide complexes (C). 100 μl of DNA complexes with tri-galactosylated lipopeptide GM246.3 or non-galactosylated lipopeptide GM212.8 was mixed with equal volume of RCA lectin (1 mg/ml) and the time dependent absorbance was measured at 460 nm. Uncomplexed ASOR was used as a positive control for the assay. Values represent the mean +/− S.D. of three samples for "A" and "C", and average of two samples for "B".

milieu. As a means of augmenting electrostatic associations, we explored the use of hydrophobic modifications to improve the formulation properties, as well as the transfection activity, of synthetic carrier and fusogenic peptides. Our hypothesis was that hydrophobic modification of the carrier and fusogenic peptides would improve the stability of the overall complex by virtue of the insensitivity of hydrophobic interactions to dissolved electrolytes. The dipalmitoylation of an additional lysine residue on a simplified "K9" condensing sequence provided the desired lipophilic targeting carrier peptide (GM246.3). The Gly-Ser spacers used in the non-lipophilic peptide were also replaced by a simplified triethyleneglycol spacer. This simplified spacer was also placed in between each galactose residue and the peptide to provide better spacing of the sugars and less steric hindrance. The data in Fig. 3A shows that substitution of GM245.3 by its more hydrophobic lipid derivative GM246.3 produced DNA particles that were more stable in 150 mM NaCl. The colloidal stability of DNA/GM246.3 complexes was also evident in 5–20% mouse serum (Fig. 3B).

To assess the surface accessibility of the galactose residues in the DNA/glycolipopeptide complexes, the galactose-specific RCA lectin from *Ricinus cumminis* was used in an agglutination assay. Specific agglutination activity was observed only with those DNA/lipopetide complexes that contained galactose or with asialoorosomucoid (Fig. 3C). These results demonstrate that galactose residues are displayed on the particle surface for receptor binding.

Hepatocyte Targeting *In Vitro*

Complexation of the DNA plasmid with tri-galactosylated condensing peptide GM245.3 produced DNA particles with hydrodynamic diameter of 127 ± 60 nm. Transfection of HepG2 cells with DNA (5 μg)/GM245.3 complexes in the presence of endosomolytic peptide GM225.1 at 1:3/1 (−/+/−) charge ratio yielded moderate reporter gene expression (Fig. 4). The levels of gene expression from cells transfected with DNA/GM245.3/GM225.1 complexes were 5–6-fold higher than those obtained with non-targeted peptide GM208.8 in the transfection complexes. These data support the hypotheses that hepatocyte transfection by plasmid/GM245.3 complexes is mediated via asialoglycoprotein receptor, and that an endosomolytic agent is required for efficient transfection following receptor-mediated endocytosis of DNA/GM245.3 complexes.

Hepatocyte Targeting *In Vivo*

Plasmid Uptake

Tail vein injection of 120 μg pCMV-CAT complexed with the hepatocyte targeting peptide GM245.3 at 1:0.5 (−/+) charge

