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Peptide-guided Gene Delivery

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Abstract

Although currently less efficient than their viral counterparts, nonviral vectors are under intense investigation as a safer alternative for gene therapy. For successful delivery, the nonviral vector must be able to overcome many barriers to protect DNA and specifically deliver it for efficient gene expression in target cells. The use of peptides as gene delivery vectors is advantageous over other nonviral agents in that they are able to achieve all of these goals. This review will focus on the application of peptides to mediate nonviral gene delivery. By examining the literature over the past 20 years, it becomes clear that no other class of biomolecules are simultaneously capable of DNA condensation, blocking metabolism, endosomal escape, nuclear localization, and receptor targeting. Based on virtually limitless diversity of peptide sequence and function information from nature, it is increasingly clear that peptide-guided gene delivery is still in its infancy.

Keywords: Peptide-guided gene delivery, fusogenic peptide, nuclear localization, targeted delivery, proteasome

Introduction

The most successful gene therapy strategies available today rely on viral vectors, such as adenoviruses, adeno-associated viruses, retroviruses, and herpes viruses. Despite their success, there are many problems associated with viruses related to immunogenicity, cytotoxicity, and insertional mutagenesis.[1-3] Although nonviral vectors are not as efficient as viral vectors, many have been developed to provide a safer alternative in gene therapy. Some of the most common nonviral vectors include polyethylenimine, dendrimers, chitosan, polylysine, and many types of peptides, which are generally cationic in nature and able to interact with plasmid DNA through electrostatic interactions.[4]

Presently there are 4 barriers that must be overcome by nonviral vectors to achieve successful gene delivery, as shown in Figure 1. The vector must be able to tightly compact and protect DNA, target specific cell-surface receptors, disrupt the endosomal membrane, and deliver the DNA cargo to the nucleus.[4] Peptide-based vectors are advantageous over other nonviral strategies in that they are able to achieve all 4 of these goals. Cationic peptides rich in basic residues such as lysine and/or arginine are able to efficiently condense DNA into small, compact particles that can be stabilized in serum.[5-7] Attachment of a peptide ligand to the polyplex will allow targeting to specific receptors and/or specific cell types. Peptide sequences derived from protein transduction domains are able to selectively lyse the endosomal membrane in its acidic environment leading to cytoplasmic release of the polyplex.[8,9] Finally, short peptide sequences taken from longer viral proteins can provide nuclear localization of condensates once they are in the cytoplasm.[10,11] Table 1 lists several peptides that are able to perform these various functions to carry out peptide-guided gene delivery.

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DNA Condensation With Poly-L-lysine and Polylysine-containing Peptides

Cationic peptides interact with the negatively charged phosphate backbone of DNA through electrostatic interactions.[69] This activity leads to small nanometer-sized particles with a net positive charge that is able to interact with cell membranes and internalize into the cell to permit gene expression. One of the first cationic peptides used to mediate gene delivery was poly-L-lysine (PLL). To increase transfection efficiency, many modifications of peptide length and sequence have been made to give several variations to the original PLL peptide.

Poly-L-lysine

The most well-known nonviral DNA condensing agent is poly-L-lysine. PLL is a synthetic repeat of the amino acid lysine, with the most commonly used PLLs ranging from a degree of polymerization (dp) of 90 to 450.[5] This characteristic makes the polymer suitable for in vivo use because it is readily biodegradable. However, as the length of the PLL increases, so does the cytotoxicity. In addition, PLL only exhibits modest transfection when used alone and requires the addition of an endosomolytic agent such as chloroquine

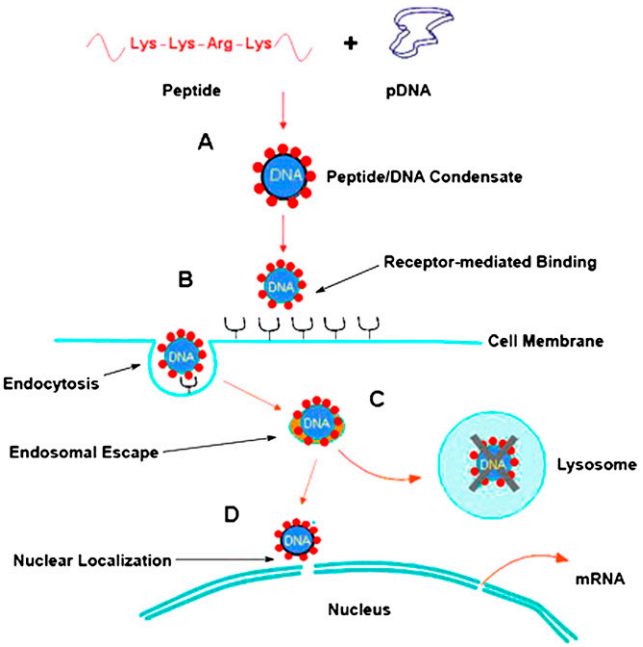

Figure 1. The 4 major barriers to peptide-guided gene delivery: the peptide must be able to (A) tightly condense DNA into small, compact particles; (B) target the condensate to specific cell-surface receptors; (C) induce endosomal escape; and (D) target the DNA cargo to the nucleus for reporter gene expression.

or a fusogenic peptide to allow for release into the cytoplasm. Another useful modification is the attachment of poly(ethylene)glycol (PEG) to the polymer to prevent plasma protein binding and increase circulation half-life of the complex.[12-14]

