Cell-penetrating peptides as delivery vectors for oligonucleotides and proteins

Studies on applications and toxicity

Peter Järver
"Education is an admirable thing, but it is well to remember from time to time that nothing that is worth knowing can be taught."

/Oscar Wilde
List of Publications

This thesis is based on the following publications, referred to in the text by their indicated Roman numerals:


III. Järver P\(^a\), EL-Andaloussi S\(^a\), Johansson H.J, Langel Ü. Cargo dependent cytotoxicity and delivery efficacy of cell-penetrating peptides- a comparative study *Biochem J.* In press


\(^a\)Both authors contributed equally to this work
Additional Publications

Järver P, Langel Ü.
The use of cell-penetrating peptides as a tool for gene regulation.
*Drug Discov Today.* 2004 May 1;9(9):395-402.

Johansson H.J., Järver P, and Langel Ü.
Cell penetrating peptides and their application in gene regulation.
In : Innovation and Perspectives in Solid Phase Synthesis & Combinatorial Chemical Libraries

Järver P, Langel Ü.
Cell-penetrating peptides-a brief introduction.

Järver P, Langel K, El-Andaloussi S, and Langel Ü
Applications of cell-penetrating peptides in regulation of gene expression
Abstract

Cell-penetrating peptides (CPPs) have for a little bit more than a decade been employed as delivery vectors for a wide range of cargoes, ranging from gold particles to entire plasmids. Although CPP are well studied and utilized in numerous publications, our knowledge about CPP mediated transport is still poor. The articles presented in this thesis all consider different aspects of CPP mediated delivery. The first two papers are evaluating and improving already known techniques. In paper I, standard polyetylenimin (PEI) transfection is improved by conjugating the CPP TP10 to the cationic polymer. In paper II, the same CPP is employed to deliver a dsDNA decoy oligo, resulting in decreased activity of the transcription factor c-Myc. The third paper is a more general overview of the delivery efficiency of well known CPPs and how the delivered cargo influences the CPP mediated toxicity. The study shows that different CPPs are suitable for different cargos and that toxic side effects depend heavily on the cargo and coupling strategy used. In Paper IV, a novel CPP, M918, is evaluated as a delivery vector for a transposon based non-viral gene therapy system. M918 display simultaneous delivery of a plasmid carrying a selection gene and a transposase into cultured cells. This is the first study where two so vastly different molecules as a cationic protein and an anionic plasmid, are simultaneously transported into cells by a peptide vector. The method might be a first step towards a safe peptide based non-viral gene therapy platform. Taken together, the results presented in this thesis might help to improve already existing techniques, increase our understandings about CPP mediated delivery and, at the same time, develop new CPP based delivery systems.
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# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bHLH/LZ</td>
<td>Basic helix-loop-helix/leucin zipper</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>bPrP</td>
<td>Bovine prion protein 1-30</td>
</tr>
<tr>
<td>C. elegans</td>
<td>Caenorhabditis elegans</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CPP</td>
<td>Cell-Penetrating peptide</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DGP</td>
<td>2-deoxy-D-glucose-6-phosphate</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double stranded DNA</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EBI</td>
<td>Endosomolytic peptide 1</td>
</tr>
<tr>
<td>E-box</td>
<td>Enhancer-box sequence</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>FP</td>
<td>Frog prince</td>
</tr>
<tr>
<td>HATU</td>
<td>2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate</td>
</tr>
<tr>
<td>HeLa</td>
<td>Henrietta Lacks</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human immunodeficiency virus 1</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HMGB-1</td>
<td>High mobility group protein B1</td>
</tr>
<tr>
<td>HOBT</td>
<td>hydroxybenzotriazole</td>
</tr>
<tr>
<td>HS</td>
<td>Heparane sulfate</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>IR/DR</td>
<td>Inverted repeat/Direct repeat</td>
</tr>
<tr>
<td>ITR</td>
<td>Inverted terminal repeat</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption/ionization– Time of flight</td>
</tr>
<tr>
<td>MDR-1</td>
<td>Multi drug resistance gene 1</td>
</tr>
<tr>
<td>MBHA</td>
<td>4-methylbenzylhydlyamine</td>
</tr>
<tr>
<td>Miz-1</td>
<td>Myc interacting Zink finger protein 1</td>
</tr>
</tbody>
</table>
mRNA  Messenger RNA
MTS  Membrane translocation sequence
Mw  Molecular weight
NADH  Nicotinamide adenine dinucleotide
Neo  Neomycin
NFκB  Nuclear factor kappa B
Ni-NTA  Nickel-nitrilotriacetic acid
NLS  Nuclear localization signal
Npys  3-Nitro-2-pyridinesulfenyl
ON  Oligonucleotide
pAntp  Penetratin
PB  PiggyBac
p-cresol  para-Cresol
PEG  Polyethylene glycol
PEI  Polyethyleneimine
Pen  Penetratin
pI  Isoelectric point
PNA  Peptide nucleic acid
PTD  Protein Transduction Domain
p-thio cresol  para-Thio-cresol
RISC  RNA induced silencing complex
RNA  Ribonucleic acid
SB  Sleeping Beauty
SB-tpase  Sleeping Beauty transposase
SCID-X1  X-linked severe combined immunodeficiency
siRNA  Small interfering RNA
SPPS  Solid phase peptide synthesis
ssDNA  Single stranded DNA
TAR  Trans activation response
TAT  Transactivator of transcription
t-Boc  tert-Butyloxycarbonyl
TE  Transposable element
TF  Transcription factor
TP  Transportan
TP10  Transportan 10
tRNA  Transfer RNA
UV  Ultra violet
wst-1  2-[4-Iodophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt
1. Introduction

Even though we know a lot about the cell, its functions, different cellular processes and, the membrane that surrounds it, we still have problems with delivering substances to the cellular interior. There are plenty of different methods and techniques developed with this purpose and this thesis is dedicated to one of these methods, Cell-penetrating peptides (CPPs). This family of peptides has throughout the passed 13 years been employed as transporters for a wide array of molecules and substances, and the aim of this thesis is to get a better understanding of these peptides, as well as trying to utilize them in new and different ways. Mainly, two different cargos have been studied, oligonucleotides (ONs) and proteins. The ON delivery stretches from short dsDNA sequences up to entire genes and plasmids. The toxicity and efficiency of various CPPs have been elucidated and by combining protein and ON delivery, CPPs have for the first time been applied as a transport vector in non-viral gene therapy.

1.1. Cellular Transport

The cell membrane, also referred to as the plasma membrane, is a lipid bi-layer common to all living cells. It holds a variety of biological molecules, primarily proteins and lipids, which are involved in a vast array of cellular processes. One of the key features of the cellular membrane is to physically separate the intracellular components from the extra-cellular environment which is vital for the maintenance of cellular functions. The plasma membrane is selectively permeable and serves as gatekeeper to what molecules, and how much, that enters and exits the cell. However, because of these astounding features, the plasma membrane can turn into an obstacle when trying to influence or alter cellular functions. This becomes very apparent when trying to treat various diseases or when trying to map out intracellular functions for a scientific cause. Very simplified, there are three ways of affecting the cell. Either through extra-cellular receptors mediating an intra-cellular response, the compound is small and has the ability to passively diffuse into the cell, or, a natural or artificial transport system is needed to facilitate cellular transport.

Mainly, cellular transport can be divided into two classes; Active and passive transport. Active transport is the mediated transport of bioactive
molecules, and other substances, across biological membranes. The process move molecules against a gradient and thus requires spending of cellular energy. Passive transport is dependent on the permeability of the cell membrane and consequently does not involve cellular energy. Instead, passive transport is dependent on the organization and characteristics of the membrane lipids and membrane associated proteins.

1.2. Cell-penetrating Peptides

Cell penetrating peptides (CPPs) make up a vastly diverse family of peptide vectors. Not only do the properties of the different peptides differ greatly, but even the family name is varying. Depending on where and what you read, CPPs are also referred to as Protein Transduction Domains (PTDs) or Membrane Translocating Sequences (MTS).

1.2.1. An introduction to CPPs

In 1994, the first Cell-penetrating peptide (CPP), pAntp, was reported in the literature (Derossi et al., 1994). The peptide derives from the third helix of the Antennapedia protein homeodomain and was later renamed to Penetratin (Pen) or pAntp. Although Pen was the earliest CPP described, the initial work on CPPs can be tracked back to 1988 when it was shown that the HIV-1 encoded trans-activator protein, Tat, which is essential for HIV-1 viral gene expression, was rapidly taken up by cells (Frankel et al., 1988; Green et al., 1988). There are earlier reports of protein delivery by cationic peptide polymers dating back to as early as 1965, though these findings are seldom referred to as the instigation of the CPP field (Ryser et al., 1965; Ryser et al., 1978; Shen et al., 1978).

Since their discovery, an ever increasing stream of CPPs have emerged (For a more comprehensive view over CPP sequences used in this thesis, see Table 1.1) but even though CPPs were introduced in 1994, still there is no unambiguous description for this stragglng family of peptides. The only distinct feature common to all CPPs is their ability to rapidly facilitate transduction across the plasma membrane. Apart from this common attribute, CPP characteristics differ vastly.
Table 1.1. CPPs employed in this thesis

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Origin</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>M918</td>
<td>MVTVLFFRLRIRRACGPRVRV-NH₂&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Protein derived</td>
<td>(El-Andaloussi et al., 2007b)</td>
</tr>
<tr>
<td>Penetratin</td>
<td>RQIKIWFQNRRMKWKK-NH₂&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Protein derived</td>
<td>(Derossi et al., 1994)</td>
</tr>
<tr>
<td>Tat 48-60</td>
<td>GPKKRRQRRPPQ-NH₂&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Protein derived</td>
<td>(Vivès et al., 1997)</td>
</tr>
<tr>
<td>TP10</td>
<td>iAGYLLGKINLKAALAKKII-NH₂</td>
<td>Chimeric</td>
<td>(Soomets et al., 2000)</td>
</tr>
</tbody>
</table>

Table 1.2. Selection of CPPs discussed in this thesis

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Origin</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPG</td>
<td>GALFLGFLGAAGSTMGAWSQPKKKRKV&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Chimeric</td>
<td>(Morris et al., 1997)</td>
</tr>
<tr>
<td>MPG&lt;sub&gt;a&lt;/sub&gt;</td>
<td>GALFLAAALSLMGWSQPKKKRKV&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Chimeric</td>
<td>(Veldhoen et al., 2006)</td>
</tr>
<tr>
<td>Pep-1</td>
<td>KETWWETWWTEWSQPKKKRKV&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Chimeric</td>
<td>(Morris et al., 2001)</td>
</tr>
<tr>
<td>Poly R</td>
<td>RRRRRR&lt;sub&gt;0+&lt;/sub&gt;-NH₂</td>
<td>Synthetic</td>
<td>(Futaki et al., 2001)</td>
</tr>
<tr>
<td>EB1</td>
<td>LIRLWSHLIHIWFQNRRLKWKKK-NH₂</td>
<td>Synthetic</td>
<td>(Lundberg et al., 2007)</td>
</tr>
</tbody>
</table>

<sup>1</sup>Originally synthesized as N-terminal free acid
<sup>2</sup>Originally synthesized with a C- and N-terminal cysteine
<sup>3</sup>C-terminal cysteamide

1.3. Uptake mechanism of CPPs

As mentioned previously, CPPs were introduced in the literature in 1994 (Derossi et al., 1994). Even though 13 years have passed and numerous of articles on the subject have been published, there is still uncertainty about the true internalization mechanism of CPPs. Pioneering studies showed in vitro translocation of D-analogue CPPs at +4°C. Based on these results it was believed that the peptides utilized a pathway that did not require a chiral receptor and was independent of cellular energy. Consequently the translocation was considered to be independent of any endosomal pathways. A direct penetration across the cellular membrane would be highly unlikely since the hydrophilic nature of CPPs prevents a passage directly through the lipid bi-
layer. So, an inverted micelle model was proposed to be accountable for the internalization (Derossi et al., 1996). Further on, it seemed that the charge interaction between CPPs and membrane bound heparane sulfate (HS) proteoglycans act as a receptor, initiating endocytotic internalization of arginine rich peptides like Tat and Arg6 (Suzuki et al., 2002; Tyagi et al., 2001), the same has later been reported for penetratin (Ghibaudo et al., 2005).

