Use of Synthetic Peptides for Non-viral Gene Delivery

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1 Introduction

The development of non-viral gene delivery systems is an important key to solving several problems occurring in viral gene delivery, such as endogenous virus recombination, oncogenic effects and unexpected immune responses. As discussed in other chapters of this book, most of the basic techniques of non-viral gene delivery systems have relied on liposome and polymer chemistries; however, peptide chemistry has also contributed strongly to this field. Peptides can be synthesized automatically or manually and their chemistry allows the design and synthesis of complicated structures, e.g. ligand-modified peptides recognizable by specific cells, dye-modified peptides to trace their locations in cells and tissues, and other "intelligent" peptides to achieve functional gene delivery. It is expected that the relative ease of peptide construction will provide researchers with a wide range of molecules as well as important information about the structural requirements for functional gene delivery. Peptides play many roles in gene delivery, such as providing a simple cationic moiety to bind DNA, acting as a ligand or facilitating the release of DNA into the cytosol, carrying a signal for transport to the nucleus, or functioning as a sensor for the regulation of gene expression in cells. In this chapter, some examples of the use of peptides in gene delivery are described, and the prospects of utilizing such peptides in gene therapy are discussed.

2 Peptides as Cationic Moieties of Gene Carriers

2.1 Oligolysines

In the early 1990s, the most commonly used DN-condensing agent for gene transfection into cells was polylysine. Its amino groups were modified with several kinds of ligands, e.g., asialoorosomucoid, transferrin, and carbohydrates, in order to achieve cell recognition and receptor mediated uptake. However, the high molecular weight

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and the polydispersity of polylysine complicated region-specific modification with ligands. To solve these problems, the use of synthesized oligolysines was reported by Gottschalk et al. (1996). They initially synthesized a peptide ($YKAK_4WK$), which had four clustered lysine moieties, as an analog of spermine, a tertamine. However, the peptide was not long enough to allow successful transfection. Having established a minimum length, $YKAK_8WK$ showed efficient gene transfection when an endosome-disruptive peptide (described below) was added. Wadhwa et al. (1997) also tested oligolysines with several chain lengths and found that 18 lysines followed by a tryptophan and a cysteine alkylated with iodoacetoamide ($AlkCWK_{18}$; $Cys(CH_2CONH_2)$-Trp-$(Lys)_{18}$) formed DNA complexes and showed efficient gene transfection into cultivated cells. In addition, a series of branched cationic oligopeptides that differed in the number and type of cationic amino acids was examined by Plank et al. (1999). They found that a minimal chain length of six cationic amino acids was required for the formation of DNA complexes that were incorporated into cells, and that a branched peptide consisting of lysine residues as cationic moiety was a weaker activator of the complement system in blood than arginine residues and high-molecular-weight polylysine.

Oligopeptides form small DNA complexes that show efficient gene transfection in cultivated cells; however, due to their lower affinity for DNA, they are not stable enough to be utilized in vivo. To stabilize the complex, peptides bound to DNA were cross-linked with glutaraldehyde (Adami and Rice 1999). After forming condensates between a 20-amino-acid peptide (CWK18) and plasmid DNA, glutaraldehyde was added. The improved stability imparted by glutaraldehyde cross-linking was demonstrated by the increased resistance of DNA condensates to serum endonucleases. Although a decrease in the magnitude of transient gene expression was determined for cross-linked DNA condensates, long lasting steady-state expression was observed. The advantage of interpeptide disulfide bonding was reported by McKenzie et al. (2000a). Disulfide cross-linking is attractive because of the release of DNA that is expected to be triggered by the reducing environment of the cytosol (Fig. 1). The authors used peptides containing multiple cysteines, such a Cys-Trp-$(Lys)_{18}$. Disulfide bond formation after condensing with plasmid DNA led a decrease in particle size of the condensates and prevented their dissociation. The cross-linked peptide DNA

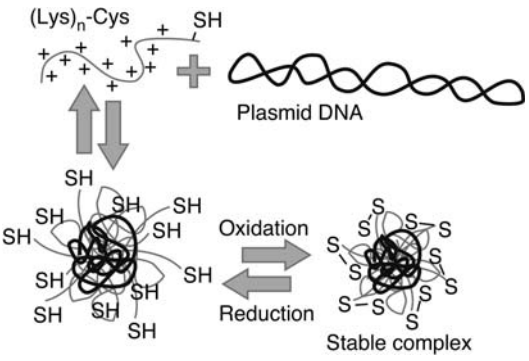

Fig. 1. Formation of cross-linked peptide-DNA complexes. The complex is formed through electrostatic interaction between cationic peptides and anionic DNA, followed by interpeptide oxidation to form disulfide bonds that stabilize the DNA complex

condensates had an higher transfection efficiency in vitro than uncross-linked ones. Furthermore, as the amount of incorporated DNA into cells was not affected by cross-linking of the peptides, it was suggested that the enhanced gene expression obtained with cross-linking was due to intracellular release of DNA triggered by disulfide bond reduction.

