Preface

The purpose of this volume of *Methods in Molecular Medicine* is to set forth examples of the great variety of techniques and applications that are now emerging in the field of nonviral gene therapy. The book emphasizes not only specific approaches to gene delivery but, in particular, the best current methods to prepare, handle, and characterize gene delivery agents. These topics are of very broad importance since gene therapy evolves from its mostly academy-based experimental and clinical research to the ever increasing number of industry-driven programs directed toward commercial development. Successful introduction of nonviral gene therapy agents into the clinic should be expected to require rigorous manufacturing and analytical methods that readily meet the regulatory guidelines under which new drug candidates are reviewed for marketing approval. Exactly what those guidelines will prove to be certainly depends on the established guidelines for review of both biological and chemical therapeutics. Additionally, many new techniques are being devised and applied to gene therapy research; these techniques will be instrumental in developing and characterizing successful gene delivery agents.

Nonviral Vectors for Gene Therapy: Methods and Protocols has two main sections. To start with, there is a series of chapters on specific protocols for the synthesis, characterization, and application of gene delivery agents. Several chapters address the topic of materials to bind with DNA to form the compact condensed phases that facilitate cellular delivery. Variations on this theme are addressed by using peptide conjugates, synthetic polymers, and lipids. Increasingly refined methods for the characterization of delivery systems and their complexes with DNA are described both as part of synthesis protocols and as separate topics. One still relatively new analytical technique is atomic force microscopy; this should rapidly gain attention for its applicability to the characterization of DNA condensation. Subsequent chapters describe approaches to gene transfer in vivo, including direct delivery by intratumoral injection or indirect delivery by cell-specific targeting of DNA complexes.

The latter section of *Nonviral Vectors for Gene Therapy: Methods and Protocols* consists of a series of review-format chapters that provide extensive additional information for those preparing and characterizing gene trans-

fer agents. These chapters contain additional information on the use of novel materials to complex DNA. In a highly detailed series of chapters from the Middaugh Laboratory at the University of Kansas, a broad range of spectroscopic techniques is discussed in the context of characterizing nonviral gene delivery agents. Finally, a short review on renal gene therapy discusses an area not well represented elsewhere in treatments of gene therapy. For these topics, the coverage presented here points out a variety of opportunities for bringing new approaches to bear on the development and application of nonviral vectors in the research lab and, eventually, in the clinic.

I regret to note that during the preparation of this book, Dr. Jean-Michel H. Vos of the University of North Carolina at Chapel Hill passed away. We are fortunate to have had him contribute to this volume. His work is being continued by his colleagues, as well as those who will draw on the Vos lab's studies in their own research.

As with any project of this nature, it would not have been possible without an incredible amount of effort and patience on the part of each and every contributor, the Series Editor, Professor John M. Walker, and Craig B. Adams at Humana Press. Thank you to everyone who has supported this project, and thank you to the readers whose interest in this volume make the efforts to produce it all worthwhile.

Mark A. Findeis

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Cationic α-Helical Peptides for Gene Delivery into Cells

Takuro Niidome and Haruhiko Aoyagi

1. Introduction

Development of nonviral gene transfer techniques has progressed, particularly the use of several kinds of cationic lipids and cationic polymers such as polylysine derivatives, polyethyleneimines, polyamidoamine dendrimers, and so on, which electrostatically form a complex with the negatively charged DNA, which can be taken up by the cells. Furthermore, targeted gene transfer has also been realized by modification of the gene carriers using cell-targeting ligands such as asialoorosomucoid, transferrin, insulin, or galactose.