However, the field changed considerably when it was reported that CPP mediated uptake was in fact endosome dependent and that harsh fixation methods drastically alter CPP uptake (Lundberg et al., 2003; Richard et al., 2003b). This brought into light that many of the reports on CPP internalization could be a result of false positive data. Since then, much attention has been given to understanding and assessing the true internalization mechanism of CPPs. It is more or less accepted that endocytosis contributes to the internalization of CPPs. A comparative study of Tat, transportan and, penetratin mediated uptake of anti-sense PNA (Nielsen, 1993) in a splice correction assay, assert that these three peptides all are internalized through endosomes, since splice correction were increased with addition of lysosomotropic agent chloroquine and decreased by low temperature (El-Andaloussi et al., 2006a). However, what type of endocytosis that is responsible for the uptake is not evident and all three endocytotic pathways have been proposed to account for Tat translocation.

Tat internalization has been shown to co-localize with transferrin and thus uptake was suggested to be dependent on clathrin mediated endocytosis (Richard et al., 2003b), which has also been suggested to be the internalization route of the entire HIV-1 derived TAT protein (Vendeville et al., 2004). Conversely, Tat fusion proteins have been reported to co-localize with caveolar markers, suggesting caveolar endocytosis to account for internalization (Ferrari et al., 2003; Fittipaldi et al., 2003). Finally, macropinocytosis has been reported to be responsible for Tat (Lundberg et al., 2006) and for Tat-Cre fusion protein (Wadia et al., 2004) uptake. Recently, Duchardt et al. reported that Tat, Pen and R6 simultaneously utilized all three mentioned endocytotic pathways for their uptake (Duchardt et al., 2007). Tat internalization has further been proposed by Tunnemann et al. to be cargo dependent, showing Tat fusion proteins (>50aa) are taken up largely into cytoplasmic vesicles whereas peptides fused to Tat (<50aa) enter cells in a manner that is dependent on membrane potential and not on endocytosis (Tunnemann et al., 2006). Similar data have been published by Curnow et al., showing that Tat directly enters liposomes at room temperature in the absence of pH gradient, ATP or other energy source. In contrast, when the peptide was attached to a 25 kDa cargo protein, it was not able to enter liposomes, although it could enter live cells through an endocytotic pathway (Curnow et al., 2005). These data are in line with our findings in paper III, showing that Tat internalization is heavily dependent on cargo size. To make things even more complicated, peptide concentration seems to influence internalization route
(Duchardt et al., 2007) and there are still reports claiming that some CPPs transduce cells in an endosome independent manner (De Coupade et al., 2005).

MPG peptide derived from the fusion peptide of HIV-1 gp41, associated with the nuclear localization signal (NLS) of SV40 and Pep-1, designed on the basis of a protein-interacting domain associated with the same NLS (Table 1.1), are both proposed to induce transient pore-like structures in the lipid bilayer and thereby facilitate delivery of several bioactive cargoes (Deshayes et al., 2004a; Deshayes et al., 2004b). As a whole, there is no definitive description of CPPs and the proper mechanism of cell entry.

It is suggested that there might be several parallel translocation pathways accounting for the uptake, and, depending on how an internalization study is preformed, the results can differ vastly.

1.4. CPP mediated delivery of bioactive cargoes

CPPs are intriguing in themselves, but their main attribute is to act as delivery vectors for other bioactive molecules. There are two strategies employable to CPP delivery. i) The vector is attached to its cargo through a linker or, in the case of peptides and proteins, associated in the same amino acid chain. ii) The peptide vector and the cargo are joined through electrostatic or hydrophobic interactions, resulting in a transducible entity. Which of these strategies to choose depends on the characteristics of the cargo and, whether the distribution is performed in vivo or in vitro. The cargoes used in this thesis are ONs and proteins, and both coupling strategies have been employed. Here, achievements so far in peptide mediated delivery of ONs and proteins will be briefly discussed

1.4.1. CPP mediated oligonucleotide delivery

The use of ON for therapeutic applications is a rapidly growing field and several methods have been developed for ON delivery, some of them are discussed in later chapters. Not only delivery of entire genes is a desired cargo, but also regulating oligonucleotides, such as decoy and anti-sense oligos and siRNA. The latter three are suitable for peptide mediated delivery and all have successfully been delivered to cells by CPPs. The anionic nature of ON makes them an ideal cargo to be delivered through co-incubation to-
gether with CPPs. However, if to be used therapeutically, a linker connecting the vector and cargo and thereby creating a more defined entity might be a more feasible approach.

1.4.2. Delivery of plasmid DNA

Although plasmid DNA is in many ways an attractive cargo, few studies on CPP delivery of entire plasmids have been reported. CPPs can be used as an enhancer of standard plasmid delivery techniques, as in paper I, and as performed by Rudolph et al. (Rudolph et al., 2003). It seems like CPPs alone are insufficient to deliver such large entities as entire plasmids (Mw in the MDa range) into cells in vitro (Kilk et al., 2005; Liu et al., 2005). In Paper IV, the novel CPP M918 is reported unable to deliver a plasmid in to cells by itself, however M918 is capable of simultaneous delivery of a cationic transposase and a plasmid in vitro.

However, Liu et al. showed in 2005 that although Tat alone has no significant transfection capability, a branched Tat structure was able to complex with plasmid DNA and displayed significant transfection capabilities in a variety of mammalian cell lines through an endocytosis-mediated pathway (Liu et al., 2005). Further, the group of Divita have developed the CPP MPG (described in Table 1 and section 1.3) that seems to have the ability to deliver entire genes into cells in vitro (Morris et al., 1999; Simeoni et al., 2003) The same is shown by, Sandgren et al. who in 2004 demonstrated that the human antimicrobial peptide LL-37 can facilitate plasmid uptake into mammalian cells in vitro (Sandgren et al., 2004). LL-37 can then possibly cause release of plasmid DNA from lysed bacteria, followed by protection of bacterial DNA from degradation and transfer of intact bacterial DNA to the nuclei of mammalian host cells. These findings can provide in a highly provocative, yet intriguing, mechanism for peptide mediated bacterial to vertebrate lateral gene transfer, explaining the origin for about 100-200 genes that are present in the human genome but absent in lower eukaryotes (Lander et al., 2001).

1.4.3. Delivery of decoy DNA

So far, only three publications have shown efficient uptake of decoy dsDNA. Except from paper II presented in this thesis, Fisher et al. published a paper in 2004 where the cellular delivery of a double-stranded ON decoy targeting NFkB was shown (Fisher et al., 2004). The delivery strategy in this paper was the same as one of the techniques employed in paper II. The CPP was conjugated via a disulfide linker to a PNA strand complementary to a 9 bases protruding 3'-terminal sequence on the decoy dsDNA. An alternative co-incubation strategy has been employed when targeting the cancer associ-
ated MDR-1 gene. The CPP MPG was co-incubated together with a dsDNA decoy mimicking the human MDR-1 promoter sequence, resulting in decreased cell viability due to decreased MDR-1 expression. (Marthinet et al., 2000) For more detailed information on decoy strategies, see chapter 1.6.

1.4.4. Delivery of anti-sense oligonucleotides

An anti-sense ON can be introduced into cells, where it specifically targets its complementary mRNA sequence and thereby inhibits protein biosynthesis. The effect is carried out through two main mechanisms, depending on the ON. i) RNase H recognizes the ON–mRNA heteroduplex and cleaves the target mRNA. ii) When targeting the mRNA, the anti-sense ON sterically inhibits translation by blocking the ribosomal machinery. Several improvements have been made in recent years in order to identify accessible target sites in the mRNA, different ON analogs with increased stability, binding affinity, and specificity (Karkare et al., 2006).

To regulate gene expression by introducing mRNA complementary ONs has become a popular approach in the field of CPPs. The technique has proven effective in several aspects, for instance, Pooga et al. showed in 1998 down-regulation of the Galanin receptor in vivo through an anti-sense ON-CPP conjugate (Pooga et al., 1998b). The same strategy has been an effective tool for decreasing viral replication. By conjugating PNA targeting the conserved HIV-1 trans-activation response (TAR) RNA stem-loop to a CPP through a disulfide bridge, the Tat-dependent trans-activation of HIV-1 can be inhibited (Turner et al., 2003; Turner et al., 2007). Morris et al. have blocked tumour growth by injecting CPP-PNA complexes (including a negatively charged PNA analog) in vivo (Morris et al., 2007). Anti-sense technologies might in some cases have an advantage over the similar, but more novel, siRNA therapies. Since siRNA mediated gene silencing is an endogenous mechanism, many genes are involved and expressed when siRNA is introduced to the cell. This might cause severe non-specific side effects (Samuel, 2004) which might be avoided by the use of anti-sense ON.

1.4.5. Delivery of splice-correction oligonucleotides

In the last couple of years it has become increasingly popular to measure the efficiency of CPP mediated ON uptake by a splice-correction assay introduced by the group of Kole in 1998 (Kang et al., 1998). The assay contains the great advantage of having a positive luciferase readout system. By binding ON to an aberrant splice site in the pre-mRNA, sterical blocking of spliceosome binding shifts the splicing pattern and a functional luciferase protein is expressed. The positive readout limits the possibility of false negative effects derived from toxicity or other negative side effects that might
influence a decreasing readout system (such as the case with most anti-sense readouts). In two separate comparative studies of CPP mediated uptake of splice-correction, the CPP transportan was shown to be the most potent delivery vector (Bendifallah et al., 2006; El-Andaloussi et al., 2006b), but as we demonstrate in Paper III, where the transportan analogue TP10 is employed, there might be a correlation between toxicity and efficiency of CPPs. By incorporating a tail of arginines to the Pen sequence, Abes et al. increased delivery of splice-correcting PNA, compared to native Pen, showing that modifications of known CPPs might increase their endosomolytic properties and thereby their delivery efficiency (Abes et al., 2007).

1.4.6. CPP mediated uptake of siRNA

In 1998, Fire et al. first described RNA interference (RNAi) in C. elegans (Fire et al., 1998). For this discovery, they were awarded the Nobel price in 2006. What they observed was that double-stranded RNAs (dsRNAs) can mediate down-regulation of gene expression more efficiently than either single-stranded sense or anti-sense RNAs. RNAi is a naturally occurring post-transcriptional phenomena (Montgomery et al., 1998) and it has since its discovery been reported to occur in numerous invertebrates and vertebrates. It is thought to have significant roles in both antiviral defense and control of endogenous gene expression (Cullen, 2002).

The responsible entity for the down regulation is a double-stranded small-interfering RNAs (siRNAs), typically in the range of 21–25 nucleotides in length with 2 nucleotide 3’-OH overhangs. Once inside the cell, the siRNA is incorporated into a riboprotein complex named the RNA-induced silencing complex (RISC) (Nykanen et al., 2001). Only one of the two strands remains bound to RISC to target a specific mRNA, and the other strand is degraded. Once bound to RISC, the single-stranded RNA guides the degradation of its homologous target mRNA by the activated RISC complex and thereby preventing the synthesis of the targeted protein.

One of the main properties of RNAi that makes it so efficient is that only a few siRNA molecules are required to induce the specific degradation of several target mRNA strands. Since siRNA acts through an active process and not through a stoichiometric mechanism, it can be used to down-regulate the expression of a wide range of genes with very high efficiency. Due to these features, RNAi is at present considered as one of the most promising tools for future treatments of several diseases.

Although siRNA seem like an ideal cargo for CPP mediated delivery, it is small and cationic and even low delivery yields should generate a great biological response, few results have yet been published (for a selection see Table 1.2). Much effort has been put in to utilize CPPs as delivery vectors for siRNA, and Lundberg et al. have shown that although TP and Pen are
able to promote uptake of siRNA through co-incubation, the biological response is absent (Lundberg et al., 2007). This might be a result of that the siRNA is trapped in endosomal compartments or that the CPPs bind their cargo too tightly. The latter seem less likely since in the same study, a CPP designed to break endosomal compartments, EB1, displayed delivery of active siRNA. The CPP MPGα has been reported to be a potent co-incubation vector for siRNA delivery and the delivery pathway has been suggested to be independent of endosomal uptake (Simeoni et al., 2003), but later the contrary has been stated, proposing endosomal uptake of MPG-siRNA complexes (Lundberg et al., 2007; Veldhoen et al., 2006).