In order to improve cross-linked peptide DNA condensates with respect to in vivo gene delivery, PEG-modified and mannose-modified peptides were synthesized. After forming condensates with DNA, the peptides were cross-linked by glutaraldehyde (Yang et al. 2001) and disulfide bonds (Park et al. 2002; Kwok et al. 2003). The DNA of these cross-linked condensates containing PEG chain and branched mannose had a longer half-life in liver, and specifically accumulated in Kupffer cells after intravenous administration; long lasting gene expression was also shown. These are excellent examples demonstrating the function of PEG as a stealthy layer that blocks non-specific binding of blood components, and of mannose as a ligand for the mannose receptor, which is specifically expressed on Kupffer cells. The combination of several functional peptides in addition to the cross-linking technique based on the oligopeptide will enable a complicated, intelligent gene delivery system to be constructed with minimum effort.

2.2 Amphiphilic α-Helical Peptides

Gene transfection into cultivated cells mediated by a cationic peptide (KALA; WEAK-LAKA-LAKA-LAKH-LAKA-LAKA-LKAC-EA) was reported by Wyman et al. (1997). The peptide was derived from influenza hemagglutinin peptide and had an amphiphilic α-helical structure. In gel retardation and ethidium bromide displacement assays, KALA formed stable complexes when the C/A (cation of peptide/anion of DNA) ratio exceeded 1 : 1. The transfection ability of KALA was determined by the expression of luciferase from its cDNA in several kinds of cells. The complex composed of KALA and plasmid DNA at a C/A ratio of 10/1 showed the highest transfection efficiency in CV-1 cells. For this high efficiency, the amphiphilic structure was an important aspect, since, in general, amphiphilic peptides have membrane perturbation activity. After uptake of the complex into cells by endocytosis, KALA disrupted the endosomal membrane, resulting in transfer of the DNA to the cytosol. This peptide provided a starting point for the construction of a family of peptides incorporating other functions to improve DNA delivery systems.

Niidome et al. (1997) also reported on the DNA-binding and transfection abilities of several amphiphilic α-helical peptides. Peptides with a large hydrophobic region could strongly bind to DNA, and the formation of large twisted-fiber-like aggregates was observed. In transfection studies, these peptide showed high gene-transfer ability into COS-7 cells.

In order to clarify the significance of the hydrophobic region in amphiphilic peptides in relation to their transfection ability, Ohmori et al. (1998) employed five kinds of peptides with a systematically varied hydrophobic-hydrophilic balance in their amphiphilic structures (Hels 13–5, 11–7, 9–9, 7–11, and 5–13) (Fig. 2). The authors evaluated the relationship between the structure, the DNA-binding ability, and the gene transfer ability of the peptides into COS-7 cells (Table 1). This study also showed that the hydrophobic region plays an important role in disrupting the endosomal

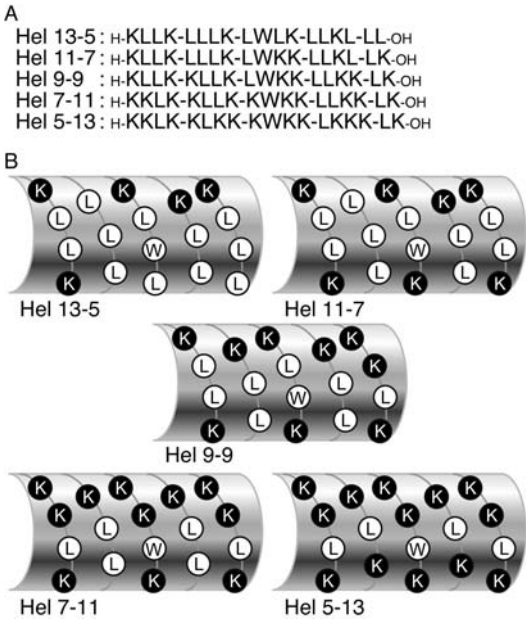

FIG. 2A, B. Structures of cationic α-helical peptides. Amino acid sequences of the designed peptides (**A**) and illustration of the amphiphilic structures of the α-helical peptides (**B**)

TABLE 1. Cationic α-helical peptides and their DNA-binding and transfection abilities