The length of the PLL can greatly influence the particle size and stability of the DNA condensate and therefore also affect the gene transfer efficiency. Higher molecular weight PLLs have a greater net positive charge and are therefore able to bind DNA tighter and form more stable complexes than low molecular weight PLLs.[15] Without charge masking by PEGylation, low molecular weight PLL complexes bind erythrocytes during circulation more so than their high molecular weight counterparts.[16] High molecular weight PLLs, however, exhibit high cytotoxicity and are complement system activators to a much greater extent than low molecular weight carrier peptides.[7]

One of the first uses of the polylysine conjugated system for nonviral DNA delivery was by Wu and Wu.[70-72] They demonstrated that a targeting glycoprotein, asialoorosomucoid (ASOR), could be coupled to the PLL carrier, which could then efficiently bind to DNA to form soluble complexes. Hepatocytes expressing the asialoglycoprotein receptor were transfected and able to express the gene product, while cells without the receptor had no detectable gene expression. With this system, Wu and Wu were able to achieve targeted delivery of PLL vectors both in vitro and in vivo.[70-72]

Polylysine-containing Peptides

Because of the polydispersity and lack of chemical control during synthesis of PLL conjugates, many researchers have turned to the development of homogenous polylysine-containing peptides. Oligolysine peptides of a defined length offer many advantages over PLL, including precise control of synthesis and homogeneity of peptide length, lower toxicity, and site-specific attachment of ligands used for cell targeting and intracellular trafficking.[5,19,73] Wadhwa et al[5] systematically varied the length of the cationic peptide Cys-Trp-Lys_n from 3 to 36 lysine residues to determine the optimal lysine chain length for both DNA condensation and transfection efficiency. Cationic peptides containing 13 or more lysine residues were able to tightly bind DNA and form small condensates ranging from 50 to 200 nm, while shorter peptides of 8 or fewer lysines formed large condensates of 0.7 to 3 μm that weakly bound DNA in a manner similar to $polylysine_{19}$. Another study by Adami et al[20] showed that a peptide containing 18 lysines was able to form stable condensates that protect DNA from degradation by both salt sonication and serum endonucleases, whereas a shorter 8 lysine peptide could not prevent DNA degradation. Based on this data, an optimal peptide sequence containing 18 lysines followed by a tryptophan and an alkylated cysteine ($AlkCWK_{18}$) produced small condensates with higher transfection efficiency than $polylysine_{19}$.[5,19] In contrast, $polylysine_{19}$ available from Sigma (St Louis, MO) was found to be a heterogeneous mixture of many short-chain oligolysines that are not capable of efficiently condensing DNA and mediating high transfection as reported by McKenzie et al.[19]

Other variations of the cationic lysine peptides include the sequence $YKAK_nWK$ by Gottschalk et al, where the central lysine cluster is varied from 4 to 12 residues to determine the minimal number of lysines required for DNA binding and gene expression. An 8 lysine cluster was able to efficiently condense DNA, but the $YKAK_8WK$ peptide-condensed DNA alone gives little gene transfer.[17] Coupling of an endosomal releasing peptide greatly enhanced gene expression of the condensates in a variety of cell lines.[17] Plank et al[18] proposed the use of branched cationic peptides, in which the number and type of cationic amino acid has been varied. A lysine was used as the branching point to extend the N-terminal cationic sequence of lysine, arginine, and/or ornithine residues that serve as the DNA-binding portion of the peptide, while the C-terminus contains a glycine spacer followed by a cysteine residue that can be used for attachment of receptor ligands or stabilizers such as PEG. This branched geometry allows flexibility of the cationic DNA binding region to interact with the phosphate backbone of DNA. A minimum sequence of 6 to 8 cationic residues was required for DNA compaction and transfection, with arginine binding slightly stronger than lysine and ornithine.[18]

Table 1. Peptides Used for Guided Gene Delivery*

Function	Peptide	Sequence	Reference
DNA Condensation	Polylysine	$K_{90\ to\ 450}$	5,7,12-16
	Polylysine-containing peptides	$YKAK_8WK$	17
		$(KKK)_2KGGC$	18
		CWK_{18}	19,20
		$CWK_{17}C$	7,21
Endosomolytic	Histidine-rich peptides	CHK_6HC	21
		CHK_6HC	22
		H5WYG	23-25
Fusogenic	Influenza HA-2	GLFGAIAGFIENGWEGMIDGWYG	26-28
	Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	29-32
	Tat (48-60)	GRKKRRQRRRPPQ	33-38
	Penetratin	RQIKIWFQNRRMKWKK	9,39,40
	Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	41,42
	GALA	WEAALAEALAEALAEHLAEALAEALEALAA	43-45
	KALA	WEAKLAKALAKALAKHLAKALAKALKACEA	46
	JST-1	GLFEALLELLESLWELLLEA	17
	ppTG1	GLFKALLKLLKSLWKLLLKA	47
	ppTG20	GLFRALLRLLRSLWRLLLRA	47
Proteasome	Gly-Ala repeat	$CWK_{18}(GA)_4$	48
Monopartite NLS	SV40 T antigen	PKKKRKV	49-52
	SV40 Vp3	KKKRK	53
	Adenovirus E1a	KRPRP	54
	Human c-*myc*	PAAKRVKLD, RQRRNELKRSP	55
Bipartite NLS	Nucleoplasmin	KRPAATKKAGQAKKKK	56
	Xenopus N1	VRKKRKTEEESPLKDKDAKKSKQE	57
	Mouse FGF3	RLRRDAGGRGGVYEHLGGAPRRRK	58
	PARP	KRKGDEVDGVDECAKKSKK	59
Nonclassical NLS	M9	NQSSNFGPMKGGNFGGRSSGPYGGGGQYFAKPRNQGGY	60
Cellular targeting	RGD	ICRRARGDNPDDRCT	61,62
	Integrin binding	PLAEIDGIELTY	63
	Secretin	HSDGTFTSELSRLRDSARLQRLLQGLV	64
	GE7 (from EGF)	NPVVGYIGERPQYRDL	65
	NL4	CTTTHTFVKALTMDGKQAAWRFIRIDTAC	66
	Neurotensin	ELYENKPRRPYIL	67
	LOX-1 binding	LSIPPKA, FQTPPQL, LTPATAI	68