<table>
<thead>
<tr>
<th>CPP</th>
<th>ON</th>
<th>Result</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>M918</td>
<td>Plasmid</td>
<td>Co-uptake of plasmid and protein</td>
<td>Paper IV</td>
</tr>
<tr>
<td>Branched Tat</td>
<td>Plasmid</td>
<td>Plasmid expression</td>
<td>(Liu et al., 2005)</td>
</tr>
<tr>
<td>TP10</td>
<td>dsDNA decoy</td>
<td>Inhibition of c-Myc</td>
<td>Paper II</td>
</tr>
<tr>
<td>TP10</td>
<td>dsDNA decoy</td>
<td>Inhibition of NFκB</td>
<td>(Fisher et al., 2004)</td>
</tr>
<tr>
<td>MPG</td>
<td>dsDNA decoy</td>
<td>Decreasing MDR</td>
<td>(Martinet et al., 2000)</td>
</tr>
<tr>
<td>TP, Rp-Pen</td>
<td>Antisense PNA-oligo</td>
<td>Inhibition of HIV-1 replication</td>
<td>(Turner et al., 2007)</td>
</tr>
<tr>
<td>Pep-3</td>
<td>Antisense PNA-oligo</td>
<td>Blocked tumor growth in vivo</td>
<td>(Morris et al., 2007)</td>
</tr>
<tr>
<td>TP, Pen, Tat</td>
<td>Slipse correction PNA oligo</td>
<td>Increased splice-correction</td>
<td>(El-Andaloussi et al., 2006b)</td>
</tr>
<tr>
<td>TP, Rp, Tat, Pen, KFF, SynB3, NLS</td>
<td>Slipse correction PNA oligo</td>
<td>Increased splice-correction</td>
<td>(Bendifallah et al., 2006)</td>
</tr>
<tr>
<td>Rp-Pen</td>
<td>Slipse correction PNA oligo</td>
<td>Increased splice-correction</td>
<td>(Abes et al., 2007)</td>
</tr>
<tr>
<td>Pen, Tat</td>
<td>siRNA</td>
<td>Down-regulation of targeted p38c MAPK</td>
<td>(Turner et al., 2007)</td>
</tr>
<tr>
<td>EB1, MPG, bPrP</td>
<td>siRNA</td>
<td>Down-regulation of targeted Luciferase</td>
<td>(Lundberg et al., 2007)</td>
</tr>
<tr>
<td>MPGα</td>
<td>siRNA</td>
<td>Down-regulation of targeted Luciferase</td>
<td>(Veldhoen et al., 2006)</td>
</tr>
</tbody>
</table>
1.4.7. CPP mediated protein delivery

Since the entire CPP research field started with the findings that the HIV-1 encoded trans-activator protein, TAT, essential for HIV-1 viral gene expression, was readily translocating the plasma membrane (Frankel & Pabo, 1988; Green & Loewenstein, 1988), it is not surprising that CPPs, and especially Tat, have frequently been utilized for protein transduction. Tat, as well as penetratin and VP22 belong to a subclass of CPPs called Protein Transduction Domains (PTDs), and by incorporating PTDs in the same aa chain as a desired protein, the protein can adopt cell penetrating properties and transduce cells both in vivo and in vitro (Fig 1.1) (Table 1.3). The first ones to apply this strategy was the group of S. Dowdy, who generated an in-frame Tat bacterial expression vector in 1998 (Nagahara et al., 1998). With this method, active Cre recombinase have successfully been delivered in vivo (Wadia et al., 2004), which is very strong evidence that CPP mediated transduction of fusion proteins is an effective delivery system. However, there are reports stating that the method does not work for protein delivery (Leifert et al., 2002), which might indicate that delivery is dependent on the properties of the protein.

As mentioned previously, Tat is by far the most common CPP to use as a fusion protein vector and together with penetratin they have in many cases proved to be effective transporters for proteins in this fashion. Very few non-PTDs have been utilized as fusion protein vectors, but polyarginine and polylysine have both been used to deliver proteins as fusion vectors in vivo (Jin et al., 2001; Park et al., 2002).

As performed in Paper III, the CPP and the protein can be linked together by other means than in the same aa chain. We utilized the properties of streptavidin and avidin that strongly bind the low weight molecule biotin to produce stable CPP-linker-protein complexes (Fig 1.1). The same strategy has been employed by Sääläk et al. to compare the efficiency of several CPPs to promote protein uptake (Sääläk et al., 2004) and by Pooga et al. to study protein uptake by transportan (Pooga et al., 2001) and by Mi et al. (Mi et al., 2000). However, since these studies use streptavidin/avidin that binds four biotinylated peptides per protein, these studies can only relatively compare the properties of CPPs, and can not be compared to the fusion protein strategy introduced by the group of S. Dowdy (Nagahara et al., 1998).

An alternative to the fusion protein strategy is co-incubation. The desired protein is simply co-incubated with the CPP, and this CPP-protein complex is then taken up by the cell (Fig 1.1). As discussed in paper III and IV, this method relies on electrostatic interactions and is heavily dependent on the properties of the protein and the employed peptide. The technique was introduced by the group of G. Divita, who designed the CPP Pep-1 (Morris et al., 2001) Pep-1 is, as mentioned previously, derived from a protein-interaction domain and is thus designed to facilitate protein transduction through co-incubation. The co-incubation strategy is attractive, since i) it is less compli-
icated to apply compared to the fusion protein approach, ii) to generate fusion proteins can in some cases disrupt the activity of the altered protein. Except for Pep-1, few studies on co-incubation of proteins have been published, but Jain et al. have shown that penetratin can increase tumor retention of single-chain antibodies *in vivo* (Jain et al., 2005), and in 2007, Myrberg et al. showed *in vitro* uptake of β-Galactosidase by a few CPPs (Myrberg et al., 2007). For an overview of CPP mediated protein delivery, see Table 1.3 for a selection of recent publications.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Protein</th>
<th>Strategy</th>
<th>Result</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pep-1</td>
<td>GFP and β-Gal</td>
<td>Co-incubation</td>
<td>GFP uptake and β-Gal activity</td>
<td>(Morris et al., 2001)</td>
</tr>
<tr>
<td>Pen</td>
<td>Single chain Ab</td>
<td>Co-incubation</td>
<td>Retention in cancer cells</td>
<td>(Jain et al., 2005)</td>
</tr>
<tr>
<td>YTA2</td>
<td>β-Galactosidase</td>
<td>Co-incubation</td>
<td>β-Galactosidase activity</td>
<td>(Myrberg et al., 2007)</td>
</tr>
<tr>
<td>M918</td>
<td>Sleeping Beauty</td>
<td>Co-incubation</td>
<td>Protein uptake</td>
<td>Paper IV</td>
</tr>
<tr>
<td>Transportan</td>
<td>GFP, Streptavidin</td>
<td>Fusion protein</td>
<td>Protein uptake</td>
<td>(Pooga et al., 2001)</td>
</tr>
<tr>
<td>Tat-HA</td>
<td>Cre</td>
<td>Fusion protein</td>
<td>Cre recombination</td>
<td>(Wadia et al., 2004)</td>
</tr>
<tr>
<td>Tat</td>
<td>Bcl-xL</td>
<td>Fusion protein</td>
<td>Neuro-protection</td>
<td>(Cao et al., 2002)</td>
</tr>
<tr>
<td>Tat</td>
<td>Bcl-xL</td>
<td>Fusion protein</td>
<td>Neuro-protection</td>
<td>(Yin et al., 2006)</td>
</tr>
<tr>
<td>Tat</td>
<td>Apoptin</td>
<td>Fusion protein</td>
<td>Apoptosis</td>
<td>(Guelen et al., 2004)</td>
</tr>
</tbody>
</table>
1.5. CPP mediated toxicity

Although extensive research about CPP mediated delivery, little attention has been brought to the toxic side effects this family of peptides might render. When toxicity has been evaluated, the methods and purposes of the studies have differed, making comparison between them complicated. Further, most of the studies done on CPP mediated toxicity have been performed on CPPs alone. Since CPPs main purpose is to act as vectors for biologically active molecules, toxic side-effects ought to be observed under these conditions in order to give a true depiction of the event. In Paper III, we show that toxic side effects caused by CPPs are highly dependent on the cargo used. In most cases, the toxic side effects are decreased if the CPPs are administered together with a cargo. However, this is not true in all cases, Jones et al. showed in 2005, that the toxicity of certain CPPs is highly cargo dependent (Jones et al., 2005). As shown in paper III a fluoresceinyl moiety coupled to Tat decreases the mitochondrial activity (and hence, also proliferation) compared to unlabeled peptide (Paper III). Further, results from paper III imply that amphipatic moment is an important factor in CPP mediated toxicity. This is the case with TP10, where the coupling position within the peptide is important for its toxic profile. By tradition, cargos have been
orthogonally coupled to TP10. This coupling strategy both increases the delivery efficiency and decreases the toxic side effects of TP10. Why this happens remains unclear, but it might be an effect of amphipaticity. When coupled orthogonally, fluorescein is attached to the cationic part of the peptide, this might reduce the amphipathicity of the peptide, and hence, the cytotoxicity. On the other hand, when coupled to the N-terminal part of the peptide, it increases the amphipathic moment and thereby increases toxic side effects. These conclusions are merely speculation, but it has previously been shown that there might be a correlation between amphipathicity and toxicity of CPPs (Drin et al., 2001; Saar et al., 2005). This observation also further supports the previously mentioned hypothesis that uptake of free peptides might deviate significantly from that of fluoresceinyl labeled ones.

1.6. Myc transcription factors and decoy strategies

1.6.1. Myc Transcription factors

Transcription factors (TFs) regulate the binding of RNA polymerase to specific DNA sequences and thereby regulate DNA conversion into messenger RNA, and eventually protein expression. TFs either enhance or repress transcription of targeted genes by inducing or blocking RNA-polymerase DNA binding. Common for all TFs is that they act through specific DNA binding motifs and TFs have been grouped in families on the basis of shared DNA-binding motifs. The critical function of these factors, together with the fact that a single TF can affect the expression of many genes, suggests that they might be an important future pharmaceutical target.

c-Myc is a bHLH/LZ (basic helix-loop-helix/leucin zipper) proto-oncogene, belonging to the Myc family of TFs. Myc proteins are over-expressed in a wide range of human cancers and when over-expressed or over-activated it increases cell proliferation through effects on chromatin structure and basal transcription. The Myc TF regulates expression of a great number of genes through binding to enhancer-box sequences (E-box) in a dimer formation together with the protein Max. Max is a ubiquitously expressed protein that also contains a bHLH/LZ motif. The dimerisation between Myc and Max is essential for Myc-DNA binding (Blackwood et al., 1991). Once bound to the E-box, the nucleosomes are acetylated through recruitment of histone acetyltransferases, which opens up the chromatin structure making it accessible for the RNA polymerase. Subsequently, the
protein heterodimer recruits a number of co-factors that regulate gene expression through a variety of mechanisms (Cowling et al., 2006).

1.6.2. Decoy oligonucleotides in therapy

Morishita and co-workers showed in 1995 that by introducing a specific dsDNA strand mimicking the promoter sequence into the cell, the activity of a specific TF can be decreased (Morishita et al., 1995). The short dsDNA can, once inside the cell, sequester its target TF and thereby prevent the TF to bind to the genomic promoter region (Fig 1.1). The method have proven efficient for in vivo inhibition of TFs like E2F (Morishita et al., 1995) and NFκB (Morishita et al., 1997).

As for many others ON based technologies, stability of the DNA strand is of high importance and to increase stability, the use of ON analogs such as PNA can be included in the decoy. Alone, PNA can not be utilized as decoy molecules (Mischti et al., 1999) but as chimeras together with DNA the effect of the decoy is intact and the stability is increased (Borgatti et al., 2003; Romanelli et al., 2001).

In treatments of chronic systemic diseases it would be necessary to administer the DNA decoy on a long-term basis. Such long-term inhibition of a vital TF might be expected to have severe physiological consequences, since the protein might also be required for a variety of other cellular pathways and responses. This is a significant drawback regarding decoy strategies and its potential for severe inhibition of normal physiological responses. Therefore, the application of decoy strategy as gene therapy might be limited to treatment of acute, TF driven conditions.

However, since the site of decoy effects is in the nucleus, bypassing the endocytotic pathways and translocation of decoy DNA from the cytoplasm to the nucleus is extremely important for the application of decoy DNA therapeutics. As mentioned previously, so far three publications report of efficient delivery of decoy DNA by a CPP in vitro. By connecting TP10 to the consensus sequence corresponding to NFκB DNA binding site, Fisher et al. were able to block the effect of interleukin-1β induced NFκB activation and interleukin-6 gene expression (Fisher et al., 2004).

The same strategy, together with co-incubation of CPP and decoy DNA, has been employed in Paper II to reduce the activity of the transcription factor c-Myc, reducing proliferation of N2a cells. Interestingly, various endocytosis inhibitors had no effect on the uptake pattern, suggesting that uptake of these complexes is not mediated via endocytosis (Paper II).

By co-incubating a decoy targeting the human MDR1 gene promoter with MPG, Marthinet et al. decreased the level of viable cells in a drug resistant cell culture treated with vinblastine, showing that the cell lines resistance to the anticancer drug was repressed by the decoy treatment (Marthinet et al., 2006).
2000). Taken together, these three studies show the potential of CPP mediated delivery of ON decoys, although no evidence for the technique to work in vivo has been presented so far.