Peptide	Sequence	DNA binding	Transfection ability
Hel 13–5	KLLK-LLLK-LWLK-LLKL-LL	+++	+++
Hel 11–7	KLLK-LLLK-LWKK-LLKL-LK	+++	+++
Hel 9–9	KLLK-KLLK-LWKK-LLKK-LK	+	+
Hel 7–11	KKLK-KLLK-KWKK-LLKK-LK	–	–
Hel 5–13	KKLK-KLKK-KWKK-LKKK-LK	–	–

membrane, which can prevent the degradation of DNA in lysosomal vesicles. Furthermore, Niidome et al. (1999a) reported that, in a deletion series of the α-helical peptides, 16–17 amino acid residues were sufficient to form a complex with DNA and to transfer it into the cells. But even if the chain length was not enough for transfection, the efficiency could be recovered by modifying the peptide with a hydrophobic chain, such as a palmitoyl group (Niidome et al. 1999b).

As an application of α-helical peptides for functional gene delivery, a galactose-modified peptide was synthesized in order to achieve receptor-mediated gene delivery into hepatoma cells (Niidome et al. 2000). The galactose-modified peptides formed complexes with a plasmid DNA and showed gene transfer abilities into HuH-7 cells, a human hepatoma cell line. Considerable inhibition of the transfection efficiency by the addition of asialofetuin, which is a ligand for the asialoglycoprotein receptor, was observed in all galactose-modified peptides.

The use of amphiphilic α-helical peptides as gene carriers for transfection into cells has the following advantages. (1) The amphiphilic peptides, which have a smaller chain length than other gene carrier peptides such as a polylysine, are still able to bind DNA and form stable complexes as the result of the strong intermolecular hydrophobic interaction. The aggregates can be internalized by endocytosis and remain resistant to digestion in lysosomal vesicles. (2) The membrane perturbation activity of amphiphilic peptides plays an important role in the escape from lysosomal degradation.

2.3 Dendritic Poly(L-Lysine)

The use of dendritic molecules as a gene carrier was reported by Haensler and Szoka (1993). Dendrimers are a new class of highly branched spherical polymers that are mono-dispersed and show high charge densities that are restricted to the surface of the molecule. These interesting features and the high flexibility of dendrimers, which allow defined molecules with functional groups to be constructed, are advantageous in the development of intelligent gene delivery systems. Previously, the synthesis of dendritic poly(L-lysine) using Boc-L-Lys(Boc)-OH as a branch unit was reported, and the properties were then described by Aharoni et al. (1982). Strictly speaking, the dendritic molecule cannot be classified in the original concept of the "dendrimer," described by Tomalia et al. in 1985, because it consists of asymmetrical L-lysine residues; nonetheless, dendritic molecules, consisting of amino acids, are expected to enable the construction of gene carriers using conventional peptide chemistry. For example, dendritic poly(L-lysine) modified by various ligands, such as a sugar chain, endosome-disrupting agents, and an intracellular targeting signal, should be readily synthesizable by selecting several protecting groups of the lysine, such as the Boc, Z, and Fmoc groups. As candidates for the gene carrier among several types of dendritic poly(L-lysine)s, block copolymers consisting of poly(ethylene glycol) and poly(L-lysine) dendrimer were tested by Choi et al. (1999, 2000). The copolymers were 50–150 nm and formed a spherical complex with DNA; they did not show any cytotoxity toward NIH3T3 cells even at higher concentrations. Shah et al. (2000) also reported the use of amphipathic asymmetric poly(L-lysine) dendrimers, in which the dendritic structure was attached to α-amino myristic acids as a hydrophobic tail. Amphipathic dendrimers bearing eight and 16 terminal amines formed stable complexes with plasmid DNA at a C/A ratio of 5/1 and allowed gene transfection into BHK-21 cells without significant cytotoxicity

The use of mono-dispersed dendritic poly(L-lysine) with a hexamethylenediamine core was reported by Ohsaki et al. (2002) (Fig. 3). In that study, the DNA-binding abilities and transfection efficiency of first- to sixth-generation dendritic poly(L-lysine)s were investigated. As shown by agarose gel shift and ethidium bromide titration assays, the dendritic poly(L-lysine)s of third generation and higher formed complexes with plasmid DNA, and the degree of compaction of the DNA was increased with increasing generation number. Fifth- and sixth-generation dendritic poly(L-lysine)s, which have 64 and 128 amine groups on the surface of the molecule, respectively, showed efficient gene transfection ability into several cultivated cell lines without significant cytotoxity.