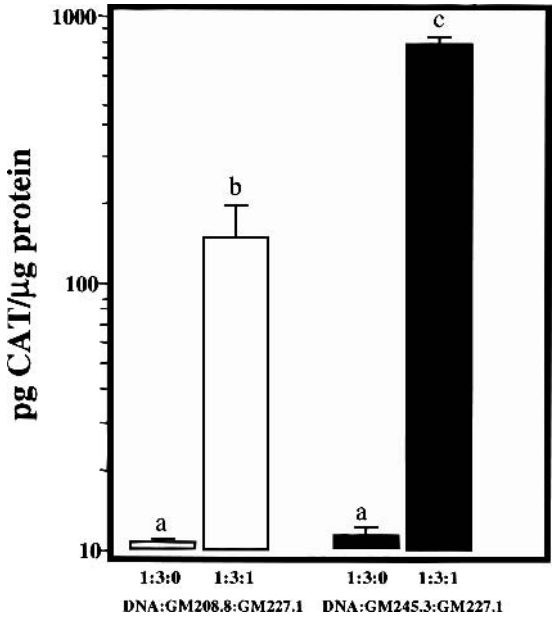


Fig. 4. Gene targeting to hepatocytes by glycopeptide-based delivery system. HepG2 cells were transiently transfected for 4 h at 37°C with 4 μg pCMV-CAT complexed with tri-galactosylated condensing peptide GM245.3 or non-galactosylated condensing peptide GM208.8 in the absence or presence of endosomolytic peptide GM225.1. Twenty-four hours after transfection cells were lysed and CAT was quantified in cell lysate by an ELISA, as described in the *Methods*. Values represent the mean +/− S.D. (n = 4).

ratio into normal rats led to plasmid uptake in liver (Fig. 5A). The liver uptake of DNA/GM245.3 complexes 2 h after injection was 10-fold higher than that obtained with uncomplexed DNA formulated in 5% glucose. Substitution of GM245.3 by its lipid derivative GM246.3 to produce more stable DNA particles yielded 100-fold higher DNA uptake when compared to DNA complexed to GM245.3. Increased DNA uptake from DNA/GM246.3 complexes could be due to the increased steric stability of DNA/lipopeptide complex in biofluids thus enhancing particle extravasation through the sinusoids into the space of Disse, followed by receptor binding. These data are consistent with the hypotheses that colloidal stability of the transfection complexes in biofluid is a crucial determinant in the efficiency of receptor-mediated gene targeting to hepatocytes (3,8). Consistent with this observation, addition of lipophilic alpha-lipoate chains to bovine serum albumin creating a local hydrophobic environment in the protein structure has been shown to inhibit albumin glycation by preventing enzyme-substrate interaction (18).

To determine if plasmid delivery from systemic administration of DNA/GM246.3 complexes was directed to hepatocytes, liver tissue was enzymatically dispersed and DNA was quantified in isolated rat hepatocytes 15 min after DNA injection. Fig. 5B shows that the hepatic uptake from DNA/peptide complexes was localized in hepatocytes. Removing the Kupffer cells and endothelial cells from liver cell mixture to obtain purified hepatocytes did not change the transfection efficiency of the GM246.3 system, suggesting that the DNA was localized in the hepatocytes. In comparison, a 6–7-fold decrease in transfection efficiency upon hepatocyte enrichment after naked DNA injection suggests a non-specific delivery.