*NLS indicates nuclear localization signal; FGF3, fibroblast growth factor 3; PARP, poly (ADP-ribose) polymerase; RGD, Arg-Gly-Asp; EGF, epidermal growth factor; and LOX-1, lectin-like oxidized LDL receptor.

The formation of stable peptide-DNA condensates is essential for gene expression. The stability of the DNA polyplex is determined by the ability of the cationic component to bind and compact DNA. Premature degradation of the DNA can occur if the polyplex dissociates in serum and exposes the DNA to metabolizing endonucleases.[6,18] High molecular weight PLL can protect DNA from rapid metabolism as shown by Wu and Wu.[70-72] To improve serum stability of short-chain oligolysine carriers, molecular cross-linking strategies have been developed. Adami and Rice[6] used gluteraldehyde to cross-link free amino groups of the peptide CWK_{18}. Following condensation of DNA with CWK_{18}, gluteraldehyde was added to the condensates resulting in the formation of 2 Schiff bases between neighboring amine groups. Both salt sonication and serum stability assays showed an improved stability with increasing amounts of gluteraldehyde cross-linking, similar to the stability of $polylysine_{1007}$. Although polylysine still gave greater levels of gene expression, cross-linked CWK_{18} condensates were able to give a 10-day steady-state expression in cell culture.[6]

Another cross-linking strategy uses the formation of disulfide bonds between the thiol groups of 2 cysteine residues. McKenzie et al[7,21] modified the CWK_{18} peptide to contain

multiple cysteine residues capable of oxidizing to form interpeptide disulfide linkages following condensation with DNA. Upon internalization, the disulfide bonds could be reversed by the cell's reducing environment, thereby releasing DNA into the cell for increased gene expression compared with gluteraldehyde cross-linking.[7,21,74] Incorporation of disulfide bonds resulted in smaller particle size and an increase in condensate stability as compared with $AlkCWK_{18}$. Maximal gene expression was obtained by a peptide with 2 terminal cysteine residues, which gave a 60-fold increase in gene expression over $AlkCWK_{18}$.[7] Because interpeptide disulfide bond formation essentially creates a polymer when condensed on DNA, McKenzie et al[21] systematically shortened the lysine sequence of the CWK_nC peptide to determine the minimal amount of residues needed for DNA condensation and gene transfer efficiency. The results indicated that 4 lysines flanked by terminal cysteines were able to condense DNA with similar particle size and transfection efficiency as a 17 lysine peptide.

Endosomolytic Peptides

Following internalization of peptide-DNA condensates by endocytosis, the polyplex must be able to escape the endosome so that the DNA can be delivered to the nucleus for gene expression. The acidic environment of the endosome has led to the design of several gene delivery peptides that become endosomolytic at a lower pH. Once inside the endosome, these peptides can either buffer against the proton pump to cause lysis or fuse with the endosomal membrane leading to pore formation.

Histidine-rich Peptides

One strategy to attain endosomal release is to buffer against the adenosine-5′-triphosphate (ATP)-dependent proton pump located in the membrane of endosomes. Polymers such as polyethylenimine (PEI) contain several secondary amines that are easily protonated in the acidic environment of the endosome. As protons are pumped in, PEI absorbs the protons leading to endosomal swelling and membrane disruption.[75,76] In an effort to translate the proton sponge activity to gene delivery peptides, histidine has been added to peptide sequences. The imidazole group of histidine has a pK_a of ~6.0, therefore allowing it to become protonated in the acidic environment of the endosome. At physiological pH the histidines will remain neutrally charged, thereby imparting selective membrane disruption in the acidic endosome.[23-25,77] Although the exact mechanism of action for histidylated peptides is still unknown, addition of the proton pump inhibitor bafilomycin A_1 leads to a decrease in endosomal lysis, which further supports the proton sponge theory.[23,24] In order to increase the gene transfer efficiency, histidine residues have been incorporated into polylysine and shorter oligolysine peptides, as well as the design of the histidine-rich H5WYG peptide.[21,22,25,78]