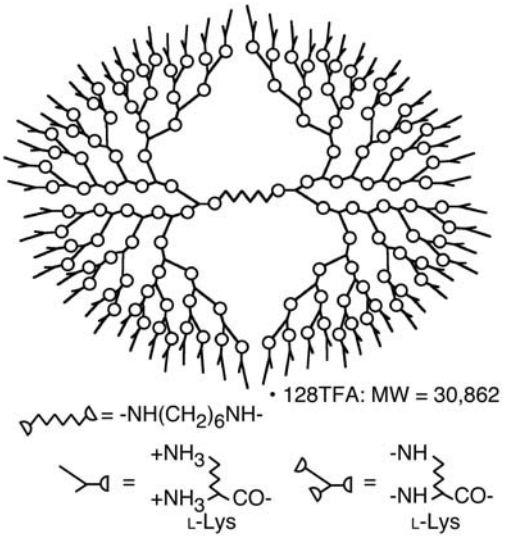

Fig. 3. Structure of sixth-generation dendritic poly(L-lysine) (KG6)

In order to understand the mechanism of complex formation of sixth-generation dendritic poly(L-lysine) (KG6) with plasmid DNA, the complex was analyzed using atomic force microscopy (Okuda et al. 2003a). After mixing for 15 min, 1- to 2-μm assemblies of complexes composed of several small particles (50–200 nm) had formed. At the same time, small, individual complexes of 50 to 500 nm were observed on a mica surface. After incubation for 2 h, only the large complexes were found on the mica surface. As the transfection efficiency of KG6 was correlated with the mean size of the DNA complexes, it was suggested that large complexes of more than 1 μm are the major species contributing to transfection in the case of in vitro transfection. With regard to the mechanism, it has been suggested that the dendrimer forms a complex with plasmid DNA, and the complex is incorporated via the endocytosis pathway. Subsequently, a part of the complex escapes from the endocytotic vesicle by the proton sponge effect of the dendrimer, similar to the case of polyethylenimine (Boussif et al. 1995), and the gene encoded in the plasmid DNA is expressed after transport into the nucleus.

Okuda et al. (2003b) also examined the effect of substituting terminal cationic groups on gene delivery into cells. KGR6 and KGH6, in which terminal amino acids were replaced by arginines and histidines, respectively, were synthesized. KGR6 bound to the plasmid DNA as strongly as KG6, whereas KGH6 showed decreased binding ability. The transfection efficiency of KGR6 into several types of cultivated cells was three- to 12-fold higher than that of KG6. The terminal guanidium groups of KGR6 are advantageous for interaction with the cell membrane or endosomal membrane and facilitate escape of the DNA into the cytosol. However, at present, more information about the efficiencies at each step of the transfection process is required to fully explain the enhancing effect of the arginines. By contrast, KGH6 showed no transfection efficiency ; however, once it was mixed with DNA under acidic conditions (pH 5.0), DNA-complexes were formed that showed high transfection efficiency, compa-

rable to that obtained with KG6-mediated transfection. The unique character of KGH6 is a basic and valuable tool that will allow pH-dependent in vitro and in vivo gene transfection systems to be constructed.

Interestingly, the DNA-complex of this dendrimer had a low zeta potential (+3 mV), and showed high transfection efficiency even in the presence of 50% serum. The neutral surface of complexes with KG6 may contribute to its inert behavior in the presence of serum. From the viewpoint of in vivo gene delivery, this feature is advantageous, since non-specific interaction with serum components are thereby reduced, and the circulation half-life of the complexes in the blood will thus be extended. Moreover, it is not necessary to modify the carrier molecule with a PEG chain in order to confer stealth-like characteristics, as with the oligolysine, described above. To confirm the stealth ability of KG6-containing DNA complexes in the blood and the potential of KG6 as a functional gene carrier that can be applied in vivo, the biodistributions of plasmid DNA in normal and tumor-bearing mice after intravenous injection of DNA complexes with KG6 or other gene carrier molecules were evaluated (Kawano et al. 2004). Southern blotting analysis revealed that plasmid DNA complexes with KG6 at a C/A ratio of 8.0 circulated in the blood for 3 h after intravenous injection. In tumor-bearing mice, plasmid DNA injected with KG6 was observed in the tumor at 60 min after intravenous injection, while no DNA was present in the tumor using DOTAP/Chol liposomes. The stealth ability of KG6-containing DNA complexes in the blood would enhance their permeability and retention (EPR) effect in the tumor. This long-lasting circulation of DNA complexes in the blood and the permeability into the tumor achieved by simple cationic molecules hold great promise regarding the successful application of gene delivery systems. Furthermore, if KG6 is modified by a ligand without loss of the original character, highly controlled gene delivery will be achieved in vivo.