![Diagram](image)

Figure 1.2: Schematic picture of DNA decoy sequestering of Transcription factors. Once inside the cell, the decoy binds its target transcription factor which is then unable to promote its function in the cell nucleus

1.7. Gene therapy for genetic disorders

Single-gene disorders are caused by a defect in a single gene. Usual examples include Huntington's disease, cystic fibrosis, and sickle cell anemia, and these disorders are often hereditary. Multifactorial disorders are caused by a combination of genes. Alzheimer's and several forms of cancer can be influenced by multifactorial disorders. When enough defects accumulate in the genome, some cells develop multifactorial disorders. These defects appear either through inheritance of mutations or acquisition of new mutations during the life of an organism, such as exposure to UV light, infection by certain viruses, spontaneous mutations, changes in copying the DNA during the aging process, et cetera. Genetic disorders can also involve entire chromosomes, such as Down syndrome, caused by replication of an entire chromosome. Finally, there are mitochondrial disorders in which the mitochondrial DNA is affected. Gene therapy typically involves the introduction of a therapeutic gene expressing a specific protein in the transfected cell to treat a single-gene disorder. Results accomplished so far suggest that gene therapy
will sooner or later alter the basics of medicine. However, despite several advancements in the field of human gene therapy, no real success has to date been achieved and only a few trials have been proposed to be successful.

Viral delivery systems are by far the most well employed vector for gene delivery. However, several drawbacks are associated with viral vectors. Firstly, they are likely to be more immunogenic and, consequently, result in a greater inflammatory response than other delivery systems. In contrast, immune and inflammatory risks of DNA-mediated gene therapy, such as transposons, are restricted to those associated with endotoxin contamination from bacterial plasmid preparations, and the compound used for delivery. Another concern with viral vectors is not as obvious and comes from the risk of contamination with either replication-competent viruses from the purification step or, endogenous defective viruses that previously have infected the recipient host. Non-viral gene therapy vectors might circumvent these problems, and DNA mediated delivery is not limited by many of the restrictions of viral vectors, such as genome size and elements required for regulation of expression and replication. Further, some viruses have a tendency to integrate near promoters and transcriptional regulating sequences, where they have an increased chance of causing undesirable effects on endogenous gene expression.

The tragic death of an 18 year old boy from Arizona caused by an adeno-virus vector used to deliver potentially therapeutic DNA to the liver in 1999 started a debate concerning the use of gene therapy (Lehrman, 1999) and the first evidence for successful gene therapy was the much criticized retroviral treatment of X-linked severe combined immunodeficiency (SCID-X1) in 2000 (Cavazzana-Calvo et al., 2000). Although successful, it was later found that three of the 17 treated children developed T-cell leukemia as a result of the viral treatment (Hacein-Bey-Abina et al., 2003). These two cases started a debate concerning the safety of viral gene therapy and the use of non-viral vectors might be a safer alternative for future trials in gene therapy.
1.8. Oligonucleotide transfection and non-viral gene delivery

In this thesis, three aspects of ON delivery are studied in three vastly different ways. In short, in paper I we aim to improve a standard method for plasmid delivery by exploiting the ability of TP10 to enhance endosomal release. In paper II the same peptide is used to deliver a short dsDNA decoy strand and, in paper IV M918 is used as a non-viral vector for delivery of a transposase and a reporter-gene.

There are several other non-viral techniques developed to facilitate delivery of ONs and entire genes into cells. The main obstacle to overcome in gene therapy is to develop a technique that delivers a desired therapeutic gene to selected tissue where proper gene expression or regulation can be achieved. An ideal delivery method should achieve at least three major criteria: i) it should protect the therapeutic ON against degradation by nucleases in the host animal, ii) it must be able to penetrate the plasma membrane and deliver the desired ON to the desired compartment, iii) it should be non-toxic to the treated individual. For stable, long term expression of a therapeutic gene it should also be able to integrate the gene into the host genome. However, in cases where the targeted cell is killed such as in gene therapy for cancer treatment or for regulating ON, the last is not essential. Here, some of the existing methods to deliver ONs and genetic material will be discussed.

1.8.1. Mechanical in vivo transfection

The most straightforward approach, and for that reason maybe the most peculiar one, is to simply bombard the cell culture or tissue with DNA covered gold particles using a gas pressured gene gun. However crude this methods seems, it has successfully been used to deliver genetic material into skin or surgically exposed tissues (Yang et al., 1990). Another way of transferring genetic material in vivo by mechanistic force is electroporation (Heller et al., 2005; Titomirov et al., 1991), where an electric current is applied over the cell culture or tissue. The current cause transient pores to form in the plasma membrane where the DNA can passively diffuse into the cell and the nucleus. Of course such a harsh method has several major drawbacks for in vivo applications. As current needs to be applied to the desired area, it has a limited effective range of about 1 cm between the electrodes which makes it difficult to transfecct cells in a large tissue area. If transfection is located internally, a surgical procedure is required to place the electrodes deep into the desired organs. Last, high voltage, and consequently thermal heating, applied to tissues can result in irreversible tissue damage.
Hydrodynamic gene transfer is a very simple method where ON or genes are introduced to highly perfused organs (such as the liver) through injection of a large volume of aqueous DNA solution into the bloodstream. This causes a brief overflow of injected DNA solution in the bloodstream that exceeds the cardiac output. As a result, the injection induces a flow of DNA solution into the liver, a rapid rise of pressure, transient liver expansion, and uptake of the DNA into the liver (Zhang et al., 2004). The efficiency of this method is the highest yet using a non-viral delivery system and about 30-40% of the hepatocytes can be transfected by only one injection (Liu et al., 1999). The method has proven to efficiently deliver a variety of compounds to the liver, including siRNA, linear DNA and plasmids (Al-Dosari et al., 2005).

1.8.2. Chemical in vivo transfection

In 1987, Felgner et al. reported that a cationic double chain monovalent quaternary ammonium lipid can bind and deliver DNA to cells in vitro (Felgner et al., 1987). Since then, several cationic lipids have been reported to enhance delivery of ONs. When mixing anionic DNA with an excess of cationic liposomes, the DNA is spontaneously condensed into small positively charged particle sometimes referred to as a lipoplex. Once the DNA is in a lipoplex complex, it is well protected from nuclease degradation and the complex is able to trigger cellular uptake and facilitate the release of ONs from intracellular vesicles before being degraded in lysosomal compartments. Although cationic lipids are very effective in vitro, their ability to deliver genetic material in vivo is limited. They have however been utilized as vectors for in vivo delivery through aerosol administration (Hyde et al., 2000) and intravenous injection, even though acute inflammation reactions were detected in the treated animals (Song et al., 1997). By conjugation of targeting ligands to these complexes it might be possible to create target-specific ON carriers (Dauty et al., 2002), but due to toxic side effects lipoplex delivery of ON is still not feasible for use in humans.

Another class of ON vectors is, as this thesis states, naturally occurring and synthetic cationic polymers. As early as 1988, poly-lysine was used for in vivo ON delivery (Wu et al., 1988) and since then several cationic polymers have been explored as carriers for in vitro and in vivo ON delivery. Most cationic polymers condense the DNA into small particles which then enters the cell via endocytosis through charge interactions with the negatively charged plasma membrane. The most well studied of these cationic polymers is probably polyethyleneimine (PEI), which has proven to be an efficient ON vector both for cultured cells and in vivo (Boussif et al., 1995). As with cationic lipids, it seems possible to promote targeted delivery of PEI-DNA complexes by coupling a ligand to the polymer (Kircheis et al.,
and as shown in Paper I, enhancement of PEI mediated delivery by conjugation of functional groups to the polymer is also an option. However, PEI is not biodegradable and in a study in 2003 on PEI toxicity, no indication for apoptosis was detected, suggesting that the polymer induces necrotic cell death (Fischer et al., 2003).

1.9. Transposable Elements

Transposable elements (TE) were discovered by Barbara McClintock (McClintock, 1950) for which she was awarded the Nobel price in 1983. TEs are mobile genetic elements that can move from one position to another within the genome. Elements that encode enzymes required for their own transposition are referred to as autonomous. Non-autonomous elements still require autonomous elements from their own or a different gene family for their movement. The transposition of DNA is central to evolution and transposable elements are an important source of the mutations on which natural selection operates. It is estimated that identifiable transposable elements make up about 40% of the genome of our own species (Lander et al., 2001). Because of their ability to insert themselves into host genomes, TEs can be seen as natural, non-viral gene-delivery vehicles and they are an important and widely used tool for germ-line transgenesis and mutagenesis in invertebrate model animals.
TEs can be divided into two main classes according to their structural organization and mechanism of transposition (Finnegan, 1989). Class I elements use an RNA-mediated mode of transposition and encode a reverse transcriptase (RT). This class is also a member of the larger family of retroid agents that also includes retroviruses. The Class II elements, the transposons, use a DNA-mediated transition through a cut and paste mechanism where a DNA strand is cut out from a chromosome and then inserted at another position within the same or in another genome (see Fig 1.2).

1.9.1. Transposons in mammals

Transposable elements in vertebrates are since long transpositionally inactive due to the vertical accumulation of mutations in their transposase genes. In our branch of evolution, transposition has not occurred in the last
50 million years. The first reactivated transposon in vertebrates is the Sleeping Beauty transposon (SB). SB was found latent in the genome of salmon fish and its DNA sequence had accumulated so many mutations so the transposon no longer jumped, but rather slept in the form of inactive non-translated DNA. The extinct functions of SB were resurrected in 1997 (Ivics et al., 1997), hence the name Sleeping Beauty. Since this SB transposon is employed in this thesis, it will be discussed in a separate paragraph later on. Here a selection of transposons active in mammalian cells will be presented and discussed.

Frog Prince

The transposase Frog Prince (FP) is like SB a class II transposon and a member of the Tc1/mariner superfamily. FP is resurrected from the frog species Northern Leopard Frog (Rana pipiens) and has approximately 50% sequence similarity to SB. Much like SB, it catalyzes cut-and-paste transposition in fish, amphibian and mammalian cell lines (Miskey et al., 2003) and FP has also been used for stable RNAi integration (Kaufman et al., 2005). The FP is, like SB, flanked by IR/DR sequences (see paragraph below), but it shows no cross-mobilization with SB. This means that even though FP and SB generates from the same family of transposases and show high similarity, they can be utilized simultaneously in the same cell without risk of cross-transposition between the two transposase recognition sites (Miskey et al., 2003).

PiggyBac

The piggyBac (PB) elements are about 2.4 kbp transposons flanked by 13 bp identical inverted terminal repeats (ITRs) and 19 bp asymmetric repeats (Li et al., 2001). The transposase is 594 aa long (Cary et al., 1989; Fraser et al., 1996) and the PB transposons insert itself into the tetranucleotide TTAA site, which is duplicated upon insertion (Fraser et al., 1996). Because of the TTAA target-site sequence, the insertion is not random as for many other transposons. PB transposition has been shown in human and mouse cell lines, and also in mouse germline cells both in vitro and in vivo. PB transposition transgenes can be up to 14.3 kbp (Ding et al., 2005) making it a valuable carrier of very long transgene inserts. Further, PB does not seem to be hampered by overproduction inhibition like SB seems to do (Wilson et al., 2007), which might be an important aspect for therapeutic use.

ΦC31

ΦC31 is not a transposon per se, but an integrase. The integrase derives from a Streptomyces phage with the same name and it has been shown to insert through recombination in a number of mammalian cell types (Groth et al., 2000). The sites between which the recombination occurs are generally identical, but ΦC31 integration naturally occurs between attP sites present in
the phage and \textit{attB} sites in the bacterial genome. In mammals there are sequences similar to \textit{attP}, named pseudo \textit{attP} sites. There are a limited number of pseudo \textit{attP} sites in mammalian chromosomes, which restricts \Phi C31 integration to specific regions in mammalian genomes. This is an important feature of \Phi C3, although positions of pseudo \textit{attP} sites might vary depending on cell type (Chalberg \textit{et al.}, 2006).

To use transposons to integrate genomic material for research purposes is not a new phenomenon, but the idea to employ them in human gene therapy is something else. Although they can stably integrate a desired gene into the human genome, there is still too little known about the host immune response towards this technique. A great deal of studies must be performed before transposons can efficiently and safely be utilized as a part of future human gene therapy.

1.9.2. The Sleeping Beauty transposon

As previously mentioned, Transposons are highly frequent in our own species, consisting of up to 40\% of our genome. However, due to absence of selection pressure, accumulation of mutations in the transposon sequence will eventually lead to inactivation of the transposon movement. Of all of the transposon remains found in vertebrates, the Tc1/mariner super-family is the best characterized (Goodier \textit{et al.}, 1994; Ivics \textit{et al.}, 1996). This family can be divided into three major types, zebrafish, \textit{Xenopus} T\text{\textsc{tx}}r and salmonid type elements (Ivics \textit{et al.}, 1996), and of the three subfamilies, the salmonid type seems to be the one that lost its transposition activity most recently in evolution (about 10-15 million years ago).