Polylysine alone cannot efficiently escape the endosome without the addition of endosomolytic agents such as chloroquine or a fusogenic peptide.[22,78] To increase the transfection efficiency of polylysine without the addition of membrane-disrupting agents, Midoux and Monsigny[78] constructed a histidine-substituted polylysine that is able to become cationic at endosomal pH. Using polylysine (dp 190), histidine is coupled by reaction with the side-chain amines of lysine to give a partially histidylated $polylysine_{190}$.[78-80] Maximal transfection efficiency was achieved with ~38% of the ε-amino groups substituted with histidine residues.[78] Acylation of the α-amino group of the histidyl residues increased the particle size of condensates and decreased transfection efficiency.[80] Pichon and coworkers have also shown that a smaller oligolysine (dp 19) can be histidylated, but it was not as efficient at transfection as the longer polylysine probably because of the inability to tightly compact DNA.[81]

Histidylation of low molecular weight disulfide cross-linking peptides has been demonstrated by both McKenzie et al[21] and Read et al.[22] Substitution of histidine for lysine in a cysteine-flanked peptide resulted in short peptides that can bind DNA, have endosomal buffering capacity, and polymerize through disulfide bonds once condensed with DNA.[21] McKenzie and coworkers[21] synthesized several sequences, varying the amount and position of histidine residues, and determined the optimal peptide for gene expression to be CHK_6HC, which gave up to a 10-fold increase in expression as compared with a non-histidine-containing control peptide. Read et al[22] also synthesized similar sequences of $CH_3K_3H_3C$ and $CH_6K_3H_6C$ and found up to a 5-fold increase in the amount of transfected cells in various cell lines as compared with transfection with PEI.

The histidine-rich peptide H5WYG designed by Midoux and colleagues is a derivative of the N-terminal sequence of the HA-2 subunit of the influenza virus hemagglutinin in which 5 of the amino acids have been replaced with histidine residues.[23-25] H5WYG is able to selectively destabilize membranes at a slightly acidic pH as the histidine residues are protonated. An anionic derivative of this peptide, E5WYG, in which the histidines are replaced by glutamic acid residues, is completely ineffective at membrane permeabilization at a pH of 6.8, while H5WYG can disrupt 50% of cells at pH 6.8 and 97% of cells at pH 6.2 within 15 minutes.[23,25] H5WYG was also able to retain its activity in the presence of serum, making it possible for use in vivo.[25]

Fusogenic Peptides

Many fusogenic peptides have also been used to promote endosomal escape and delivery of DNA cargo to the cytosol and/or nucleus. Generally short sequences of only 20 amino acids are needed for membrane destabilization, and they usually contain a high content of basic residues.[8] Many cell-penetrating peptides (CPPs) are derived from the transduction domains of proteins that interact with cell membranes, including Tat, melittin, penetratin, transportan, and INF peptides.[8,9,82,83] There are also several synthetic amphipathic peptides such as GALA, KALA, JTS-1, and ppTG20 that are able to traverse membranes. These peptides adopt an α-helical structure at endosomal pH leading to hydrophobic and hydrophilic faces that can interact with the endosomal membrane to cause disruption and pore formation.[9,26]

One of the oldest and most well studied CPPs is the INF peptide. In an effort to mimic viral mechanisms of endosomal escape, Wagner et al designed short peptides derived from the N terminus of the influenza virus hemagglutinin HA-2.[27,28] These peptides contain acidic residues that are protonated upon a decrease in pH, allowing the peptide to form an α-helical structure. When conjugated to polylysine, DNA polyplexes containing the influenza peptide were able to mediate significant gene expression in vitro from 10- to 10 000-fold increase over condensates without the fusogenic peptide.[26-28]

Melittin is a 26 amino acid peptide whose sequence is derived from the venom *Apis mellifera* (honey bee). The peptide has strong amphipathic character that is able adopt an α-helix conformation and is well known for its cytolytic activity. Studies by Ogris et al[29] using melittin conjugated to PEI show a strong increase in gene expression in a variety of cell lines over transfection with PEI alone. Melittin, however, can be highly toxic as demonstrated by Boeckle and coworkers. These investigators compared C- versus N-terminally linked melittin-PEI conjugates and found that by changing the site of linkage they could change the membrane lytic activity of the peptide at different pHs. At neutral pH, the C-terminally linked conjugate was highly membrane lytic and therefore toxic to cells, while at endosomal pH the N-terminally linked conjugate showed an increased lytic activity and promoted higher endosomal escape of the gene delivery vector.[30] Modification of the C-terminally linked melittin peptide by replacement of the neutral glutamine residues (Gln25 and Gln26) with glutamic acids can increase the membrane lytic activity of these conjugates at endosomal pH.[31] Melittin has also been developed into a gene delivery peptide capable of condensing and cross-linking on DNA.[32] With the addition of lysine residues for added positive charge and terminal cysteine residues for polymerization, the hemolytic melittin peptide can be used to condense DNA into particles capable of transfecting various cell lines with an efficiency equal to that of PEI. By stabilizing the melittin through disulfide bonds, the peptide is not released until it is introduced into the reducing environment of the endosome, therefore protecting the cell from the cytolytic activity of the free melittin peptide.[32]

Another peptide derived from a viral sequence is the Tat (48-60) sequence from the human immunodeficiency virus 1 (HIV-1) protein. The entire Tat protein is 86 amino acids in length and contains a highly basic region required for translocation activity. The peptide fragment from residues 37 to 72 contains the basic region along with an α-helical structure that is capable of internalization into several cell lines.[33-35] It was later discovered that the α-helix (residues 27-47) is not necessary for activity of the peptide. The minimally active sequence of the Tat (48-60) peptide contains a 9 amino acid stretch of basic residues that are required for the membrane lytic activity.[33,34,36,37] Rudolph and coworkers have shown that multimers of the Tat peptide can efficiently condense DNA and produce a 6- to 8-fold increase in transfection over control peptides.[38]