3 Peptides as Ligands

3.1 RGD Peptide

Studies of the bioactivity of RGD (arginyl-glycyl-aspartic acid tripeptide motif) peptide have a long history (reviewed by Ruoslahti 2003). Initially, Pierschbacher and Ruoslahti (1984) found that the RGDS (L-arginyl-glycyl-L-aspartyl-L-serine) peptide promoted cell adhesion. When included in extracellular matrix proteins, such as fibronectin, vitronectin, fibrinogen and laminin, this peptide motif was recognized by integrins, a family of cell-surface proteins (Pytela et al. 1985a, 1985b). The $\alpha 5\beta 1$- and αV-containing integrins were particularly efficient in recognizing RGD peptide sequences (Hynes 1992). As the integrins act as receptors for cell adhesion on the substratum and in cell-cell interactions, RGD peptide and its analogues showed several bioactivities, such as the induction of apoptosis by endothelial cells (Brooks et al. 1994a) and the inhibition of angiogenesis (Brooks et al. 1994b; Buerkle et al. 2002). It is expected that RGD peptides will not only act as tools for studying the basic mechanisms of cell adhesion, cell-cell interaction, metastasis and angiogenesis, but that they will also function as therapeutic agents for the treatment of diseases, such as thrombosis and cancer. Recently, the imaging of tumors using RGD peptides modified with ^{18}F and ^{64}Cu was reported (Chen et al. 2004; Haubner et al. 2004).

The application of RGD peptide in gene delivery was reported by Hart et al. (1995) and Harbottle (1998). They used a peptide, $[K]_{16}$RGD peptide ($[K]_{16}$GGCRGDMFGCA), containing the RGD motif and a DNA-binding domain of 16 lysine residues as a gene carrier. The peptide was oxidized to allow formation of intramolecular disulfide bridges that increase its affinity for integrins. Experiments carried out using several cell lines showed that the peptide bound to fibronectin and vitronectin integrin receptors and formed nuclease-resistant complexes with DNA. The transfection efficiency was enhanced by the addition of the RGD motif to $[K]_{16}$ peptide, whereas an RGE motif had no effect, indicating that the transfection was dependent on integrin (Colin et al. 1998).

Erbacher et al. (1999) modified polyethylenimine (PEI) with RGD peptide. In their study, thiol-derivatized PEI was conjugated to the integrin-binding peptide CYGGRGDTP via a disulfide bridge. This PEI-RGD conjugate formed 30- to 100-nm toroidal particles whose surface charge was close to neutral, as a consequence of the shielding effect of the prominent zwitterionic peptide residues. Following in vitro transfection, the expression of PEI-RGD was 10- to 100-fold higher in integrin-expressing epithelial (HeLa) and fibroblast (MRC5) cells than that of PEI without the RGD motif and with the RGE motif. The PEI-RGD system was further improved by the addition of a PEG chain between them. PEG-ylation of cationic polymers reduces non-specific binding to cell surfaces. A cyclic peptide, ACDCRGDCFC (Suh et al. 2002) and a tetra peptide, RGDC (Kunath et al. 2003) were modified into PEI via a hydrophilic poly(ethylene glycol) (PEG) spacer. Insertion of the PEG chain kept the DNA complexes of the conjugates neutral, whereas complexes consisting of RGD-PEI and PEI had a positively charged surface. In in-vitro transfection experiments using endothelial cell lines, RGD-PEG-PEI conjugates showed integrin dependent gene expression. Although an obvious effect of PEG-ylation was not observed in the in vitro system, the charge shielding effect of PEG would no doubt be advantageous in in-vivo gene therapy, especially for systemic injection.

RDG-modified lipid-protamine-DNA lipopolyplex (LPD) was prepared by Harvie et al. (2003). The incorporation of PEG-ylated lipid into LPD complex decreased their in vitro transfection activity. In order to restore particle binding and specifically target LPD formulations to tumor cells, a lipid-RGD peptide conjugate DSPE-PEG-ACDCRGDCFCG was synthesized and then incorporated into LPD formulations. The resultant LPD-PEG-RGD showed significantly increased binding and uptake compared to that obtained with an LPD-PEG formulation. Moreover, transfection of LPD-PEG-RGD was specific for integrin-expressing cells. The combination of RGD with liposome-based systems could also be effective in targeted and systemic gene delivery.