Due to their wide spread among species in nature (Plasterk, 1996), it is possible to speculate that the Tc1/mariner transposase family is not restricted to specific host species for its activity. Although wide spread, all elements isolated from several fish species have accumulated mutations through evolution to the extent that they have since long lost their activity. In an attempt to reactivate the activity of this transposon Ivics \textit{et al.} aligned isolated defective transposase elements creating a genetic archetype sequence of the extinct gene. The method proved to be a success, and in 1997, 10 million years after its extinction, the resurrected transposase SB was introduced in the literature (Ivics \textit{et al.}, 1997). The SB transposon system consists of two distinct parts that are both mutually exclusive for transposition to occur. \textit{i)} The synthetic transposase gene (SB-t\text{\textsc{pase}}) and \textit{ii)} a salmonid type element carrying the regions recognized by the transposase (see Fig 1.2) (Ivics \textit{et al.}, 1997). The genetic elements recognized by the SB-t\text{\textsc{pase}} flank the transposon and consist of 200-250 bp long inverted repeats (IRs). Within each IR, there are two 15-20 bp long direct repeats (DR) that act as binding sites for the
transposase and together these regions are referred to as IR/DR. These four DR binding sites within the ID/DR pairs are not identical (the outer DRs are two bp longer) but they are all essential for successful transposition (Ivics et al., 2004).

When the transposase and the transposon are present in a cell simultaneously, four major steps lead to the excision of the genetic sequence from one locus and later integration of the sequence at a different locus. These steps are i) binding of the SB-tpase to the IR/DRs ii) complex formation where the two ends are joined by the transposase iii) excision from the donor site iv) integration at another locus within the same or in another genome (Fig 1.2).

The integration site of SB is greatly specific at the actual sequence level and takes place at TA dinucleotides, which are duplicated upon insertion and flank the integrated DNA element. Even though the TA insertion is specific at the sequence level, it can be considered to be random from a genomic perspective (Vigdal et al., 2002), and successful attempts of trying to direct SB transposon insertion at a genomic level have been carried out in human cells (Ivics et al., 2007; Yant et al., 2007).

Transposition of SB has shown to be efficient in a wide variety of cells and species and evolutionary distance from the salmonid origin of the transposase does not seem to be the crucial factor, since transposition has been shown to be more efficient in human cells than in lower organisms (Ivics et al., 2004). It seems like the recipient cell is of importance for efficient transposition. In 2003 it was suggested that the nuclear protein, HMGB1 (High-Mobility Group protein B1) that binds ssDNA and unwinds dsDNA, is a host-encoded cofactor for SB transposition. It was shown that transposition was severely reduced in mouse cells deficient in HMGB1 expression, and that the effect could be reversed by transient over-expression of the HMGB1 protein. Further the negative effect was partially reversed by over-expression of the analogue HMGB2, but not with HMGA1 (Zayed et al., 2003). SB-tpase has also been suggested to interact with host factor Miz-1 (Myc-interacting zinc finger protein 1) and thereby down-regulate cyclin D1 expression in human cells. The transposase associated down-regulation of cyclin D1 results in a prolonged G1 phase and retarded growth of transposase-expressing cells (Walisko et al., 2006).

1.9.3. The Sleeping Beauty transposon in gene therapy

As with many other therapeutic techniques, delivery is a key issue in gene therapy and transposons might be an important tool for future gene therapy research. When SB was introduced in 1997 it opened up a new field in gene therapy. Since then several publications have shown efficient insertion by SB of several genes in a broad range of cells (Izsvak et al., 2000), including human cell lines (Geurts et al., 2003), and it has also been found
active in vivo (Carlson et al., 2003; Yant et al., 2000). Since the SB system can be based on the sole use of plasmid DNA, any available plasmid DNA delivery system can be utilized for its delivery. Further mRNA can be used as a transposase source, limiting the risk of spontaneous integration of the SB-tpase gene in the host genome (Wilber et al., 2006). Another advantage of the SB system is the ability to integrate very large strands of genetic material without severe loss of activity. At 6 kbp, there is a 50% decrease of insertion. This range is enough to deliver 70-80% of the coding sequences in the human genome (Geurts et al., 2003), and by improving the activity of the transposase by mutagenesis, the size of the insert will probably be of less importance.

A number of publications in recent years show SB as a potential tool for future gene therapy. It has been used to complement both factor VIII and factor IX deficiencies in hemophilic mice (Ohlfest et al., 2005; Yant et al., 2000) and has shown efficiency in the treatment of murine tyrosinemia type I (Montini et al., 2002). Further it has been delivered to the liver by hydrodynamic administration, prolonging the production of insulin in the liver of type I diabetic mice (He et al., 2004). In 2006 it was shown that SB can stably transfect primary human T-cells (Huang et al., 2006) and the next year, correction of murine fumarylacetoacetate hydrolase (FAH) deficiency was shown in transplanted hepatocytes (Wilber et al., 2007). These reports show that the SB system might have the ability to work as a future non-viral gene therapy platform, if it is combined with a proper delivery system.
2. Aims of the study

Papers and results presented in this thesis all consider different aspects of peptide mediated delivery. Our aims were to improve and evaluate existing methods and also aspire to develop new ways of employing CPP strategies. All four papers have distinctive approaches to peptide mediated delivery

- Paper I: Improve established polyethyleneimine transfection by conjugating the CPP TP10 to the polymer backbone

- Paper II: Utilize TP10 as a vector for a dsDNA decoy targeted against transcription factor c-Myc

- Paper III: Evaluate three well characterized CPPs; Tat, Penetratin and TP10, by means of their delivery efficiency and toxic side effects using commonly utilized cargoes

- Paper IV: Apply the novel CPP M918 as a vector for transposase based non-viral gene delivery
3. Methodological Considerations

Methods used in this thesis are described in each paper. In this chapter the practical and theoretical aspects of the methods will be discussed.

3.1. Peptide synthesis and purification

The ability to stepwise build peptides on solid phase was first introduced in 1963 by Robert Bruce Merrifield (1921-2006) (Merrifield, 1963) for which he was awarded the Nobel price in 1984. Solid phase peptide synthesis (SPPS) allows the synthesis of natural peptides which are difficult to express in bacteria. Further, the incorporation of unnatural amino acids, backbone modification, and the synthesis of D-peptides, makes SPPS the standard method to produce peptides.

In brief, small polymer beads are covered with linkers on which peptide chains can be built in a stepwise manner. The linker is designed so that it will retain covalently bound to the peptides until cleaved when desired by a strong acid (in the case of t-Boc SPPS, hydrogen fluoride acid). Amino acids with reactive side chains are protected by protective groups where needed.

The overwhelmingly important consideration compared to other synthesis methods is the ability to generate extremely high yield in every step. For example, if each step were to have 99% yields, a 20-amino acid peptide would be synthesized in 80% final yield, if each step were 90%, it would be synthesized in merely 10% yield.

The peptides employed in this thesis were synthesized in a stepwise manner in a 0.1 mmol scale on an automated peptide synthesizer (Applied Biosystems, Model 433A) using t-Boc solid-phase peptide synthesis strategy. tert-Butyloxycarbonyl amino acids were coupled as hydroxybenzotriazole esters to a p-methylbenzylhydroxylamine (MBHA) resin to obtain C-terminally amidated peptides. This modification makes peptides more stable in a biological environment, and further adds one positive charge to the peptide. Peptides containing a formyl protected tryptophan residue were deprotected in 20% piperidine in DMF for 1 h. Peptides used for uptake studies were labeled N-terminally and TP10 was also labeled orthogonally on Lys$^7$ with carboxyfluorescein using 5 eq. 5,6-carboxyfluorescein, 5 eq. 1,3-diisopropylcarbodiimide, 5 eq. HOBt and 20 eq. N,N-diisopropylethylamine dissolved in dimethyl sulfoxide (DMSO)/dimethylformamide (DMF) 1:1 over night. Biotin was coupled as hydroxybenzotriazole ester to the N-terminus of tat and penetratin or to the ε-amino group of lys$^7$ of TP10.

The peptide was finally cleaved from the resin using liquid HF at 0 °C for 1 h in the presence of p-cresol (1:10). Peptides containing sulfur were
cleaved in the presence of p-cresol and p-thio-cresol (0.5:0.5:10). Peptides were purified using reversed phase HPLC and molecular weight was determined by MALDI-TOF mass spectrometry using Perkin Elmer prOTOF™ 2000 MALDI O-TOF Mass Spectrometer (Perkin Elmer, Sweden).

3.1.1. PNA synthesis

PNA was synthesized basically in the same fashion as abovementioned peptides. The difference being that the resin was downloaded to about 0.1-0.2mmol/g compared to about 1mmol/g for peptide synthesis. The reason for this being that PNA is more bulky than amino acids and tends to aggregate during synthesis. Additionally, 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) was employed as coupling reagent. To allow for disulfide conjugation between the PNA and CPPs, a cysteine was introduced in the PNA C-terminal.

3.1.2. Peptide-PNA conjugate setup

To create CPP-PNA conjugates linked by a disulfide bond, 3-nitro-2-pyridinesulphenylated TP10 was co-incubated over night with cystein coupled PNA in a mixture of DMSO/DMF/acetic acid (2/2/1, pH 5.5). The conjugate was purified and molecular weight was defined as previously described. When performing mass spectrometry, three peaks were identified; One peak corresponding to the peptide, one to the PNA and, finally one corresponding to the Peptide-PNA conjugate. Nevertheless, the complex was considered to be pure since only one peak was detected when analyzing the conjugate by analytical reverse-phase HPLC. The three peaks detected by mass spectrometry is most likely an effect derived from disulphide bond degradation caused by the high energy laser produced by the mass spectrometer.

3.2. Selection of CPPs

There are numerous CPPs described in the literature and almost every lab working with CPs have their own preferred CPP, often designed in their lab. By tradition our lab has worked with Transportan (TP), which is a chimeric peptide consisting of parts from the neuro-peptide galanin and the wasp venom mastoparan and was introduced by M. Pooga et al. in 1998, (Pooga et al., 1998a). More recently, we are working with the deletion analog of TP, named TP10 (Soomets et al., 2000). In the first two papers TP10 is utilized in two different ways. In paper I, TP10 is used to enhance the
efficacy of traditional PEI transfection. By conjugating the peptide to PEI, or by adding it to the transfection mixture, the transfection efficiency was increased several fold. The increase was most pronounced at lower concentrations allowing decreased amounts of PEI, without loss of delivery yield. TP10 was shown to be an efficient vector of short dsDNA in paper II, where the CPP was either covalently attached, or mixed with, DNA decoy targeting the oncogenic transcription factor c-Myc. Introduction of the decoy significantly reduced cell proliferation in vitro. In both papers, the uptake mechanism was shown to be mainly endocytotic.

In paper III, a comparative study between three of the major CPPs is performed. Selected CPPs are Penetratin, Tat and abovementioned TP10. Penetratin, or pAntp, is the first discovered CPP, deriving from the third helix of the homeobox-protein Antennapedia. Tat, probably the most extensively studied CPP, was introduced a few years later than Penetratin. Tat originates from the HIV-1 transcription factor with the same name. Different variants of Tat have been presented in the literature, but here the 47-57 variant is used. The reason for selecting these three CPPs, besides the fact that they are well characterized and frequently used, is their diverge properties. Penetratin is 16 residues long and carries a net positive charge of 7. Tat on the other hand is highly cationic carrying 8 positive charges and only 11 amino acids. TP10 is 21 residues long and contains only 4 positive charges. Further, TP10 is highly amphiphatic and has a hydrophobic N-terminal tail. By comparing both the transfection yield as well as the long and short term toxicity of the three peptides with various cargoes, a comprehensive image of the vast difference in delivery properties was established.

In 2007, S. EL-Andaloussi et al. introduced a new CPP M918, derived from the tumor suppressor protein p14ARF (El-Andaloussi et al., 2007a). In paper IV, M918 is employed as a vector for simultaneous delivery of both the transposase Sleeping Beauty and a donor plasmid.

3.3. Cell culture

Different cell lines have different properties and respond differently to treatments. Therefore it is important to analyze experiments in more than one cell-line in order to draw any general conclusions from an experiment. Further, one can not be certain that one cell-line is the same in two different laboratories. Age, cell passage number, and, cultivation milieu is just some of the parameters that can change the features of a specific cell-line. All cell lines used are well characterized and can easily be cultured in common media containing serum and antibiotics.