Recently, novel gene transfer techniques have been reported, in which an amphiphilic α-helical peptide, containing cationic amino acids is used as a gene carrier into cells. Wyman et al. ***(1)*** employed a peptide, KALA (WEAK-LAKA-LAKA-LAKH-LAKA-LAKA-LKAC-EA), which is derived from the sequence of the N-terminal segment of the HA-2 subunit of the influenza virus hemagglutinin involved in the fusion of the viral envelope with the endosomal membrane. This peptide showed several functions in the transfection process, e.g., condensing DNA and causing an endosome-membrane perturbation, which enables it to deliver the incorporated DNA to the cytosol, which is essential for efficient transfection. Similarly, the authors also found the transfection technique, which is mediated by some amphiphilic α-helical peptides (e.g. Ac-LARL-LARL-LARL-LRAL-LRAL-LRAL-$NHCH_3$ [4_6] and KLLK-LLLK-LWKK-LLKL-LK [Hel]) as shown in **Table 1** ***(2–5)***. After that, for the purpose of refining of the peptide structure, we investigated the influence of the peptide chain length on gene transfer ability. As a result, 16 and 17 amino acid residues were sufficient to form aggregates with the DNA,

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Table 1
Structures of Amphiphilic α-Helical Peptides

Peptide	Sequence	Chain length	Cationic charge
4_6	Ac-LARL-LARL-LARL-LRAL-LRAL-LRAL-NH_2	24	6
$4_6\Delta8$	Ac-LARL-LRAL-LRAL-LRAL-NH_2	16	4
Hel	KLLK-LLLK-LWKK-LLKL-LK	18	7
HelΔ1	LLK-LLLK-LWKK-LLKL-LK	17	6

and transfer the DNA into the cells in the deletion series of 4_6 and Hel, respectively (**Table 1**; ***6***). In addition, the authors succeeded in constructing a multiantennary galactose-modified peptide containing four galactose residues that serve for efficient binding to the asialoglycoprotein receptor on hepatoma cells ***(7)***.

This chapter focuses on synthesis of the peptides and a method of gene transfer using them. As is well known, a peptide is readily synthesized because of the development of an automatic peptide synthesis apparatus and reagents for synthesis. From this point of view, it is expected that the gene transfer method mediated by the peptide is easily accepted by many researchers taking part in the gene therapy study.

2. Materials

2.1. Peptide Synthesis

1. Peptide synthesis apparatus (ABI 431A, PE Biosystems).
2. Fmoc-Lys(Boc) preloaded Wang resin, Rink amide resin (100–200 mesh) (Calbiochem-Novabiochem, CA).
3. Fmoc protected amino acids, 2-(1H-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU), *N*,*N*,-diisopropylethylamine, NMP, dichloromethane (DCM), piperidine (Watanabe Chemical, Hiroshima, Japan).
4. Thioanisole, *m*-Cresol, ethandithiol, trifluoracetic acid (TFA), acetonitrile (Wako Chemicals, Osaka, Japan).
5. High-performance liquid chromatography (HPLC) apparatus (Hitachi L7100 System, Tokyo, Japan).
6. Reverse-phase (RP)-HPLC column (YMC-Pack C4, ϕ10 × 150 mm, Kyoto, Japan).
7. Matrix-assisted Laser Desorption Ionization-Time of Flight-Mass Spectra (MALDI TOF-MS) apparatus (Voyager DE STR, PE Biosystems).

2.2. Preparation of Plasmid DNA

1. Plasmid DNA, which contains a luciferase gene and SV40 promoter (PicaGene control vector, PGV-C), was purchased from Toyo Ink (Tokyo, Japan).
2. Plasmid DNA (pCMVluc), containing a luciferase gene under control of cytomegalovirus enhancer/promoter, was prepared by removing the *Bgl*II and

*Hin*dIII insert of the plasmid PGV-C (Toyo Ink), and ligating with the *Bgl*II and *Hin*dIII fragment from the pRc/CMV (Invitrogen), which contains cytomegalovirus promoter.

3. Closed circular plasmid DNA was purified by ultracentrifugation in cesium chloride gradients. The plasmid preparations showed a major band of closed circular DNA and minor amount (<20%) of nicked plasmid.