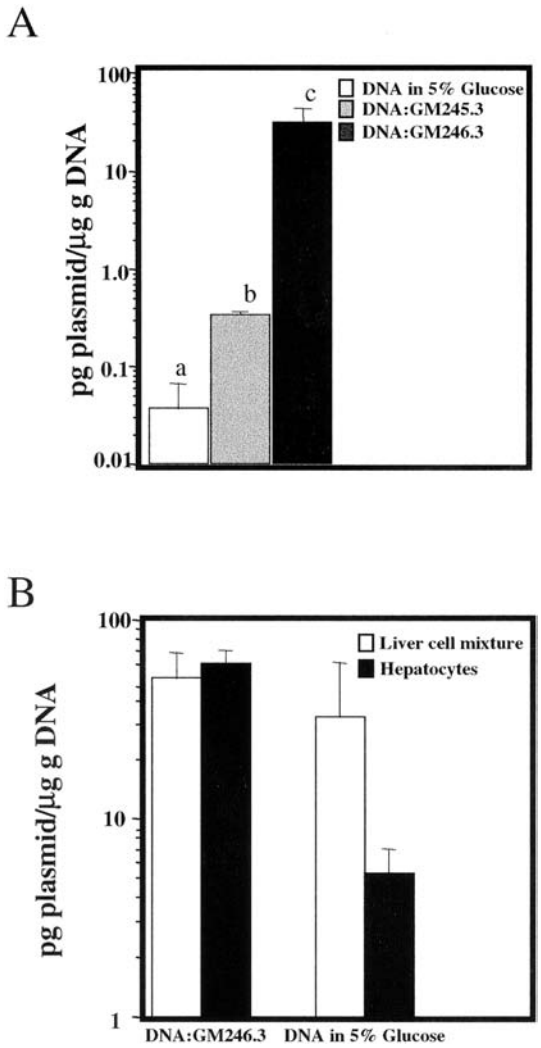


Fig. 5. Plasmid uptake by liver and hepatocytes after tail vein administration of DNA/glycopeptide complexes. Rats were injected with 120 μg of pCMV-CAT complexed with tri-galactosylated peptide GM245.3 or tri-galactosylated lipopeptide GM246.3. Liver was collected 2 h later for DNA quantitation in tissue extract (A), or perfused with collagenase solution for DNA quantitation in isolated hepatocytes (B), by a quantitative PCR assay. Values represent the mean +/− S.D. (n = 5). Bars with different superscripts in parentheses are statistically different ($p < 0.05$) using *t*-test.

Gene Expression

Systemic administration of DNA (120 μg/1.2 ml)/GM246.3 peptide complexes (1:0.5 (−/+)) into mice yielded CAT expression in liver (Fig. 6A). The levels of CAT expression measured 2 days after DNA injection were 40-fold higher than those obtained with DNA complexes with non targeted lipopeptide GM212.8 or with uncomplexed DNA injected in 5% glucose. Addition of an endosomolytic lipopeptide GM227.3 into DNA/peptide complexes (1:0.5:1 (−/+/−)) significantly enhanced the transfection efficiency. The levels of CAT expression obtained with DNA/GM246.3/GM227.3 peptide complexes were ~130-fold higher than those obtained with DNA complexes with non-targeted peptide GM212.8 or uncomplexed DNA plasmid formulated in 5% glucose. The levels of gene