HeLa cells are undoubtedly the most frequently used cell line and they are derived from cervical cancer cells obtained from Henrietta Lacks, who
died from her cancer in 1951. Neuro-2a was established from a spontaneous tumor of a strain A albino mouse. C3H/10T1/2 was isolated in 1972 from a line of C3H mouse embryo cells and the CHO-K1 cell line was derived as a subclone from the parental CHO cell line initiated from a biopsy of an ovary of an adult Chinese hamster in 1957. Cultivation of the cell lines are described in the corresponding papers.

3.4. Flow cytometry

Flow cytometry is a powerful method for counting, examining and sorting cells suspended in a stream of fluid. It allows for simultaneous analysis of several physical and/or chemical characteristics of cells flowing through an optical detection apparatus. By detecting specific properties, flow cytometry can distinguish a large number of single cells by means of size, apoptosis, proliferation, protein expression and a many of other features. In paper I, flow cytometry is used to analyze eGFP expression in cells either conventionally transfected with PEI, or transfected with PEI and TP10.

3.5. Quantification of CPP mediated uptake

To study the actual amount of CPP that translocates from the extracellular environment and gain access to the cytoplasm, nucleus or any other organelle is not an easy task. The most commonly used method is to utilize a fluorescent marker cargo covalently attached to the peptide vector. The basis for this is that the method is quick, easy and economical. However, it does not discriminate between peptides caught in endosomal vesicles, membrane associated and, actual translocated peptides/cargo. In paper I and IV the translocation is assured by a positive readout, meaning that if the plasmid cargo has not entered the cell no protein expression will occur. In paper II, on the other hand, the readout is negative. If the decoy cargo targeting the TF c-Myc is ferried into the cytoplasm/nucleus, it will bind its target protein disabling it from promote expression of c-Myc regulated genes. This results in a decrease of proliferation, which could be the results of several biological events and not only a decrease of c-Myc regulated gene expression. The negative readout makes translocation verification troublesome compared to a positive readout method.

In paper III, translocation is only verified by fluorescent cargo. As discussed above, this method does not discriminate between actual translocation and cargoes caught in endosomal vesicles and/or membranes. However, when studying the literature one finds that there are sufficient verifications for CPP translocation. For example, the Cre recombinase fusion protein system developed by S. Dowdy (Wadia et al., 2004) and the splice-correction
assay developed by R. Kole (Kang et al., 1998) are both excellent models employing a positive read out to confirm cargo translocation. Taken together, biological activity assays (both positive and negative) and fluorescence translocation assays provide substantial evidence that CPPs are efficient vectors for a vast spectrum of cargoes.

3.6. Protein expression and purification

The ability to express and purify native and recombinant proteins is of tremendous importance in current molecular biology. There are several different methods to produce proteins, including SPPS previously discussed in this chapter. Although, SPPS is a convenient method to produce shorter peptides, utilization of bacteria is a less cumbersome method for production of long amino acid chains.

In paper IV Rosetta™ cells, a strain of the archetypal bacterium for biochemists E. coli, and the lac-operon dependent expression vector pET15b are used for production of recombinant transposase. The choice of bacterial strain and expression vector can greatly affect the efficiency of protein expression. The Rosetta™ strain is protease-deficient and carries a lac-permease (lacY) mutation which allows for even entry of IPTG (allolactose analogue) into all cells in the population. This leads to a concentration-dependent IPTG induced protein expression and by adjusting IPTG concentration, expression can be regulated from very low level expression up to fully induced expression levels. Lower expression level may enhance the solubility and activity of difficult proteins. Further, the Rosetta™ strain carries the pRARE plasmid, which encodes tRNAs for mammalian codons that rarely occur in normal E. coli. This enhances the expression levels of proteins otherwise limited by species specific codon usage.

By using the pET15b vector for protein expression, a His-tag is N-terminally introduced to the expressed protein. This tag simplifies protein purification greatly, but also alters the native amino acid sequence of the protein which can disturb protein function. The purification of His-tagged proteins is a straightforward method using Ni-NTA resin that binds polyhistedins with high affinity. After washing the tagged protein is competitively eluted using increasing concentrations of imidazol.
3.7. Western Blot and protein detection

Western blot originates from the laboratory of G. Stark at Stanford. The name Western blot was given to the technique by W. N. Burnette in 1981 (Burnette, 1981) and is a word play on the DNA counterpart, Southern blot. Traditionally Western blots are used to measure protein expression levels at a given time point in a cell lysate. In paper IV the method is used to evaluate CPP mediated translocation of the transposase Sleeping beauty.

By exposing cell cultures to a CPP-protein complex or CPP-fusion proteins and then perform a Western blot using Sleeping beauty specific antibody, the amount of Transposase taken up by the cells can be measured. The amount of translocated transposase was indexed to the structural protein tubulin to control protein levels between cell lysates. This practice ensures correction for the amount of total protein on the membrane in case of errors or incomplete transfers or uneven loading of lysates.

As discussed in the chapter about CPP uptake quantification, since the Western blot was performed on total cell lysate this technique does not discriminate between membrane-bound transposase, transposase trapped in endosomal vesicles or if it actually is in the cytoplasm/nucleus. However, the CPP used in these experiments, M918, has been shown to translocate ONs to the nucleolus with biological effects. Further, both CPPs Tat and Pep-1 have been utilized as protein vectors where the translocated protein has retained its activity post delivery (see corresponding sections in chapter 1.4).

3.8. Methods to study CPP toxicity

When studying transfection and methods of cellular translocation, uptake yields often get the center of attention. However, the toxic side effects these methods might render are just as important as effective delivery. As discussed in paper III, it is important to consider both the acute and long term toxicity, since they do not necessarily correlate. Varying CPPs have vastly different toxic properties. While some display severe cellular damage at 10μM, such as TP10, some peptides are more or less non-toxic at concentrations up to 50μM, as in the case of Penetratin (paper III). There seem to be some correlation between amphipathic nature of peptides and toxicity. Additionally, CPPs displaying high levels of toxicity are often very efficient delivery vectors.

When studying transport over biological membranes, the most obvious cause of toxicity would be acute perturbation of the cellular plasma membrane. In this thesis two different methods with essentially the same purpose are used to quantify membrane disturbance, 2-deoxy-D-glucose-6-phosphate
(DGP) leakage (paper I, II and IV) and lactate dehydrogenase (LDH) leakage (paper III).

DPG leakage assay

![Diagram of DPG leakage assay]

*Fig 3.1: Schematic picture of the deoxy-D-glucose membrane leakage assay. Deoxy-D-glucose is introduced to the cell by glucose carriers and phosphorylated by hexokinase inside the cell. The phosphorylated product can not leave the cell if the plasma membrane is intact.*

In the DPG leakage assay (Fig 3.1) cells are loaded with a radioactively labeled glucose analogue, \(^{[3]}H\)-2-deoxy-D-glucose (DG). The analogue is introduced to the cell by glucose carriers and phosphorylated by hexokinase when inside the cell. The product, DGP, is unable to exit the cell if the plasma membrane remains intact (Walum et al., 1982). When treated with CPP of interest, the efflux of DGP to the supernatant is a direct measurement of the peptide induced plasma membrane perturbation.
**LDH leakage assay**

![Diagram of LDH leakage assay](image)

Fig 3.2: Schematic picture of the LDH membrane leakage assay. LDH released from the cell through damaged membranes is measured in the supernatant using an enzymatic assay where reazurin is converted into the end product resorufin.

The enzyme LDH is responsible for the interconversion of lactate and pyruvate with concomitant exchange of NAD⁺ and NADH. LDH released from the cell through damaged membranes (Fig 3.2) is measured in the supernatant using an enzymatic assay where reazurin is converted into the end product resorufin, which can be quantified by measuring fluorescence at 560Ex/590Em. LDH is a rather large protein (MW~140 kDa), so the LDH leakage assay is not as sensitive as the DGP assay.

**Wst-1 Proliferation assay**

![Diagram of Wst-1 proliferation assay](image)

Fig 3.3: Schematic picture of the wst-1 proliferation assay. The wst-1 salt is cleaved to soluble formazan dye by the succinate-tetrazolium reductase, which exists in active mitochondria. The formazan dye formed is then a measurement of viable cells.
The wst-1 assay is a way to monitor mitochondrial activity and long term toxicity in a cell culture (Ishiyama et al., 1996). Although high mitochondrial activity often, but not necessarily, is a sign of proliferating cells the assay is frequently used as a measurement of proliferation. In brief, cells are treated with tetrazolium salt (wst-1). The salt is cleaved to soluble formazan dye by the succinate-tetrazolium reductase, which exists in mitochondrial respiratory chain and is active only in viable cells (Fig 3.3). Total activity of this mitochondrial dehydrogenase in a sample is elevated with the increase of viable cells. As the increase of enzyme activity leads to an increase of the production of formazan dye, the amount of formazan dye is related directly with the number of metabolically active cells. The formazan dye formed by metabolically active cell can be quantified by measuring its absorbance. Hence, the absorbance of formazan dye solution is in direct proportion to the number of viable cells.
4. Results and discussion

The four articles that constitute the basis of this thesis are all considering different aspects of CPP mediated delivery of ONs and/or proteins. In paper I, II and IV focus lies on delivery applications of specific cargos and in paper III a more general view of delivery and toxicity is presented. In this chapter, the essential findings of each paper will be highlighted and discussed.

4.1. The CPP TP10 can enhance efficiency of standard PEI transfection (Paper I)

PEI, transfection has been a standard method for the introduction of plasmids into cells in vitro for a long time. Although efficient, it can render toxic side effects to cells. In paper I, we aspired to see whether TP10 can, alone or joined to a plasmid via PNA, promote plasmid uptake. Further TP10 was studied as an enhancer of standard PEI transfection and whether TP10 alters route of PEI mediated uptake.

Results show the inability of TP10 to carry plasmids across the cell membrane both in complex with plasmid and as a single peptide linked to the plasmid via a PNA oligomer (Fig 4.1A, B). However, despite the incapacity to condense DNA by itself, TP10 enhances uptake of PEI condensed plasmids (Fig 4.1 D). By conjugating PEI and thereby also the DNA/PEI complex surface to TP10 (Fig 4.1 E), presence of at least 10 nM of free TP10, results in increased levels of reporter gene expression. If TP10 is covalently attached to the DNA/PEI complexes the concentration can be magnitudes lower compared to free peptide. If higher concentrations of TP10 were used transfection was decreased compared to moderate concentrations. Although TP10 merely contains four positive charges it can still compete with PEI for binding to DNA phosphates. At TP10/PEI ratio 24/1, almost every fourth positive charge comes from TP10 and this could be enough to impede PEI/DNA complex formation.

Cells transfected by standard PEI method had a higher count of cells expressing the eGFP 24 h post treatment. However, cells transfected by the TP10–PNA modified protocol, where a TP10-PNA conjugate is linked to the plasmid (Fig 4.1 C), gave a smaller population of cells expressing higher levels of the reporter gene. After 2 days the TP10-PNA modified protocol had surpassed the standard PEI protocol in both cell number and reporter gene expression. The difference can be explained either by changed uptake...
mechanism or by different kinetics of reporter gene expression. Since experiments performed with endocytosis modifiers suggest that the TP10/PEI mediated uptake mechanism remains unchanged compared to standard PEI transfection, the later explanation seems more likely.

Bearing in mind that the uptake mechanisms seem identical and the 72 h TP10–PNA protocol gives a greater population of eGFP expressing cells, the difference in eGFP expression after 24 h is difficult to explain. Higher expression levels of eGFP should mean either that a higher copy number of plasmids have been delivered or that transcription is enhanced by PNA. Less eGFP expressing cells must be an effect of either slower nuclear delivery or transcriptional inhibition by PNA. Since a mixture of unconjugated TP10 and PNA do not show the same profile, the effect requires the TP10-PNA conjugate. The observed fact can be explained if the conjugate forms large plasmids aggregates. Plasmid-TP10-PNA aggregates would be very large complexes that might need longer time to reach the nucleus. Once the aggregate has reached the nucleus, a higher number of plasmids will be expressed resulting in an increased eGFP expression.

Observed improvements of eGFP expression at lower PEI concentration could be explained by buffering of endosomal pH by TP10 resulting in increased endosomal escape. At low PEI concentrations the TP10 amino groups could interfere and improve the observed transfection yield. At higher PEI concentrations, the peptide influence decreases, resulting in reduced effect of the peptide.

When co-administered in PEI transfection, either as TP10–PNA or as TP10–PEI conjugate, the peptide is more likely internalized through PEI induced endosomes compared to addition of free peptide. Consequently, both conjugates result in higher uptake levels, comparable to addition of 100-fold higher concentrations of free TP10.