Penetratin is a short peptide derived from the third α-helix of the homeodomain of Antennapedia, a *Drosophila* transcription factor. It is 16 amino acids in length and is able to efficiently translocate across membranes as well as the full length homeodomain protein.[39,40] Penetratin has been used to transfer and transfect a variety of compounds, including peptides, peptide nucleic acids, antisense oligonucleotides, and double-stranded DNAs.[9,40]

Transportan is a 27 amino acid CPP that is synthesized by joining the N-terminal sequence of the neuropeptide galanin with mastoparan, a pore-forming wasp-venom peptide, through a single lysine residue.[41] This peptide is able to penetrate several cell types and, once inside, it accumulates in the nucleoli. The lysine linker provides a suitable attachment point for a variety of macromolecules for membrane translocation.[41,42]

The 30 amino acid peptide GALA is an amphipathic peptide that transitions from random coil to α-helical as the pH is lowered from 7.6 to 5.0.[43] It has a repeating unit of glutamic acid-alanine-leucine-alanine (GALA) that gives it its amphipathic character. At physiological pH, the glutamic acid residues exhibit charge repulsion, which is neutralized as the pH is lowered allowing it to adopt the α-helix structure.[43-45] At endosomal pH, GALA is able to bind to bilayer membranes and induce leakage of phosphatidylcholine vesicles. Reversal of the positions of the glutamic acid and leucine residues (LAGA) results in loss of the α-helical structure and therefore a decrease in vesicle leakage.[44] Truncated peptides of 1, 2, 3, or 4 GALA repeats were still able to form an α-helix but did not induce leakage when protonated.[43] Even though GALA has membrane lytic activity,

with its anionic nature it is not able to bind and compact DNA for efficient transfection. Replacement of some alanines with lysines and a reduction in glutamic acid content gives the resulting cationic KALA peptide that is able to condense DNA, induce membrane leakage, and mediate gene expression. As pH is decreased, KALA behaves in the same manner as its anionic counterpart to transition from random coil to α-helix and is therefore able to retain its membrane lytic activity. Condensates prepared with KALA are able to transfect a variety of cell lines without the addition of membrane disruptive agents.[46]

JTS-1, a novel amphipathic peptide developed by Gottschalk and coworkers, was able to form an α-helix dominated by strong nonpolar amino acids on the hydrophobic face and glutamic acid residues on the opposite hydrophilic face. It lysed both phosphatidylcholine liposomes and erythrocytes at a pH of 5, and showed an 8-fold higher hemolysis activity than INF-7.[17] As with GALA, JTS-1 is negatively charged at neutral pH and unable to bind the phosphate backbone of DNA. To overcome this problem, all glutamic acid residues of JTS-1 were substituted with either lysine or arginine resulting in the 2 peptides ppTG1 and ppTG20, respectively. Both peptides retained the α-helix conformation along with membrane lytic activity and were also able to compact DNA.[47] They are able to mediate DNA transfection in vitro in a variety of cell lines as well as in vivo in mice following intravenous injection.[47]

In order for efficient gene delivery to occur, the peptide component of the condensate must be able to aid in the release of intact DNA from the endosome. Histidine-rich oligolysine peptides have been developed to buffer against the endosomal proton pump and are able to show an increase in gene expression relative to non-histidine-containing control peptides. These peptides are especially effective in vitro when a high stoichiometry of peptide can be dosed but are not as useful in vivo when dilution occurs as the condensates are distributed throughout the circulation. Fusogenic peptides, on the other hand, bind to the endosomal membrane to create pores. At low pH, they adopt an amphipathic conformation that allows them to fuse with the membrane for endosomal escape of the polyplex. Cationic fusogenic peptides are optimal for in vitro experiments as they are able to condense DNA for transfection. However, anionic fusogens are preferred in vivo because of their greater activity at lower pHs than their cationic counterparts, which is necessary in the endosomal environment for more efficient gene delivery.

Modulation of Proteasome Activity

The proteasome presents another possible barrier to the efficient delivery of nonviral vector systems. Proteasomes are multi-subunit enzymes responsible for the degradation of many cytosolic proteins such as damaged or misfolded proteins, degradation of cyclins for cell cycle control, destruction of transcription factors that control cell differentiation, and processing of foreign proteins for generation of cellular immune responses.[48,84,85] Duan and coworkers have shown that the proteasome plays an important part in the metabolism of viral delivery vectors. The addition of tripeptide proteasome inhibitors was able to increase the gene expression of the adeno-associated virus (AAV) vector by 50-fold in cell culture.[86] Because of their similarity in size and susceptibility to serine proteases, Kim et al[48] hypothesized that peptide delivery systems could possibly be metabolized by the proteasome as well. When simultaneously treated with proteasome inhibitor, a 30-fold increase in luciferase expression could be observed in 2 different cell lines, thereby implicating the proteasome as a contributor in the degradation of the peptide DNA condensate.