2.3. Cell Culture and Gene Transfer into Cells

1. COS-7 cells (a monkey kidney cell line, RCB accession no. RCB0539), HeLa cells (a human cervix, RCB accession no. RCB0271) and CHO cells (a Chinese hamster ovary, RCB accession no. RCB0285) (RIKEN Cell Bank, Tsukuba, Japan).
2. HuH-7 cells (a human hepatoma cell line, JCRB accession no. JCRB0403) (Health Science Research Resources Bank, Osaka, Japan).
3. Dulbecco's modified Eagle's medium, RPMI 1640, media supplements, and heat-inactivated fetal calf serum (IWAKI Glass, Chiba, Japan).
4. PicaGene luminescence kit (Toyo Ink).
5. Luminometer (Maltibiolumat LB9505, Berthold, Germany).

3. Methods

3.1. Peptide Synthesis

As shown in **Table 1**, the authors recommend two kinds of peptides, $4_6\Delta8$ and HelΔ1, which were refined from two original α-helical peptides, 4_6 and Hel, respectively ***(6)***.

3.1.1. Elongation of Peptide Chain on Resin

Peptides can be manually synthesized by the stepwise elongation of Fmoc protected amino acid on Rink amide resin (for 4_6; 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin, 0.43 mmol/g resin) or Fmoc-Lys(Boc) preloaded Wang resin (for Hel; Fmoc-Lys(Boc)-*p*-benzoyloxy alcohol resin, 0.58 mmol/g resin) in 0.1 mmol scale as described by Fields et al. ***(8)***, and in Catalog & Peptide Synthesis Handbook of Calbiochem-Novabiochem. The Fmoc amino acid derivatives used are as follows: Ala, Arg(Pbf), Leu, Lys(Boc), and Trp. The coupling protocol is shown as follows:

1. Wash: 2 mL DCM (3×).
2. Wash: 2 mL NMP (3×).
3. Wash: 2 mL of 20% piperidine/*N*,*N*,-dimethylformamide (1×).
4. Deprotection of Fmoc group: 2 mL 20% piperidine/DMF (15 min).
5. Wash: 2 mL NMP (5×).
6. Coupling of amino acid; Fmoc-amino acid (0.3 mmol), HBTU (0.3 mmol), HOBt (0.3 mmol), and *N*,*N*,-diisopropylethylamine (DIEA) (0.6 mmol) in 3 mL NMP:DMF (1:1) (15 min).
7. Wash: 2 mL DMF (3×).
8. Kaiser test ***(9)***: When the coupling is incomplete, the protocol is repeated from **step 6**.

As a matter of course, it is possible to elongate the peptide chain using automatic peptide synthesizer (PE Biosystems, ABI 431A). In this case, the peptides are also synthesized by FastMoc method in 0.1 mmol scale. Even if single coupling of amino are applied for all elongation steps, satisfactory peptides in purity are obtained. In the case of modifying by acetyl group to N-terminal of peptide, 4_6, the Fmoc-deprotected peptide on resin is treated with 1 mmol acetic anhydride in 2 mL NMP for 20 min. After the resin is washed by DCM and methanol, they are dried *in vacuo*.

3.1.2. Deprotection and Cleavage of Peptide Resin

The protecting groups and the resin are removed with TFA (2.0 mL) in the presence of *m*-cresol (60 µL), ethandithiol (180 µL), and thioanisole (360 µL), in a round flask. After swirling at room temperature for 60 min, the resin is removed by filtration under reduced pressure and washed by TFA. Twenty milliliters of cold diethylether is added to the filtrate, then, the flask can be cooled with ice to further assist precipitation. Crude peptide is isolated by filtration under reduced pressure and washed by cold diethylether.

The crude peptides can be purified by RP-HPLC on a YMC-Pack C4 column (ϕ10 × 250 mm) with a linear gradient of water–acetonitrile containing 0.1% TFA. Peptide $4_6\Delta8$ and HelΔ1 are eluted at about 80 and 55% of acetonitrile, respectively. The authors recommend that if possible, the crude peptide be passed through a column of Sephadex G-10 or G-15 (ϕ10 × 250 mm) with 10% acetic acid before purification by RP-HPLC to avoid damaging the HPLC column. The peptide fractions are collected, then lyophilized. The yields obtained after purification are about 80 (34 µmol) and 100 mg (36 µmol) in the cases of $4_6\Delta8$ (as 4TFA salt) and HelΔ1 (as 6TFA salt), respectively. The purified peptides can easily be identified by MALDI-TOF-MS ($[M + H]^+$ = 1874.4 [$4_6\Delta8$], 2105.7 [HelΔ1]).