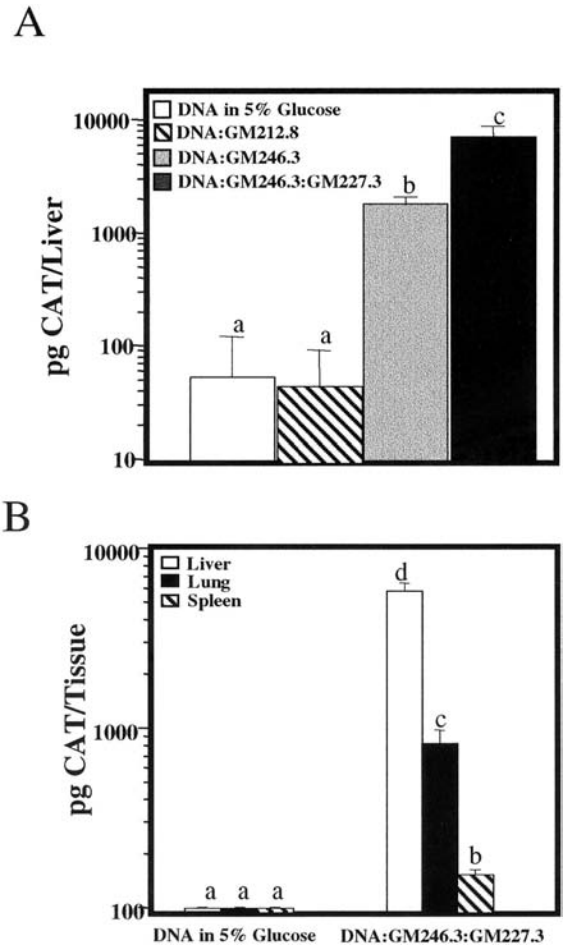


Fig. 6. Levels and liver-specificity of gene transfer by tail vein administration of DNA/glycopeptide complexes. pCMV-CAT (120 mg) was complexed with non-targeted lipopeptide GM212.8 or tri-galactosylated lipopeptide GM246.3 2/1 GM227.3 endosomolytic lipopeptide at 1:0.5:1 (2/1/2) molar charge ratio, or formulated in 5% glucose. The DNA formulations were administered intravenously by tail vein injection and the tissue were collected 48 h later for measurement of CAT expression. Comparison of targeted and non-targeted peptides and naked DNA formulations for CAT expression in liver (A). Biodistribution of CAT expression from DNA/tri-galactosylated lipopeptide/endosomolytic lipopeptide complexes in liver, lung, and spleen (B). Values represent the mean 1/2 S.D. (n 5 5±8). Bars with different superscripts in parentheses are statistically different (p , 0.05) using *t*-test.

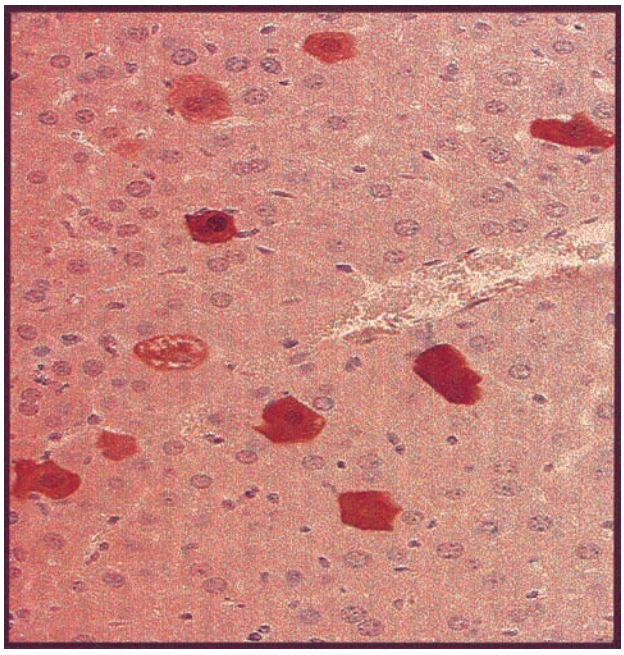


Fig. 7. Immunohistochemical localization of CAT expression in liver 48 h after tail vein administration of DNA/glycopeptide complexes.

expression in liver were 6-fold and 50-fold higher than those obtained in lung and spleen, respectively, (Fig. 6B), demonstrating the liver specificity of the targeted DNA/glycopeptide gene delivery system. CAT immunohistochemistry showed that CAT expression from DNA/GM246.3/GM227.3 complexes was localized exclusively in the hepatocytes (Fig. 7). Tail vein injection of 120 mg DNA in 5% glucose did not reveal CAT positive cells in liver tissue.

It is concluded from the above data that systemically administered DNA/GM246.3/GM227.3 peptide complexes were able to access hepatocytes for receptor binding and endocytosis. A significant enhancement in liver transfection by the endosomolytic peptide shows that plasmid translocation from endosomes to hepatocyte nucleus is required for efficient transfection following receptor-mediated endocytosis (3,8,18). The endosomolytic peptide is designed to form alpha-helices at low pH, by protonation of the glutamic acid residues, to expose a hydrophobic face comprised of only strongly apolar amino acids and a hydrophilic face mainly dominated by the glutamic acid residues. This structural conformation favors the partitioning of the amphipathic peptides, or potentially clusters of these, into the endosomal membrane, thus affecting the release of the endosomal contents into the cytoplasm. (3,8). At physiological pH, the negative charge of the glutamic acid residues maintain the peptides in a random coil conformation, preventing them from destabilizing biological membranes. The activity of endosomolytic peptides on biological membranes has been shown to be pH-dependent in a hemolytic assay, erythrocyte hemolysis only occurring at acidic pH and not at physiological pH (9,11).