To protect peptide DNA condensates from proteasome metabolism, a novel series of peptides were designed based on the glycine-alanine repeat sequence of the Epstein-Barr virus (EBV) nuclear antigen-1 (EBNA1). EBNA1 contains a 238 amino acid stretch, consisting of only glycine and alanine residues, that is able to prevent proteolysis.[87-89] Sharipo et al[88] have been able to show that a minimum 8 amino acid Gly-Ala sequence is needed to retain the protective activity. Based on these data, Kim and coworkers[48] designed a gene delivery peptide containing an intrinsic Gly-Ala repeat to provide protection to the peptide DNA condensate from premature degradation by the proteasome. Varying lengths of a Gly-Ala repeat were synthesized on the C-terminal end of the CWK_{18} peptide, which were then used to condense DNA and transfect various cell lines. Over a log increase in gene expression was observed with a Gly-Ala repeat of 4 in HepG2 cells over the control peptide CWK_{18}, indicating a protective mechanism provided by the Gly-Ala repeat against metabolism of the peptide carrier by the proteasome.

Nuclear Localization Peptides

Upon escaping the endosome, the condensed DNA must be efficiently trafficked to the nucleus, where the uncoated DNA must be released for gene expression to occur. Transport into the nucleus generally occurs through nuclear pore complexes (NPCs). However, the inner diameter of an NPC is only ~9 nm, so free diffusion of condensates, which are much larger in size, is unlikely. To ensure nuclear targeting, many nuclear localization signal (NLS) peptides have been developed that target the DNA to the nucleus and allow entry through the NPCs by active transport.[26,90,91] NLSs are characterized by short clusters of basic amino acids that are recognized and bind to cytoplasmic receptors known as importins.[10,11] Nuclear import of macromolecules is a 2-step process involving the binding of the NLS to the NPC followed by translocation through the pore.[10]

NLS peptides can be associated with their DNA cargo through ionic interactions, covalent attachment to the DNA with a chemically active group, or site-specific attachment using a peptide nucleic acid (PNA) clamp.[92] If the NLS peptides have a sufficient net positive charge, they can interact with the negatively charged DNA with or without another condensing agent to enhance nuclear targeting of the polyplex. However, with this method there is the possibility of the NLS dissociating from the DNA before it has reached the nucleus. NLS peptides can also be covalently attached to DNA through chemically active or photoactive groups. This form of attachment is nonspecific and may occur at any location on the DNA including the gene of interest, which may lead to inhibition of reporter gene expression. To avoid these issues, PNA clamps can be used for specific attachment of the NLS sequence to plasmid DNA.[26,92-94] With all of these methods, the number of NLS peptides added per plasmid can affect the success of nuclear targeting. Zanta et al[95] demonstrated that a single NLS attached to DNA is sufficient for nuclear entry and that several NLS peptides would inhibit transport into the nucleus.

Nuclear Localization Signal Sequences for DNA Delivery

Although there are some exceptions, NLS sequences can generally be separated into monopartite or bipartite peptides that contain either 1 or 2 clusters of basic (Lys or Arg) amino acids, respectively.[26] The most well known and popularly used NLS in the field of gene therapy is from the large tumor antigen of the simian virus 40 (SV40). This monopartite peptide has a minimum 7 amino acid sequence of PKKKRKV that is required for its NLS activity and is able to increase the speed of nuclear uptake of plasmid DNA by up to 100-fold faster than a reversed NLS sequence.[49-51] A single mutation of the second positively charged lysine with a neutral asparagine completely abolishes the nuclear transport activity of the peptide.[52]

Another study used noncovalent attachment of the NLS to DNA to show improvement in gene expression. The M9 sequence of the heterogeneous nuclear ribonucleoprotein (hnRNP) A1 is a 38 amino acid sequence used by Subramanian and coworkers to increase nuclear import of plasmid DNA. The M9 sequence was conjugated to a cationic peptide for DNA condensation and used to transfect cells, resulting in a 10-fold increase in reporter gene expression as compared with a scrambled M9 control sequence.[60]

Nuclear Localization Signal Sequences for Protein Delivery

While many other NLS sequences have been identified, they have mainly been used to study the nuclear import of proteins. The following sequences are known to translocate cargo to the nucleus and could perhaps find utility in the field of gene delivery. Attachment of these sequences to plasmid DNA could lead to enhanced gene expression in the same way that the previous 2 peptides have helped target DNA to the nucleus.

Two different monopartite NLS sequences have been derived from SV40, with the second NLS peptide from the SV40 structural protein Vp3. It has been found by Gharakhanian and coworkers that the 35 amino acids on the C-terminal end of the Vp3 protein are essential for its nuclear targeting. Within the 35 residue sequence is a stretch of 5 basic amino acids identical to the NLS peptide of the SV40 T antigen and several other stretches with significant homology to several histones.[53]

Another monopartite NLS sequence comes from the adenovirus E1a gene product. The proteins encoded by the E1a gene have 2 separate domains capable of nuclear targeting. The more rapid of the 2 is a 67 amino acid stretch located at the carboxyl terminus of the protein. Truncation of this sequence to the pentapeptide KRPRP is sufficient to retain nuclear accumulation of an attached cargo to the same extent as the native E1a sequence.[54]