3.2. Preparation of Peptide–DNA Complex

Complex of peptide and DNA is prepared by mixing 2.5 µg plasmid DNA (PGV-C) with the peptides at peptide:DNA charge ratio of 2.0 in serum-free medium. The authors protocol is shown as follows:

1. Prepare the DNA solution: 2.5 µg plasmid DNA in 250 µL serum-free culture medium.
2. Prepare the peptide solution: 1.6 m*M* (as cationic groups) peptide aqueous solution. Practically, 930 µg $4_6\Delta8$ • 4TFA or 743 µg HelΔ1 • 6TFA are dissolved in 1.0 mL sterilized water.
3. 10 µL Peptide solution is added to 250 µL plasmid DNA solution.
4. Stand for 15 min at room temperature.

3.3. Gene Transfer Protocol into Cultured Cells

1. Plate cells in 24-well tissue culture dishes (ϕ16 mm) at 1×10^5 cells/well and grow overnight in an atmosphere of 5% CO_2 at 37°C.

2. Wash twice with 1 mL serum free medium.
3. The peptide–DNA complex as described above is poured gently to the cells.
4. After incubation for 3 h at 37°C, 1 mL medium containing serum is added.
5. After incubation for 12 h at 37°C, the medium is replaced with 1 mL fresh medium containing serum and the cells are further incubated for 24 h.
6. Harvest of cells and luciferase assays are performed as described in the protocol of PicaGene luminescence kit using a luminometer.
7. The protein concentrations of the cell lysates are measured by Bradford assay using bovine serum albumin as a standard. The light unit values shown in the figures represent the specific luciferase activity (relative light units/mg protein), which is standardized for total protein content of the cell lysate. The measurement of gene transfer efficiency is performed in triplicate.

To date, the authors have tested gene transfer efficiencies of the peptides into several cell lines, such as COS-7, HeLa, CHO, and HuH-7 cells. **Figure 1** shows the results for each cell line. To compare the efficiencies of the peptides to a commercially available gene transfer agent, we employed Lipofectin (Gibco-BRL). As a result, the efficiencies of the peptides were similar to or about 10-fold lower than those of Lipofectin. Except for the case of needing a large amount of expression product, it can be said that the peptides are enough to use as a gene carrier. On the other hand, the peptides showed different efficiencies depending on the cell lines. These peptides can be chosen according to the cell lines.

The authors also evaluated cytotoxic activities of the peptide–DNA complexes, using Alamer Blue™, under the same conditions as those in the transfection procedure. As a result, little cytotoxic activity of the complexes could be observed. However, when the complex is prepared at peptide:DNA charge ratio of 4 and more, considerable cytotoxic activities are observed, which the authors consider to result from membrane perturbation activities originated from the amphiphilic structures of the peptides ***(3,5)***.

3.4. Active Targeting of Gene into Hepatoma Cells Using Galactose Modification of Peptide

As described in the Introduction, peptide is easily available because of the development of its synthetic technology. Therefore, this allows design and synthesis of functional gene carrier molecules such as carbohydrate-modified peptide for targeted gene delivery. Furthermore, peptide-based gene carrier enables construction of well-defined molecules, which cannot be achieved by polymer-based molecule. Here is introduced a synthesis of a multiantennary galactose-modified peptide and its application to a human hepatoma cell line.

3.4.1. Preparation of Multiantennary Galactose-Modified Peptide

The synthesis method is described as follows and is summarized in **Fig. 2**.