3.2 Other Peptides

In addition to RGD peptides, the use of peptides bound to other types of integrin has been reported. A linear or cyclic PLAEIDGIEL, which binds to $\alpha 9\beta 1$-integrin, expressed in lung epithelia, hepatocyte and muscles, was connected to a DNA-binding moiety of 16 lysine residues (Schneider et al. 1999). In that case, specific gene delivery could only be achieved with the the cyclic form of the peptide. However, inclusion of a cationic liposome, lipofect-amine, into the peptide/DNA complexes the efficient gene transfer of both peptides with significant targeting specificity.

The serpin-enzyme complex receptor (SECR) was successfully targeted for gene delivery using peptide ligands covalently linked with oligolysine (Patel et al. 2001). The authors found that the peptide $[K]_{16}$CSIPPEVKFNKPFVFLI forms small complexes with DNA and showed high transfection efficiency in an Huh-7 human hepatocyte cell line expressing the receptor.

Recently, several peptides that can function as targeted ligands have been found in a phage display library. By repeating bio-panning on target tissues or cell lines, specific phage peptides can be screened and the peptide sequences subsequently identified. Peptide SIGYPLP was found to target vein endothelial cells and the phage was used as a vector for gene delivery (Nicklin et al. 2000). Other targeting peptides include: THALWHT, for targeting to human airway epithelia (Jost et al. 2001); CNGRC, for targeting to tumor (Colombo et al. 2002); CSRPRRSEC, CGKRK and CDTRL, for targeting to the neovasculature (Hoffman et al. 2003); and CGNKRTRGC, for targeting to tumor lymphatic and tumor cells (Laakkonen et al. 2004). Further accumulation of a wide variety of targeted peptides will allow the construction of tailor-made functional gene carriers that can be targeted to specific tissues.

4 Peptides as Tools for Endosome Disruption

4.1 Acidic Peptides

The endocytotic pathway is a major route of non-viral gene delivery into cells´, except for the method using DNA-encapsulated liposomes. Following uptake, most of the DNA complexes containing the gene carriers are degraded in acidic endocytotic vesicles or returned to the outside of the cells by exocytosis. Therefore, escape from the endosome to the cytosol is a critical step in efficient gene delivery and expression. To promote escape, endosome-disruptive peptides, derived from the amino-terminal sequence of influenza virus hemagglutinin HA-2, have been applied (Wagner et al. 1992; Midoux et al. 1993; Plank et al, 1994). The peptides were found as a membrane fusion domain of HA-2, which fused the virus and endosomal membrane in host cell, that is, the peptide domain had membrane perturbation activity that was triggered by an acidic environment. Furthermore, designer model peptides, which have anionic amino acids and take on an α-helical structure, have also been reported (Haensler et al. 1993; Plank et al. 1994; Ohmori et al. 1997, 1998). Such endosome-disruptive peptides introduce the DNA into the cytoplasm prior to fusion of the endosome with the lysosome. They contain several acidic amino acids, as shown in Table 2, and their α-helical structures, formed under acidic conditions, arise from protonation of the glutamic carboxylates at acidic pH, which decreases repulsion of the negatively charged side chains of glutamic acids. As a result, the peptides exhibit membrane-perturbation activities (Fig. 4). In the gene delivery protocol in vitro, anionic peptide was covalently conjugated to cationic carrier molecules, such as a polylysine, and the DNA complexes of the conjugates were then added into the cell culture medium (Wagner et al. 1992; Haensler et al. 1993). Alternatively, the anionic peptides were mixed with complexes of DNA and cationic carriers, and the resulting complexes were used in transfection (Plank et al. 1994; Midoux et al. 1993; Gottschalk et al. 1996; Ohmori et al. 1997, 1998). By the addition of anionic endosome-disruptive peptides, the transfection efficiencies of cationic gene carriers improved dramatically.

Table 2. Anionic endosome-disruptive peptides

Peptide	Structure
Peptide1, INF3	GLFE-AIAG-FIEN-GWEG-MIDG-GGC
Peptide2	GLFG-AIAG-FIEN-GWEG-MIDG-GGC
INF5	$(GLFE\text{-}AIEG\text{-}FIEN\text{-}GWEG\text{-}nIDG)_2$-K
INF7	GLFE-AIEG-FIEN-GWEG-WYG
GALAcys	WEAA-LAEA-LAEA-LAEH-LAEA-LAEA-LEAC-AA
GALA	WEAA-LAEA-LAEA-LAEH-LAEA-LAEA-LAAL-AAGG-SC
GALA-INF1	GLFG-AIAG-FIEN-GWEG-LAEA-LAEA-LAAL-AAGG-SC
Peptide I	GLFE-AIAE-FIEG-GWEG-LIEG-CA
JST-1	GLFE-ALLE-LLES-LWEL-LLEA
4_3E	LAEL-LAEL-LAEL