The magnitude of gene transfer to liver by intravenous administration of DNA/GM246.3/GM227.3 peptide complexes was dependent on DNA dose (Fig. 8A). Lowering the DNA dose from 120 mg to 60 mg or 30 mg resulted in a dose-dependent drop in gene expression by 1.7-fold and 3-fold, respectively. These differences were not statistically significant. The liver transfection was sensitive to injection volume. Lowering the formulation volume from 1.2 ml to 0.6 ml resulted in 10±15 fold reduction in DNA uptake and expression (Fig. 8B). We do not know the exact mechanism by which the injection volume affects the efficiency of DNA/GM246.3/GM227.3 peptide complexes. It has been hypothesized that the high injection volume minimizes interaction of serum proteins or opsonins with the transfection complexes by dilution effect thus further enhancing colloidal stability of the complexes in blood circulation. High injection volume could also cause osmotic changes in liver sinusoidal area causing a transient opening of the sinusoids and may allow a better penetration of transfection complexes into the space of Disse and subsequently a higher receptor

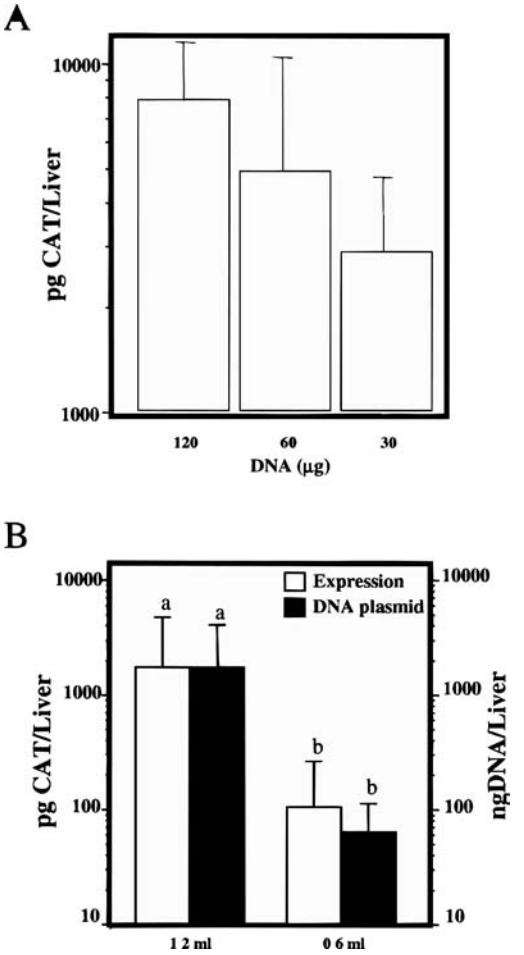


Fig. 8. Effect of DNA dose and injection volume on CAT expression in liver 48 h after tail vein administration of DNA/glycopeptide complexes. pCMV-CAT was complexed with tri-galactosylated lipopeptide GM246.3 containing 30 μg, 60 μg, or 120 μg DNA in the presence of endosomolytic lipopeptide GM227.3 (1:0.5:1 (−/+/−)) and administered in 1.2 ml volume into tail vein of mice (A). Effect of injection volume (0.6 ml and 1.2 ml) on the levels of CAT expression in the liver after tail vein injection of DNA/GM246.3/GM227.3 complexes containing 60 μg DNA (B). Values represent the mean +/− S.D. (n = 5). Bars with different superscripts in parentheses are statistically different ($p < 0.05$) using *t*-test. The differences between groups in "A" were not statistically significant at "*p*" value of 0.05.