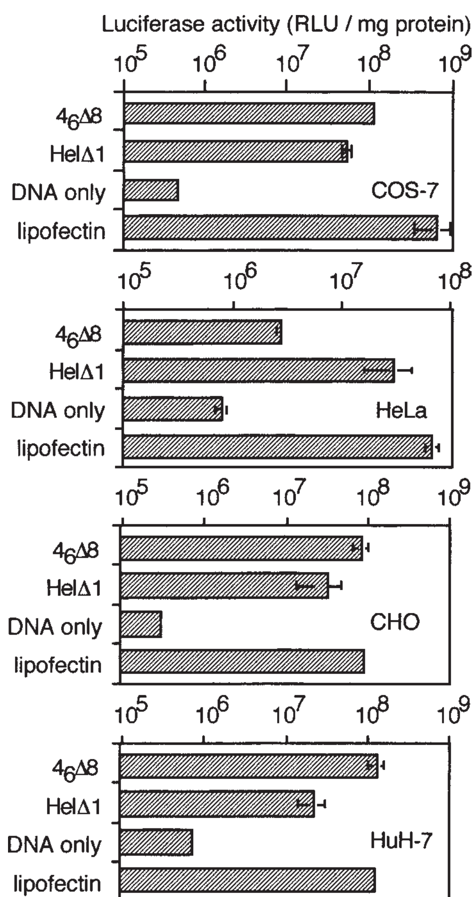

Fig. 1. Gene transfer efficiencies of the peptides into several cell lines.

1. α-Helical peptide portion can be synthesized by ordinary Fmoc solid-phase method on Rink amide resin (*see* **Subheading 3.1.1.**).
2. After coupling Fmoc–2-(2-aminoethoxy) ethanol as a linker, Fmoc-βAla-Lys(Fmoc) is coupled twice. Fmoc groups of secondary coupled Fmoc-βAla-Lys(Fmoc) are removed by piperidine. At this step, the peptide has four amino groups in the molecule.
3. After cleavage from resin and deprotection, the peptide is purified by RP-HPLC as similar condition to the case of peptide 4_6 (*see* **Subheading 3.1.2.**)
4. The amino groups of the peptide can be modified with lactose by aldimine formation, followed by reduction with $NaBH_3CN$ of the secondary amines as follows. The purified peptide (11 μmol, 50 mg as 10TFA salt) is mixed with a solution of lactose (530 μmol, 190 mg as monohydrate) in 500 μL 10 m*M* aqueous