In summary, coupling TP10 to PEI or DNA via a PNA linker or adding free TP10 to PEI/DNA complexes increases transfection yields. Increase is not due to a switch in internalization mechanism, but rather by improved endosomal release (Fig 4.1).
Figure 4.1: TP10 and PEI mediated Plasmid uptake. A) TP10 anchored to a plasmid through a PNA linker does not facilitate uptake. B) Neither does TP10 alone when co-incubated with the plasmid. C) When TP10 is anchored to a plasmid through a PNA linker in a PEI transfection. Uptake is increased. D) The same is true when TP10 is co administered with PEI. E) TP10 covalently bound to PEI increases transfection compared to PEI alone.

4.2. Transduction of a CPP-DNA decoy complex targeting c-Myc reduces cell proliferation in vitro (Paper II)

The ability to inhibit function on DNA binding proteins by introduction of DNA decoys can, as discussed in chapter 1.6, have several therapeutic benefits. In paper II, TP10 was used as a delivery vector for a dsDNA decoy targeting the oncogenic transcription factor Myc. TP10 was able to deliver the decoy both anchored to the decoy via a disulphide bond to a nine bases long PNA linker (Fig 4.2A) and co-incubated with the CPP to form a non-
covalent complex (Fig 4.2B). The mechanism of internalization was established to be independent of endosomal formation.

Even though the TP10–PNA Myc decoy (Fig 4.2A) only slightly increased cellular uptake, the decoy had a strong biological effect decreasing cellular proliferation. If comparing amount of decoy delivered to the biological effect, the decrease in proliferation was more pronounced when decoy was transduced via TP10–PNA conjugate than via non-covalent TP10 decoy complex (Fig 4.2B). However, decoy delivered by complex formation had a stronger overall effect on proliferation. One can speculate that the DNA decoy and the cationic TP10 form stable complexes. These complexes are delivered into the cell but the strong interaction remains, making it more difficult for the decoy to bind its target protein. When anchored to the peptide via TP10-PNA, delivered DNA is not shielded and interaction with the target protein is not hindered. Consequently, even though more DNA decoy entities are delivered into the cell through non-covalent complex formation (about 20 million Myc decoy/cell), decoys delivered via TP10-PNA conjugates (at most about 400,000 Myc decoy molecules/cell) are more accessible to the target Myc protein. Delivery of the DNA decoy was not affected by endosomal inhibitors and hence uptake seems to be independent of endosomal formation.

![Image of diagram](image)

*Figure 4.2: CPP mediated uptake of an E-Box containing dsDNA decoy targeting the TF Myc. A) The decoy is connected to the peptide through a CPP-PNA disulfide conjugate. B) The decoy is co-incubated together with a CPP prior to cell treatment*
4.3. Delivery efficacy and toxicity of CPPs are cargo dependent (Paper III)

Although development of efficient vectors for delivery across biological membranes is a very important aspect in modern molecular biology and pharmaceutical research, the toxicity displayed by these delivery vectors is just as significant. In paper III, three well characterized CPPs (Tat, Pen and TP10) were evaluated by means of their delivery efficiency and toxic side effects. The study was performed using three diverse cargos; fluorescein, dsDNA, and protein. By comparing both delivery yield, as well as acute and long-term toxicity displayed by the three peptides a comprehensive image of their intrinsic features as vectors was presented.

Cytotoxicity displayed by free peptides was assessed in HeLa and CHO cells. When analyzing membrane disturbance and mitochondrial activity, using an LDH leakage assay and the wst-1 assay, both Tat and Pen showed no toxicity up to 50 μM concentrations. Conversely, TP10 displayed long-term side effects in HeLa and CHO cells at 20 μM concentration. Further, TP10 induced LDH leakage (approximately 20 %) at 10 μM concentration. These data suggest that cationic peptides are less cytotoxic than amphiphatic peptides at higher concentrations, which is in line with other publications investigating the toxic profile of free peptides (Drin et al., 2001; Saar et al., 2005).

The most common way to determine delivery yield of a CPP is to measure translocation of fluorescein labeled peptides. On a routine basis, peptides have been labeled N-terminally, with the exception of transportan and its analog TP10, that have been labeled orthogonally on Lys^{13} or Lys^{7}, respectively (Pooga et al., 1998a). By measuring uptake of fluorescein-labeled CPPs, TP10 was determined to be the most efficient CPP and the uptake of Tat was nearly undetectable.

As previously mentioned, uptake studies does not give an accurate picture unless it is combined with toxicity studies. Pen shows no signs of toxicity when fluoresceinylated in any of the two assays used. Tat, in contrast, displays slightly higher membrane leakage as free peptide but, interestingly, proliferation is reduced significantly already at 20 μM concentration as labeled peptide. The discrepancy highlights the importance of using both membrane leakage and long-term toxicity assays, as they do not necessarily correlate. TP10 behaves very differently depending on coupling position of the fluoresceinyl moiety. When attached orthogonally, delivery yield is increased and toxic side effects are decreased. When coupled N-terminally translocation is dramatically reduced and both acute and long term toxicity is increased. This might be explained by increased amphiphatic properties. When coupled N-terminally, the already hydrophobic nature of the peptide
tail is increased, resulting in membrane destabilization and elevated cytotoxicity. When coupled orthogonally, fluorescein is attached to the cationic part of the peptide, possibly reducing the amphipathic and toxic nature of the peptide. It is important to consider that since the fluorescein moiety apparently influence the cell viability, it is possible that it also alters the uptake levels of peptides. To use radioactive labeled CPPs could give a more accurate depiction of correlation between uptake yields and toxicity.

Using carboxyfluorescein as a cargo molecule provides a valuable tool as means of evaluating cellular peptide uptake, however, it is not a biologically relevant cargo. Therefore, to further investigate the potential of using CPPs to deliver biologically relevant molecules, dsDNA similar to that used in paper II was utilized as cargo. In agreement with uptake observed for fluorescein labeled peptides, TP10 was the most potent vector. Tat displayed somewhat higher translocating activity compared to fluorescein labeled peptide. When evaluating toxicity rendered from dsDNA delivery, both penetratin and Tat are virtually nontoxic up to 50 μM concentrations. Intriguingly, it appears that the DNA cargo decreases the toxic side effects of TP10. The observed decreased toxicity could be explained by electrostatic and hydrophobic interactions between peptide and DNA, making the CPP less exposed to cellular membranes.

Finally, the three CPPs were assessed for their ability to deliver proteins. To get a comprehensive study of protein translocation, two diverse methods to improve protein uptake were applied. Either, the CPPs were co-incubated with proteins, or biotinylated CPPs were premixed with proteins to form stable CPP-protein complexes. Two different proteins, streptavidin and avidin, were included in the study. The two proteins used exhibit similar and dissimilar properties. Most obvious is their ability to form tight interactions with biotin. Less apparent but not less important in this study is their difference in pI value; streptavidin 5.5 and avidin 10.5.

When comparing data from co-incubation studies on streptavidin, Pen is as effective as TP10 to promote cellular uptake. No significant difference in uptake was observed for avidin with or without peptide co-incubation, most probably because avidin has a cationic nature that prohibits electrostatic interactions with positively charged CPPs. Again, Pen and Tat show no toxic side effects. Also the toxic side effects of TP10 seem to decrease together with protein compared to free peptide, suggesting that peptide-protein interactions, as in the case of DNA, somewhat shield the cellular membrane from perturbation. However, a strong reduction in cell viability was observed 24 h post streptavidin treatment, concluding that streptavidin is toxic if applied for longer periods of time. Interestingly, when co-incubating streptavidin with increasing concentrations of TP10, the long-term toxicity clearly decreases, suggesting that the peptide forms stable complexes with protein that remain unaltered inside cells and thereby shield the cytotoxic epitopes of the
protein. This phenomenon was not observed for Tat or Pen, indicating that these peptides might form less stable complexes with streptavidin.

When comparing uptake of stable CPP-protein complexes (biotinylated CPPs pre-incubated with streptavidin or avidin) we observed a surprising result. TP10 and Tat are then both efficient vectors. Strikingly, Tat efficiently internalizes to cells when conjugated via biotin to proteins but is poorly internalized as fluorescein coupled peptide. One plausible explanation for this behavior could be that Tat utilizes different internalization routes depending on its cargo properties. Fluoresceinylated Tat may have the ability to pass directly through the plasma membrane as suggested in (Tunnemann et al., 2006), but the uptake is not high enough to be visualized. In contrast, when attaching a large cargo to the peptide the uptake mechanism is changed to endocytosis, as suggested in (El-Andaloussi et al., 2006a; Richard et al., 2003a; Wadia et al., 2004).

Results from paper III strongly show that different peptides are suitable for different cargos and that the uptake yield of one cargo does not assure that the same CPP is effective in delivery for all bio-active molecules. In addition, results show that the cytotoxicity of peptides is highly dependent on cargo used and cargo coupling position within the peptide.

4.4. The CPP M918 can simultaneously transduce Sleeping Beauty transposase and a donor plasmid in vitro (Paper IV)

In paper IV, the aim was to develop a peptide based delivery method to translocate the SB transposase (SB-tpase) as a protein together with a donor plasmid into cells in vitro. The method is attractive since it avoids the use of DNA as a source for SB in transposon mediated gene therapy. By producing the transposase in a bacterial strain and later apply it as a protein circumvents the possible risk of spontaneous integration of the transposase gene in the treated cell.

Two different CPPs, Tat and M918, (for sequences see Table 1.1) were evaluated for their ability to deliver the SB-tpase together with a transposon donor plasmid carrying a Neomycin resistant gene into cells in vitro (Fig 4.3). Further, since Tat is derived from a shuttling protein (and therefore is a so called peptide transduction domain, PTD) and Tat fusion proteins have frequently been utilized to facilitate cellular uptake of proteins both in vivo and in vitro (Nagahara et al., 1998; Wadia et al., 2004), the Tat amino acid
sequence was introduced N-terminally to SB-tpase with the purpose to generate a transposase with cell-penetrating properties, Tat-SB-tpase (Fig 1.1).

Surprisingly, the Tat-SB-tpase fusion protein showed no signs of uptake in CHO cells. This was rather unexpected, since Tat fusion proteins, as previously mentioned, have been reported to readily translocate into cells. Neither did Tat promote any uptake of SB-tpase when the peptide and the protein were co-incubated prior to cell treatment. The low protein uptake by Tat through complex formation is plausibly a result of electrostatic repulsion between the highly cationic peptide (for sequence, see Table 1.1) and the SB-tpase (theoretical pI 10.6). However, cellular uptake was monitored by western blot and low yields of the transposase might have been able to cross the membrane, but not sufficient to be detected. Due to this result, it was not unexpected that when co-incubation of Tat and SB-tpase together with the transposon donor plasmid was preformed, no uptake of SB-tpase or the plasmid was detected.

![Diagram showing uptake of SB-tpase and donor plasmid by CPPs](image)

**Fig 4.3:** Uptake of SB-tpase and donor plasmid by CPPs. Tat does not facilitate SB-tpase uptake neither as a fusion protein nor when co-incubated with the transposase. M918 conversely display efficient cellular uptake of the transposase and simultaneous uptake of an antibiotic resistance carrying plasmid and the SB-tpase.

M918 conversely displayed efficient uptake of SB-tpase in both CHO and HeLa cells. Although the peptide is cationic like Tat, M918 has more hydrophobic amino acid residues compared to Tat (for sequence, see Table 1.1). These hydrophobic residues can probably facilitate complex formation between the peptide and the transposase and is therefore a more efficient protein transport vector when using co-incubation strategy. This can be com-
pared with previously discussed Pep-1, which has been designed with the purpose to complex bind proteins by hydrophobic interactions (Morris et al., 2001) (for sequence see .J 1).

Protein uptake by M918 seems to be more efficient at lower CPP concentrations. SB-tpase uptake is increased about 4.5-fold at 1:125-250 ratios (10-20 μM peptide concentration) compared to protein alone, and decreases with higher CPP concentrations. Further, higher CPP concentrations cause slight membrane perturbation and this side effect might influence the detected uptakes.

The important use of trypsin to degrade extra cellular proteins and peptides becomes very evident in this study. Without this step, the detected protein levels are several folds higher than the actual protein uptake.

The SB-tpase seems to alone be taken up by the cells. This is probably an effect derived from the interactions between the negatively charged plasma membrane and the positively charged transposase, and does not necessarily indicate membrane translocation. If so, this might show that even though a trypsination step is included in the uptake study, all the extra cellular proteins are not fully degraded.

When performing co-translocation with both SB-tpase and donor plasmid, similar concentrations and ratios as in protein transduction seem to be most efficient for the SB-tpase delivery. Again, lower concentrations appear to translocate more protein than higher concentrations. Although uptake of the transposase is most efficient at 1:125 ratio (protein:CPP), colony formation 14 days post transfection is most apparent at 1:500 ratio (40 μM peptide concentration).