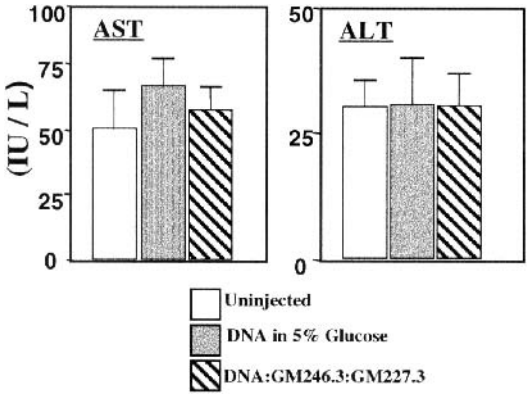


Fig. 9. Serum ALT and AST levels after tail vein administration of DNA/glycopeptide complexes. pCMV-CAT (60 μg) was formulated in 5% glucose or complexed with tri-galactosylated lipopeptide GM246.3 with the endosomolytic lipopeptide GM227.3 (1:0.5:1 (−/+/−)). Blood samples were collected 48 h after plasmid injection and ALT and AST levels were quantified in serum. Values represent the mean +/− S.D. (n = 4–6). Means were not statistically different (*t*-test).

binding. As shown in Fig. 3, DNA/GM246.3 peptide complexes are stable at physiological salt concentration and in dilute serum, therefore, other factors such as plasma clearance rate, blood flow rate, and/or activation of complement system may influence the colloidal and steric stability of plasmid/peptide complexes *in vivo*. Recent studies have also shown the necessity of high injection volume (1–3 ml) to achieve systemic gene transfer using uncomplexed DNA in mouse liver (20–21). The glycopeptide-based hepatocyte targeted gene delivery systems presented in this study have an advantage over uncomplexed DNA due to their hepatocyte specificity, since uncomplexed DNA also targets non-hepatocyte cells (Fig. 5 and ref. 20), and better transfection efficiency (Fig. 6).

Tolerability of DNA/Glycopeptide Complexes

The systemic administration of DNA/GM246.3/GM227.3 peptide complexes was well tolerated by animals since no adverse effects were detectable. Analysis of serum ALT and AST levels (Fig. 9) and gross tissue examination after tail vein administration of DNA/GM246.3/GM227.3 complexes into mice did not show noticeable differences from control groups. These results demonstrate that the glycopeptide-based hepatocyte targeted gene delivery systems described in the present study are safe for systemic administration.

CONCLUSIONS

Gene targeting to hepatocyte by systemic administration of a non-toxic, biodegradable, non-antigenic glycolipopeptide-based plasmid delivery system is described. The prototype glycopeptide gene delivery system for hepatocytes is based on the structure and function of the liver and principles of cellular uptake and intracellular trafficking of DNA plasmid. The systemic administration of DNA/peptide complexes yields hepatocyte-specific gene delivery and expression. These peptide-based hepatocyte gene targeting systems are advantageous over high-molecular weight macromolecules or polylysines, which are generally highly charged, have a propensity to elicit immune response and are often cytotoxic. Further improvement in colloidal and steric stabilization of the prototype system is necessary to enable a significant decrease in dose and injection volume before this system can be considered for clinical applications.

ACKNOWLEDGMENTS

We would like to thank the following groups or individuals at VALENTIS, INC. for their assistance: Manufacturing and QA/QC groups for providing DNA plasmid, and Dr. Michael Fons for his valuable comments on this manuscript.

REFERENCES

1. D. K. F. Meijer and G. Molema. Targeting of drugs to the liver. *Semi. Liver Disease* **15**:202–256 (1995).
2. F. D. Ledley, R. M. Adams, H. E. Soriano, G. Darlington, M. Finegold, R. Lanford, D. Carey, D. Lewis, P. A. Baley, S. Rothenberg, M. Kay, M. Brandt, R. Moen, W. F. Wnderson, P. Whitington, W. Pokorny, and S. L. C. Woo. Development of