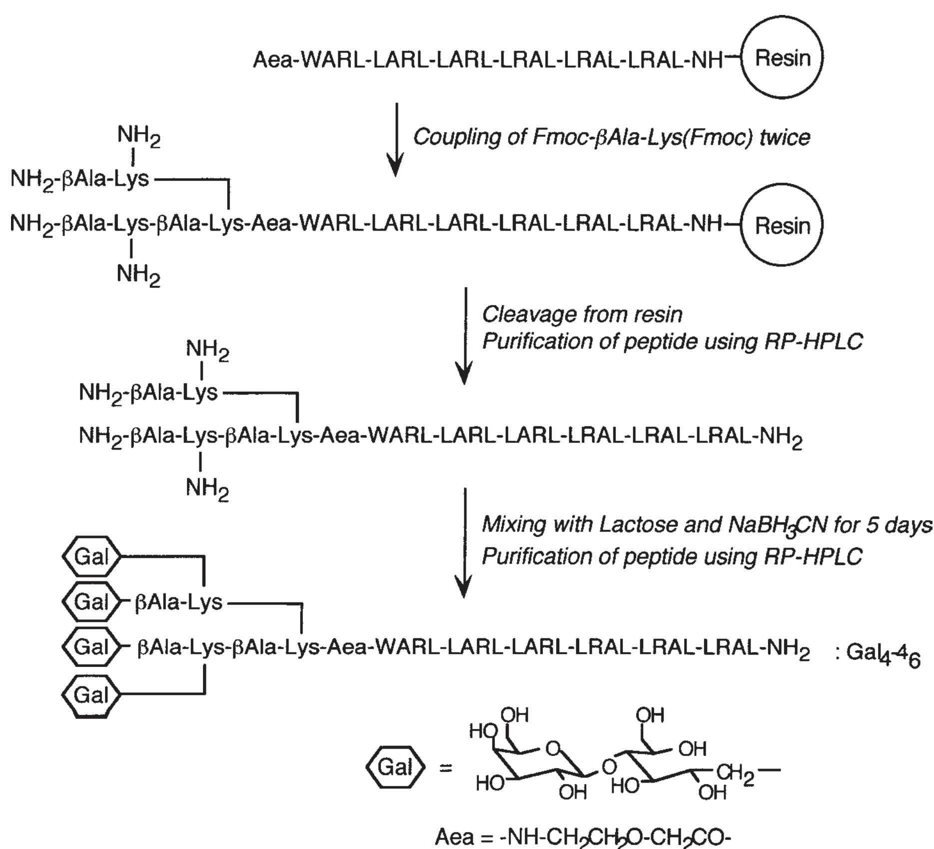

Fig. 2. Outline of synthesis of galactose-modified peptide, Gal_4–4_6.

sodium acetate, pH 5.0, at 37°C, then $NaBH_3CN$ (44 μmol, 2.8 mg each) is added to the solution at 12-h intervals. After total incubation for 60 h at 37°C, the resultant galactose-modified-peptide is purified by RP-HPLC. Yield is 20 mg (38%).

5. Identification is performed by MALDI-TOF-MS ($[M + H]^+ = 4816.7$). The peptide concentration in solution is determined from UV-absorbance of Trp at 280 nm in a buffer containing 6 *M* Gu • HCl (ε = 5500).

3.4.2. Gene Transfer Using Galactose-Modified Peptide into Hepatoma Cell Line, HuH-7

Protocol for gene transfer into HuH-7 cells, a human hepatoma cell line, is similar to that described in **Subheadings 3.2.** and **3.3.** Complex of peptide and DNA is prepared by mixing 2.5 μg plasmid DNA (pCMVluc) with Gal_4–4_6 at peptide:DNA charge ratio of 2.0 in serum free medium.

1. Prepare the DNA solution: 2.5 μg plasmid DNA in 250 μL serum free RPMI 1640.

2. Prepare the peptide solution: 1.6 m*M* (as cationic groups) peptide aqueous solution. Practically, 1.5 mg Gal_4–4_6 • 10TFA is dissolved in 1.0 mL sterilized water.
3. 10 μL peptide solution is added to 250 μL plasmid DNA solution.
4. Stand for 15 min at room temperature.
5. The peptide–DNA complex as described above is poured gently on to the cells, which are washed twice with 1 mL serum-free RPMI 1640, beforehand. Following procedures are same to **steps 4–7** in **Subheading 3.3.**

As a result of measurement of luciferase activity in HuH-7 cells, the activity of Gal_4–4_6 was 300-fold higher than that of 4_6. In addition, the authors could confirm that the DNA complex of Gal_4–4_6 was internalized into the cells via the asialoglycoprotein receptor (*see* **Subheading 3.5.**).

This chapter introduced a galactose-modified peptide containing 2-(2-aminoethoxy)ethanol and βAla as a linker, which allows the galactose residues to be easily recognized by the receptor. However, the authors found that a Gal_4–4_6 derivative without any linker showed a high efficiency into HuH-7 cells, as similar to that of Gal_4–4_6. Insertion of linker into the peptide system is not always necessary for recognition of the galactose residues by the receptor.

3.5. Commentary

In the authors' series of studies, it has become clear that the hydrophobic region on the amphiphilic structure of the peptides plays an important role in binding to the plasmid DNA and formation of aggregates with the DNA. It is likely that the hydrophobic region of the peptides induces stable oligomers with a well-defined number of monomers by self-association with their hydrophobic faces in aqueous solution. As a result, the oligomer would behave like a polycation, which can form aggregates with DNA **(Fig. 3)**. Furthermore, the authors indicate that the hydrophobic region is also important for the disruption of the endosomal membrane in the cell, which can transfer the incorporated DNA to cytosol and prevent the degradation of the DNA in the lysosomal vesicles ***(3,5)***.