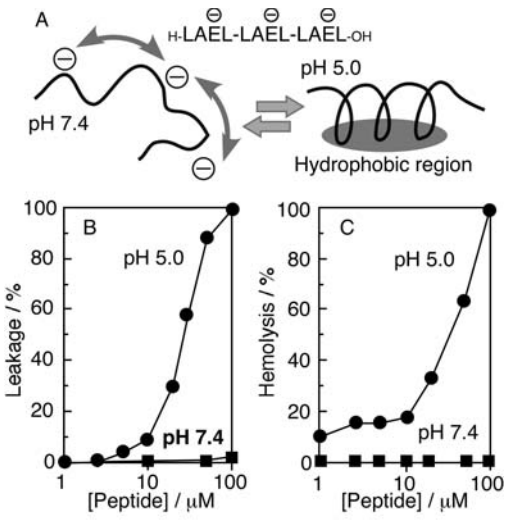

Fig. 4. The conformational change of an acidic peptide (LAEL-LAEL-LAEL) (**A**) and its pH-dependent liposome disruption (**B**) and hemolytic (**C**) activities

4.2 Peptides Containing Histidine Residues

Midoux et al. (1998) designed a peptide, H5WYG (GLFHAIAHFIHGGWHGLIHG-WYG), that undergoes a dramatic conformational change between pH 7.0 and 6.0 that correlates with the protonation of the histidyl residues. Thus, the peptide shows membrane perturbation activity at a slightly acidic pH but not at neutral pH. Addition of the peptide to a transfection system mediated by glycosylated polylysine led to a significant increase in gene expression. By contrast, McKenzie et al. (2000b) synthesized a peptide, Cys-His-$(Lys)_6$-His-Cys, by substituting histidine for some of the lysine residues in the peptide Cys-Trp-$(Lys)_{17}$-Cys. The new peptide provided buffering capacity that enhanced in vitro gene expression in the absence of chloroquine. As the pKa of the histidine residues is similar to the pH in endosomal vesicles, the histidines have a proton sponge effect (Boussif et al. 1995). From this viewpoint, the enhancing

effect of the peptide, H5WYG may have originated from the buffering effect of the histidine residues in the peptide, in addition to its membrane-perturbing activity.

5 Peptides as Signals for Transport to the Nucleus

Gene expression from the transgene in plasmid DNA requires transcription of the DNA to mRNA, which occurs in the nucleus. Inefficient entry of DNA into the nucleus is a major limiting step in non-viral gene delivery systems. In the case of dividing cells, the DNA is internalized when the nuclear membrane is reconstituted. However, there are serious problems in the case of non-dividing cells, in which entry into the nucleus is thought to occur only through the nuclear pore complex. Therefore, in order to achieve active transport to the nucleus, nucleus localizing signal (NLS) peptides have been widely used. Recent efforts have been summarized in excellent reviews (Bremner et al. 2001; Tachibana et al. 2001; Cartier 2002). In most cases, NLS peptides are conjugated with a gene carrier, such as PEI or cationic liposome. In order to function as a NLS peptide, the carrier should not dissociate from the DNA in the cytosol. In the case of gene carriers that release DNA in the cytosol or whose time of endosome escape is mediated by membrane fusion, as in the case of lipoplex (Xu et al. 1996), NLS peptide modification is of no use. Moreover, it is hard to imagine that this type of DNA complex can transport through the nuclear pore. To clarify this problem, Zanta et al. (1999) directly modified DNA with a NLS peptide. An oligonucleotide cap modified with a NLS peptide (PKKKRKVEDPYC) was synthesized, and the cap was then ligated to a linearized luciferase gene. By modifying with the peptide, transfection was remarkably enhanced, whereas no enhancement of the control peptide (PKTKRKVEDPYC) was observed, suggesting that enhancement was due to importin-mediated translocation.

Although direct modification with NLS peptide is a straightforward strategy to achieve highly efficient gene expression, construction of the gene is complicated, costly, and time-consuming. Recently, a convenient method of DNA modification with NLS peptide was reported by Zelphati et al. (2000). They used an NLS peptide linked with a peptide nucleic acid (PNA), which can hybridize with plasmid DNA by forming a triplex strand invasion complex. The resulting DNA contained the NLS peptide via the PNA chain. This technique has been made commercially available as GeneGrip, from Gene Therapy Systems (San Diego, CA, USA). Bremner et al. (2004) prepared several NLS-modified DNAs using this system and compared their characteristics. Although the synthesis of PNA-peptide hybrid is relatively complicated, even for researchers in the field of gene therapy, the technique offers an easy approach to introducing not only the NLS onto plasmid DNA but also other functional peptides and groups without disturbing transcriptional activity.