a clinical protocol for hepatic gene transfer: Lessons learned in preclinical studies. *Ped. Res.* **33**:313–320 (1993).
3. A. P. Rolland. From Genes to Gene Medicines: Recent advances in non-viral gene delivery. *Crit. Rev. Ther. Carrier Systems* **15**:143–198 (1998).
4. R. J. Stockert. The asialoglycoprotein receptor: relationships between structure, function, and expression. *Physiol. Rev.* **76**:591–609 (1995).
5. G. Y. Wu, J. M. Wilson, F. Shalaby, M. Grossman, D. A. Shafritz, and C. H. Wu. Receptor-mediated gene delivery *in vivo*. *J. Biol. Chem.* **266**:14338–14342 (1991).
6. G. Y. Wu, and C. H. Wu. Liver-directed gene delivery. *Adv. Drug Del. Rev.* **12**:159–167 (1993).
7. J. Frese, C. H. Wu, and G. Y. Wu. Targeting of genes to the liver with glycoprotein carrier. *Adv. Drug Del. Rev.* **14**:137–152 (1994).
8. L. C. Smith, J. Duguid, M. S. Wadhwa, M. J. Logan, C. H. Tung, V. Edwards, and J. T. Sparrow. Synthetic peptide-based DNA complexes for nonviral gene delivery. *Adv. Drug. Deliv. Rev.* **30**:115–131 (1998).
9. S. Gottschalk, J. T. Sparrow, J. Hauer, M. P. Mims, F. E. Leland, S. L. Woo, and L. C. Smith. A novel DNA-peptide complex for efficient gene transfer and expression in mammalian cells. *Gene Ther.* **3**:448–457 (1996).
10. M. Nishikawa, S. Takemura, Y. Takakura, and M. Hashida. Targeted delivery of plasmid DNA to hepatocytes *in vivo*: optimization of the pharmacokinetics of plasmid DNA/galactosylated poly (L-lysine) complexes by controlling their physicochemical properties. *J. Pharmacol. Exp. Ther.* **287**:408–415 (1998).
11. J. G. Duguid, C. Li, M. Shi, M. C. Logan, H. Alila, A. Rolland, E. Tomlinson, J. T. Sparrow, and L. C. Smith. A physiochemical approach for predicting the effectiveness of peptide-based gene delivery systems for use in plasmid-based gene therapy. *Biophys. J.* **74**, 2802–2814, 1998.
12. G. Barany and R. B. Marrifield. Solid phase peptide synthesis. *In The Peptides*: Analysis, Synthesis, Biology, Acad. Press, New York (1980).
13. S. A. Kates. In "Peptides, Chemistry, Structure, Biology, Proc. 13th American Peptide Symposium". R. S. Hodges and J. D. Smith (eds.), Escom, Leiden, 1994, pp. 113.
14. Haensler, J. and Schuber, F. Preparation of neo-galactosylated liposomes and their interaction with mouse peritoneal macrophages. *Biochim. Biophy. Acta* **946**:95–105 (1988).
15. Otvos, T., Wroblewski, K., Kollat, E., Perczel, A., Hollosi, M., Fasman, G. D., Ertl, H. C. J. and Thurin, J. Coupling strategies in solid-phase synthesis of glycopeptides. *Peptide Res.* **2**:362–366 (1989).
16. K. Anwer, K. A. Earle, M. Shi, J. Wang, R. J. Mumper, B. Proctor, K. Jansa, H. C. Ledebur, S. Davis, W. Eaglstein, and A. P. Rolland. Synergistic effect of formulated plasmid and needle-free injection device for genetic vaccines. *Pharm. Res.* **16**:889–895 (1999).
17. A. L. Schwartz, S. E. Fridovich, and H. F. Lodish. Kinetics of internalization and recycling of the asialoglycoprotein receptor in a hepatoma cell line. *J. Biol. Chem.* **257**:4230–4237 (1982).
18. T. Kawabata, and L. Packer. Alpha-lipoate can protect against glycation of serum albumin, but not low density lipoprotein. *Biochem. Biophys. Res. Commun.* **203**:99–104 (1994).
19. S. I. Michael, and D. T. Curiel. Strategies to achieve targeted gene delivery via the receptor-mediated endocytosis pathway. *Gene Ther.* **1**:223–232 (1994).
20. F. Liu, Y. K. Song, and D. Liu. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* **6**:1258–1266 (1999).
21. G. Zhang, V. Budker, and J. A. Wolff. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum. Gene Ther.* **10**:1735–1737 (1999).