The human c-*myc* protein also contains 2 regions that are able to target the nucleus. C-*myc* is a short-lived phosphoprotein found in the nucleus that is 439 amino acids in length. Peptide fragment M1 from residues 320 to 328 has the sequence PAAKRVKLD, while peptide fragment M2 contains residues 364 to 374 with the sequence RQRRNELKRSP. Both sequences are able to target their cargo to the nucleus, although M1 is much faster and more efficient than M2. Deletion of the M1 peptide from the c-*myc* sequence results in a protein that is mostly, but not completely, cytoplasmic in distribution, while M2 deletion does not result in an observable difference in the normal nuclear targeting of the protein. These results indicate that M1 probably serves as the main NLS for the c-*myc* protein.[55]

Bipartite NLS sequences contain 2 essential domains of basic amino acids separated by a spacer that is generally 10 to 12 amino acids in length.[26] One of the most well characterized bipartite NLSs is the *Xenopus* protein nucleoplasmin, which has 2 clusters of basic amino acids separated by a 10-residue spacer sequence. Nucleoplasmin has the sequence KRPAATKKAGQAKKKK, which partially resembles the SV40 T antigen NLS with the lysine stretch at its carboxyl terminus. However, the full length peptide is needed for sufficient nuclear targeting of attached cargo. Attachment of the short basic segment from the C-terminus to serum albumin only results in partial nuclear accumulation; however, efficient targeting is achieved with the full 16 amino acid peptide.[56]

Also from *Xenopus* comes the bipartite NLS derived from the N1 protein. Kleinschmidt and Seiter showed that the 24

amino acid sequence of the N1 NLS can be divided into 2 required nuclear uptake sequences of VRKKRKT and AKKSKQE joined by a 10 amino acid spacer to give the overall peptide VRKKRKTEEESPLKDKDAKKSKQE. Mutation of a single amino acid in either signal sequence results in decreased nuclear uptake of N1, whereas a lysine-to-threonine mutation of the spacer region does not influence nuclear accumulation. As with nucleoplasmin, neither signal sequence alone is able to mediate efficient nuclear targeting, but the full length peptide is needed for sufficient accumulation in the nucleus.[57]

The mouse fibroblast growth factor 3 (FGF3) protein contains a bipartite NLS motif. Kiefer and coworkers narrowed down the location of the NLS sequence from 5 possible regions in the protein sequence to the arginine-rich site located in the N-terminal half of the protein. The 2 basic regions of the peptide are designated NLS1 with the sequence RLRR, and NLS2 made up by the amino acids RRRK. The 2 cationic clusters are joined by a 16 amino acid spacer giving the entire NLS sequence of RLRRDAGGRGGVYEHLGGAPRRRK. Point mutations in either or both of the NLS regions resulted in decreased or abolished nuclear accumulation, respectively.[58]

Poly (ADP-ribose) polymerase (PARP) is a protein involved in DNA repair that contains a classical bipartite NLS sequence as well. Schreiber et al have defined the NLS sequence as corresponding to residues 207 through 226 of the PARP protein with the sequence KRKGDEVDGVDECAKKSKK. As with all other bipartite peptides, the 2 basic stretches are crucial for activity but not able to mediate nuclear uptake on their own. Experiments show that the 11 amino acid spacing between the basic clusters can be varied in length without affecting activity as long as K222 remains in the correct position.[59]

Peptide Ligands for Targeted Delivery

One of the major challenges that determines the efficacy of nonviral vectors is specificity for a target site.[96] Many peptides can function as ligands to target nonviral gene delivery systems to specific cell and/or tissue types within the body and enhance the delivery of nonviral vectors. These peptides can be used to condense the DNA if they contain enough positively charged basic residues, but in general they are covalently attached to another condensing agent and function only for targeting purposes. Peptide ligands can be short sequences taken from larger proteins that are the essential amino acids needed for receptor recognition, such as the epidermal growth factor (EGF) peptide used to target cancer cells.[65,97] Other synthetic peptide ligands have been identified by phage display, including the ligands used to target the lectin-like oxidized LDL receptor (LOX-1).[68]

The integrins are one of the most well studied and targeted families of receptors in gene delivery. Integrins are cell-surface receptors found on all cell types that have many important roles in the attachment of cells to the extracellular matrix, cell migration, cell-cell interactions, and signal transduction.[98] The receptors are heterodimeric and composed of multiple α- and β-subunits. Integrin binding peptides generally have short sequences of around 6 amino acids and can either bind a wide variety of integrin receptors or be specific for a single integrin. Some integrin-binding peptides have a conserved Arg-Gly-Asp (RGD) motif and generally will bind to many different integrins.[98] Other ligands, such as the peptide PLAEIDGIELTY obtained by phage display, bind specifically to the $\alpha_9\beta_1$-integrin.[63]

Several peptides with the conserved RGD sequence have been developed to target integrin receptors. Hart and colleagues have developed several RGD-containing peptides that include a short polylysine tail for binding to plasmid DNA. The polylysine tail is able to efficiently condense DNA as compared with a polylysine standard lacking the integrin-binding peptide. These peptides have a cyclic RGD domain that is able to efficiently recognize the integrin receptor in a variety of cell types. Competitive inhibition studies with free RGD peptide proved that uptake of the RGD-polylysine-DNA vector is integrin mediated.[99,100]

Another RGD peptide used to target integrin receptors is molossin, from the venom of the American pit viper *Crotalus molossus molossus*, which has the sequence ICRRARGDNPDDRCT. Attachment of a 16 amino acid lysine chain to the N-terminus provides DNA binding capabilities to the peptide. The integrin binding motif, RGDNP, can be modified to RGENP to serve as a control peptide as it will completely abolish all integrin-binding activity.[61,62] Collins and Fabre[61] used the polylysine-molossin peptide to transfect the corneal endothelium in rabbits. With the addition of chloroquine as an endosomolytic agent, 30% of the endothelial cells were transfected, but with the use of a fusogenic peptide nearly 100% transfection was achieved.