In order to clear detail translocation pathway of the DNA in the cell, the authors evaluated effects of several endocytosis inhibitors on the gene transfer efficiencies of peptide 4_6. Treatment with cytochalasin B, which depolymerizes the microfilaments of actin and blocks the uncoated pit-mediated endocytosis (macropinocytotic process) ***(10)***, reduced to 25% of the original efficiency of 4_6; no effect was observed in the case of treatment with chlorpromazine, an inhibitor of clathrin-dependent, receptor-mediated endocytosis ***(11)***. Furthermore, *N*-ethylmaleimide (NEM), which inhibits fusion between endosomes at an early stage of the endocytic pathway ***(12)***, reduced to 50% of the original efficiency, and the microtubule-depolymerizing agent, nocodazole, which interferes with transport from the early to late endosome mediated by endocytic carrier vesicles ***(13)***, increased the efficiency of the peptide by sixfold. From

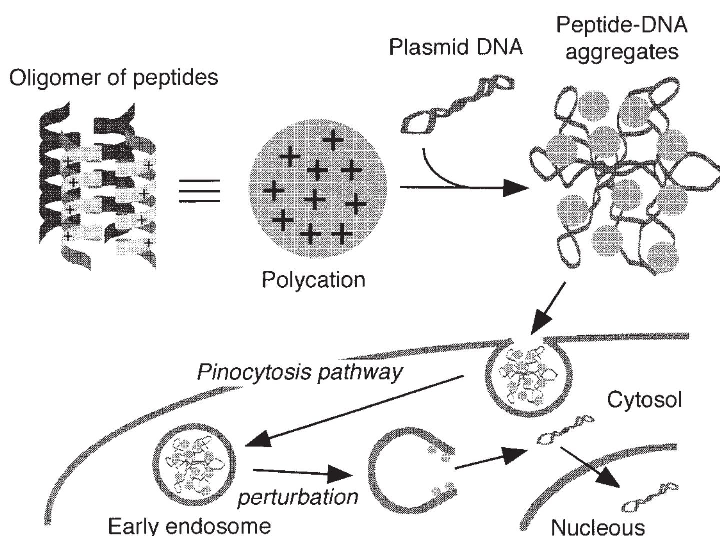

Fig. 3. Formation of peptide–DNA complex and its transfer pathway into cell.

these results, it is reasonable to suppose that the complexes of peptide 4_6 and plasmid DNA were internalized by macropinocytotic process, and a part of the complexes could be translocated from the endosomal compartment to the cytosol during an early endosome step **(Fig. 3)**. However, the other part of the complexes would be translocated into degradation compartments such as late endosome and lysosome, where the complex could no longer be translocated to the cytosol. In order to enhance gene transfer efficiency, it will be necessary to consider active translocation to the cytosol of the DNA complex. Because there is now no information for translocation into nucleus of the complex, elucidation of this transfer mechanism in the cell will give a clue to construct a novel gene carrier with higher efficiency.

The authors have also studied the transfer pathway of the DNA complex with Gal_4–4_6. At first, the competitive effects of asialofetuin, which is internalized into hepatoma cells via the asialoglycoprotein receptor-mediated endocytosis, and fetuin, which is not recognized by the receptor, on the transfer efficiencies of the peptides were examined. As a result, the transfer efficiency of Gal_4–4_6 is reduced to 1% of the original efficiency in the presence of the asialofetuin, but no effect was observed in 4_6. However, fetuin showed weak effect compared with asialofetuin.

In addition, the authors evaluated effects of several endocytosis inhibitors on the gene transfer efficiencies of Gal_4-4_6. Treatment with chlorpromazine significantly reduced the efficiency; no significant reduction was observed in the case of treatment with cytochalasin B. This result suggested that the

internalization of Gal_4–4_6 was mediated by the clathrin-dependent, receptor-mediated endocytosis. Furthermore, NEM reduced to 50% of the original efficiency, and nocodazole increased the efficiency by twofold. From these results, DNA complex with the galactose modified peptide, Gal_4–4_6, would be translocated from the endosomal compartment to the cytosol during an early endosome step as in the case of 4_6.

Finally, to apply these galactose-modified peptides for targeted gene delivery in vivo, it is necessary to further examine the cell selectivity using several cell lines, stability in blood, capture by the reticuloendothelial system, and so on. When these points are solved, this delivery system will be one of the powerful tools for gene therapy.

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