6 Peptides as Sensors for Functional Gene Delivery

Peptides can be used as sensors that respond to cellular conditions. Each tissue expresses hundreds of genes, many of which are tissue specific, for example, gene expression in vascular smooth muscle cells is different from that of endothelial cells. Furthermore, there are large differences in gene expression between normal and

abnormal tissues, such as inflammatory and tumor tissues. If a peptide was able respond to a specific protein in a target tissue and control gene delivery in the cells, then a tissue-specific gene expression system could be developed.

Katayama et al. (2002) focused on substrate peptides for cAMP-dependent protein kinase (PKA) and caspase-3 as sensors. The enzymes act as mediators of intracellular signals in the regulation of gene expression and apoptosis, respectively. Extraordinary activation of these enzymes is known to play a role in many diseases, such as melanoma, prostate tumor, and colon cancer for PKA, and hepatitis, Alzheimer's disease, Parkinson's disease, and other various nerve-denaturing diseases for caspase-3. Thus, these enzymatic activities are important determinants of the cellular condition. In the report of Katayama et al. (2002), the peptides were incorporated into polymers; specifically, graft-type copolymers were synthesized using methacryloyl-peptide monomer and acrylamide with radical copolymerization (Fig. 5A). In the case of the PKA system, polymer containing the substrate peptide (ALRRASLG) formed complexes with plasmid DNA via electrostatic interactions due to the cationic net charge of the peptides. Once PKA phosphorylates the peptide at the serine residues, the net charge of the peptide is reduced, and the polymer is then expected to release its DNA (Fig. 5B). PKA-dependent DNA release was observed by agarose gel retardation assay. In cell-free transcription and translation systems, the addition of PKA to the DNA complex of the polymer triggered gene expression from the plasmid DNA. After transfection of the complex into NIH 3T3 cells, the addition of forskolin, which actives PKA, significantly enhanced expression of the reporter gene. In the caspase-3

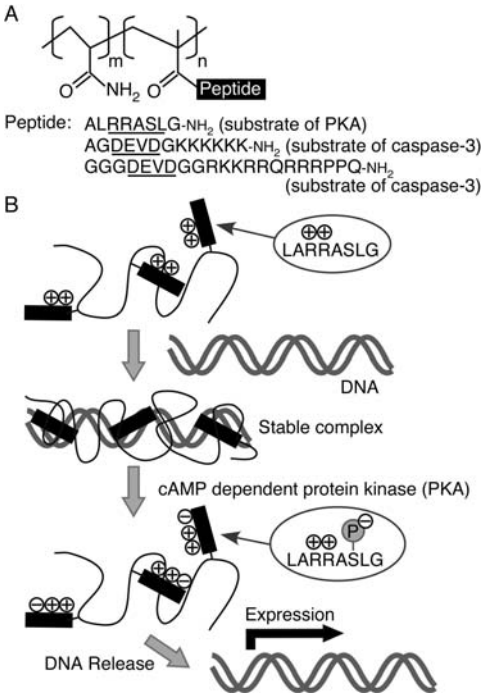

FIG. 5. **A** Structures of the polymer-peptide conjugates. **B** cAMP-dependent protein kinase(PKA)-dependent DNA release

system, a peptide containing a substrate for caspase-3 (AGDEVDG) and a cationic sequence (KKKKKK or GRKKRRQRRRPPQ; Tat peptide) for DNA binding was used as a pendant peptide on the polymer. When the DEVD portion of the peptide is recognized and cleaved by caspase-3 at the C-terminus of the second Asp (D), the cationic portion is released from the polymer. Therefore, polymer-covered DNA is released by the addition of caspase-3, allowing transcription of the gene. In a cell-free in vitro transfection system stimulated by staurosporin, controlled DNA release and gene expression mediated by caspase-3 were achieved. These systems are the first examples of an artificial gene regulation system controlled by a cationic polymer containing a peptide sensor.

7 Prospects

As described in this chapter, peptides have several applications in gene delivery. Advanced techniques of peptide synthesis make it possible to design and precisely synthesize complicated peptides, such as a multifunctional peptide containing an additional PNA chain and a ligand. In addition, the molecular science of bioactive peptides has progressed. Basic research on peptide, such as their physical characteristics, their detailed structure-function relationships, the mechanism of their biological activity, and further screening of functional peptides using a peptide library, will provide a large number of tools for use in gene delivery. Thus, peptide science will connecting a wide variety of research fields and will strongly contribute to the development of functional gene delivery systems.

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