A third integrin-binding peptide is a novel peptide discovered by phage display that is specific for the $\alpha_9\beta_1$-integrin receptor. Described by Schneider et al,[63] this peptide has the sequence PLAEIDGIELTY and is derived from the third fibronectin type III repeat (TNfn3) of tenascin C. Tenascin C is a specific ligand for the $\alpha_9\beta_1$-integrin receptor (which is highly expressed in human airway epithelia) and is of particular interest to the field of cystic fibrosis (CF) gene therapy. Treatment of cells with this peptide resulted in a complete displacement of the entire TNfn3 protein, indicating the shortened sequence is capable of cellular targeting.

Also of interest to those studying CF is the use of secretin as a targeting ligand for nonviral gene therapy. Both biliary and pancreatic duct epithelia express the secretin receptor

(SR) and make it an exceptional target for CF patients with gastroenterological problems arising from the disease. McKay and coworkers[64] have conjugated the 27 amino acid secretin peptide to a linear form of polyethylenimine, which provides endosomal buffering and release of the complex for targeted delivery to SRs. Using this vector, a 10-fold greater increase in transfection was observed in cells expressing SR than in cells without SR, which was inhibited by competing secretin peptide.

Another important target for nonviral vectors is tumor cells in an effort to replace conventional cancer therapies. However, the peptide must be highly specific for the tumor cells and not be able to transfect and exhibit the toxic effects of its cargo in normal cells. Many human tumors, such as hepatocellular carcinoma (HCC), overexpress epidermal growth factor (EGF) receptors, making EGF a ligand of choice for tumor targeting.[65,97] Complexation of part or all of the EGF sequence with DNA results in a vector capable of efficient targeting and expression in cancer cells having the EGF receptor, as indicated by a 2-fold or more increase in expression in the tumor relative to other organs. Expression of the vector can be reduced by addition of free EGF, indicating uptake is EGF receptor mediated.[97]

Several peptide ligands have been derived from sources that target the central nervous system in hopes to provide targeted gene therapy for several neurological disorders such as Alzheimer's and Parkinson's diseases. Zeng et al designed a 29 amino acid sequence (designated NL4) corresponding to amino acids 80 through 108 of loop 4 of the nerve growth factor (NGF) polypeptide as a ligand to target the NGF receptor TrkA. Attachment of a 10 amino acid polylysine tail (NL4-10K) to the C-terminal end of the peptide allows for DNA binding. Studies show that NL4-10K complexed with DNA is able to increase reporter gene expression in PC12 cells (TrkA-positive), but not able to induce expression in TrkA-poor, TrkB-positive, or TrkC-positive cells.[66]

Neurotensin (NT) has been used as a targeting ligand to neural cells by Martinez-Fong and coworkers. Conjugated to poly-L-lysine through a *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) linkage, it is able to bind DNA and target the high-affinity neurotensin receptor (NTRH). Cell lines expressing NTRH, such as N1E-115 and HT-29, show expression of the gene products when transfected with the NT-SPDP-polylysine complexes, while cell lines lacking NTRH (COS-7 and L-929) are not transfected. Simultaneous treatment with free NT or the NTRH antagonist SR-48692 prevents uptake of the complex, thereby showing the targeting specificity of the ligand for the neurotensin receptor.[67]

Phage displays can be very useful in the discovery of novel peptide sequences that target a specific cell receptor. White et al used this method to search for a peptide specific for LOX-1. Up-regulation of LOX-1 in endothelial cells is associated with dysfunctional states such as hypertension and atherosclerosis, whereas normal venous and arterial vascular endothelia only express low levels of LOX-1. To identify sequences specific for LOX-1, peptide recovery was quantified following transfection in either LOX-1-positive or LOX-1-negative cells. Using this technique, 3 peptides, LSIPPKA, FQTPPQL, and LTPATAI, were identified as potential candidates for further study in this system.[68]

Conclusion

Peptide-guided nonviral gene delivery has clear advantages over other gene therapy strategies. Unlike viral vectors, peptides have reduced cytotoxicity and immunogenicity along with a much greater biodegradability. Peptides can also serve many functions that other nonviral vectors cannot perform alone. There are peptides to condense DNA into compact particles, disrupt the endosomal membrane, escape proteasomal degradation, traffic DNA cargo to the nucleus, and target polyplexes to specific receptors. These properties may all be part of a single peptide sequence or a combination of peptides chemically conjugated together to form a vector capable of packaging and targeting DNA for efficient delivery. Over the past 20 years, peptide-guided gene delivery has progressed from the use of heterogeneous mixtures of polylysine to the precise synthesis of defined-length homogenous peptides. The use of peptides as nonviral delivery vectors is still in its infancy and has the potential to grow exponentially as new peptides are discovered that have the ability to achieve these goals alone or in combination with other systems.

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