The two entities being transported have very different properties. The transposase is cationic and has a molecular mass in the kDa range. The plasmid on the other hand is anionic and its molecular mass lies in the MDa range. That the two should display identical uptake patterns is highly unlikely. However, since M918 is insufficient to deliver the plasmid without the presence of the transposase, it is likely that they are both taken up by the same pathway. It is apparent that M918 together with SB-tpase and pT/Neo causes slight membrane perturbation (about 20 % deoxyglucose leakage) after 60 min at 40 μM, which possibly contributes to efficient plasmid delivery and, consequently Neomycin (G418) resistance, 14 days post transfection. This is the first study where two so vastly divergent molecules as a cationic protein and a plasmid, are concurrently translocated into cells via a peptide vector. As discussed previously, to deliver SB-tpase protein and donor plasmid simultaneously via CPP mediated delivery have several advantages over other methods such as viral delivery. The most obvious being the use of protein as a source for the transposase instead of DNA. This modification eliminates the risk of spontaneous integration of the transposase gene into the affected cells genome, which could have devastating effects in vivo. The same objective has been achieved by using mRNA as the SB-tpase
source (Wilber et al., 2006), but in this study we show that M918 is a potent vector for simultaneous uptake of both SB-tpase and transposase donor plasmid in vitro.
5. Conclusions

The four papers that constitute the framework of this thesis all consider different aspects of oligonucleotide and protein delivery. Here, the main findings in each paper are here accentuated and described.

- **Paper I:** By combining CPP technology with commonly used Polyethylenimine (PEI) transfection, increased transfection efficacy was achieved. Improvement was especially evident at lower PEI concentrations, consequently decreasing toxic side effects cause by the treatment.

- **Paper II:** Decoy strategies aim to bind transcriptional factors (TFs) in the cytoplasm, disabling them from entering the nucleus and thus hinder transcription initiation. By introducing dsDNA coding for the binding site of the oncogenic TF c-Myc to the cytoplasm via CPP transduction, cell proliferation was significantly decreased.

- **Paper III:** Different CPPs have distinctively different properties by means of length, charge and hydrophobic moment. We studied three of the major CPPs and their ability to transport oligonucleotides and proteins into different cell lines. The results show that different peptides are suitable for different cargos and that the uptake yield of one cargo does not assure that the same CPP is effective in delivery for all bio-active molecules. Further, we show that the cytotoxicity of CPPs is highly dependent on cargo used and cargo coupling position within the peptide. These data could serve as guidelines to develop CPPs for future pharmaceutical research.

- **Paper IV:** Transposable elements have emerged as a promising candidate for human non-viral gene-therapy. Sleeping Beauty is to date one of the most efficient transposases in mammals. Sleeping Beauty transposase has so far been mostly been delivered to cells via a DNA source. This might cause spontaneous integration of the transposase gene and cause fatal damage to the affected cell. Hence, it would be advantageous to employ a non genetic source for the transposase. We developed a CPP based method where the transposase and a donor plasmid are delivered simultaneously and thereby eliminating the risk of spontaneous integration of the transposase gene, making it a safe and non immunogenic alternative to viral gene-therapy.
5.1. Populärvetenskaplig sammanfattning på svenska


I publikation I, används CPPn TP10 till att förbättra en väl utvecklad metod för transport av en främmande gen in i cellkärnan. Samma CPP används i publikation II till att transportera ett så kallat decoy DNA in i cellen. Decoy DNA har en förmåga att binda till, och därmed inaktivera, ett specifikt protein som är ansvarigt för uttrycket av en genfamilj. Publikation III är en mer generell översikt över hur effektiva vissa CPPer är för transport av olika substanser och hur de negativa bieffekterna är beroende av vilken CPP som används. I publikation IV används en nyutvecklad CPP, M918, till att samtidigt transportera en främmande gen och ett protein in i cellen. Proteinen i fråga är ett så kallat transposas, som har förmågan att klippa ut en gen från ett ställe och sedan klistra in den igen i på ett annat ställe i en kromosom. Tekniken skulle kunna användas som ett alternativ i icke-viral genterapi och därmed minska riskerna med att använda virus som transportörer för terapeutiska gener.

Sammanfattningvis syftar denna avhandling till att förbättra tidigare välkända molekylärbiologiska metoder, ge en bättre förståelse över hur CPPer beter sig samt ligga till grund för utvecklandet av nya CPP baserade tekniker.
6. Acknowledgments


Först och främst vill jag så klart tacka min handledare, Úlo. Med idel positivitet och uppmuntrande ord som ”Dåligt, som vanligt” och ”Peter, måste jag döda dig?” har han guidat mig igenom den här tiden och för det är jag honom evigt tacksam. Minst lika viktiga har mina medförfattare varit, främst Samir och Sandra. Utan er assistans, hjälp och idéer hade det inte blivit någon avhandling för min del. Tack!


Ett gemensamt tack till Úlos grupp bestående av; Helena, Ulla, Emelia, Maria, Maarja, Karti, Marie-Louise (tack för att du läste igenom avhandlingen), Pernilla, Meeri, Tina, Yang, Küllikki, Mats (you’re truly incredible with machines) Kalle och Ursel (a special thanks to you two, for introducing me to the wonderful world of SPPS) Margus och Matjaž (thank you for discussions and the inspiration), Elo och Kent i Tartu. Tack för all hjälp på och utanför labbet, alla fester och all Champange (Tack igen, Úlo)

Alla andra doktorander som jag träffat under min tid på Neurokemi: Linda, Linda, Victoria, Veronica, Malin, Johanna, Caroline (du är begåvad, kom ihåg det), Sofia, Katarina, Ove, Micke, Helena, och Karolina (tack för alla kramar och allt kul). Tack för att det varit så
självklart att kunna be vem som helst om hjälp, med vad som hels, när som helst och hur som helst. Ni har alla bidragit till att min tid som doktorand är en tid att minnas.

Mina vänner från studietiden på Södertörn. Främst Ulrika, Johan, Lasse, Ivo, Jonas och Ina, samt mina handledare på Södertörn; Lotta och Marco.

Alla andra vänner, alla de som hjälpt mig utanför studier och Neurokemins väggar. Ni är många och minst lika viktiga. Många dyker nog upp i flera sammanhang, men det är svårt att hålla isär er. Vårt fotbollslag, Tjetja. För alla måndagar i Kristineberg: Il capitano Mätz, Sanna o Poppe, Jenny (åh, Jenny)... ni är för många och jag kan inte rabbla upp alla, men vi har haft kul där på grusplanen och i bastun efteråt. Mer fotboll... Mitt kära BK Borsten, för alla söndagar med folköl och oliver på Zikken. "Vi e blåa som blåbär, och gula som gulbär..."

De Bittra Pojkarna; Petter, Henrik, Olle och Jacke. För all musik, all folköl, all starköl och alla kvällar i Stadshagen.

My newfound friends in Estonia; Christoph, Roman, Liina, Külliik ja Marten, and Wonderful Piret

Allt och alla på och runtomkring Nada, mitt andra vardagsrum. Tack för att jag har fått dansa och husera fritt bakom skivspelarna på lördagar. Andreas, Sandra, Joel, Lillis, Simon, Åke... Igen, det blir det för många. Ett speciellt tack till Olle för att du fanns där när det behövdes, utan att veta om det.


Andra vänner; Anders- den bästa vän man kan få, Wiborg- för alla julafinar med Papa D och all paintball, Saga- mitt egna spektakel, Monica- min själfrände, Dansk-Henrik- tycker du ska komma hem snart, jag saknar dig, Julia- Peter and Juul 4ever, Boel- den vackraste av vänner, Kalle o Pontus och alla vänner som har kommit genom er, Emma- för vår tid tillsammans och för att du gjorde mig till en bättre människa.

Min familj; Inger och Anders- ni finns alltid där och det vet jag. Mormor- för att du är finast i hela världen, Johan- den bästa bror man kan ha. Att ha er bakom mig är allt man kan önska sig. Allt annat blir sekundärt i jämförelse. Tack
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Paper I
Evaluation of transportan 10 in PEI mediated plasmid delivery assay

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Received 18 August 2004; accepted 9 December 2004
Available online 26 January 2005

Abstract

Cell-penetrating peptides (CPPs) are novel high-capacity delivery vectors for different bioactive cargoes. We have evaluated the CPP transportan 10 (TP10) as a delivery vector in different in vitro plasmid delivery assays. Tested methods include: TP10 crosslinked to a plasmid via a peptide nucleic acid (PNA) oligomer, TP10 conjugation with polyethyleneimine (PEI), and addition of unconjugated TP10 to standard PEI transfection assay. We found that without additional DNA condensing agents, TP10 has poor transfection abilities. However, the presence of TP10 increases the transfection efficiency several folds compared to PEI alone. At low concentrations as 0.6 nM, TP10–PNA constructs were found to enhance plasmid delivery up to 3.7-fold in Neuro-2a cells. Interestingly, the transfection efficiency was most significant at low PEI concentrations, allowing reduced PEI concentration without loss of gene delivery. No increase in cytotoxicity due to TP10 was observed and the uptake mechanism was determined to be endocytosis, as previously reported for PEI mediated transfection. In conclusion, TP10 can enhance PEI mediated transfection at relatively low concentrations and may help to develop future gene delivery systems with reduced toxicity.

Keywords: Transportan 10; Cell-penetrating peptides; Polyethyleneimine; Transfection

1. Introduction

Developing strategies for effective gene delivery is a field of high priority in biomedicine. Still, after many years of research, gene therapy applications in humans have not managed to proceed into common
use. The main delivery problem is the lack of safe transfection of recombinant DNA into desired target tissues/cells. All known gene delivery vectors in use today fail to meet the requirements for an ideal system [1]. Viral vectors are primarily limited by host immunoresponse [2] and non-viral systems are limited by either low transfection efficiency or high cytotoxicity. In general, the more efficient the delivery systems are, the higher the toxic side effects become [3].

PEI has successfully been used in gene delivery without any additional components [4-6]. However, accumulation of PEI and its fragments causes damage to cell metabolism. At higher concentrations, it may also mediate disruption of intact cell membranes. Therefore, improvements that allow reduced PEI concentration are necessary before applying the technique in mainstream gene therapy.

Short, synthetic peptide-based vectors are suggested to be the most biocompatible and economical candidates for future gene delivery systems [3,7,8], but, so far, they show lack of delivery efficiency and tissue targeting. Different approaches can be used to improve peptide vectors and we here show three possible methods of enhanced peptide mediated transfection.

Highly positively charged peptides may electrostatically interact with plasmids and thereby reduce the negative net charge of the DNA [9-11]. The DNA/peptide complex becomes neutral or even positively charged, which enhances interactions with negatively charged cell membranes. Additionally, the positive charges of peptides condense native supercoiled plasmid DNA into smaller, more compact particles [10]. Taken together, these features may facilitate peptide mediated gene delivery. Phage libraries allow high throughput screening for DNA interacting peptides [12,13] making it easy to establish appropriate peptide sequences.

Another approach applicable for peptide mediated gene delivery is to link them to a polycation. Ogris et al. have crosslinked the antimicrobial peptide melittin to the polycation PEI [14]. While PEI functions as a DNA neutralizing and condensing agent, melittin lyses endosomes and thereby increases the probability for DNA delivery into the nucleus. In order to achieve cell specific gene delivery, extracellular receptor ligands can be employed to direct the DNA-peptide complex to the desired tissue or cell type (reviewed in Ref. [15]).

A third approach is to crosslink peptides to DNA via sequence specific hybridisation. The so-called bioplex technology uses a chimera or conjugate of desired peptide and peptide nucleic acid (PNA) oligomer [16-18]. The PNA oligomer is able to replace one DNA strand in double-stranded helices [19,20] and thereby link peptide and DNA together. This technique efficiently links the peptide to the desired positions in the DNA and the complex structure is well characterized. The SV40 nuclear localization signal (NLS) sequence was the first peptide demonstrated to be effective when using the bioplex strategy [21].

A family of peptides capable of trafficking across plasma membranes is cell-penetrating peptides. Cell-penetrating peptides are 7-30 amino acid long peptides that are able to translocate various macromolecules across the plasma membrane [22,23]. Transportan, for example, has been demonstrated to deliver streptavidin-colloidal gold conjugates into the cell, which corresponds to delivery of a globular protein with the approximate molecular weight of 1 MDa [24]. The ability of transportan to translocate large hydrophilic molecules through hydrophobic biological membranes makes it attractive as a gene delivery vector. However, transportan exerts intracellular unwanted side effects like GTPase activity [25]. Therefore, recently designed analogues of transportan (including TP10) that have less side effects and still are efficient vectors, are more interesting than transportan as potential vehicles for gene delivery [26].

In the field of gene delivery, CPPs have been demonstrated to deliver plasmids [11,27], improve viral transfection [28], and they have also been shown to increase liposome uptake [29]. In these studies the HIV-1 transcription factor Tat[47-57], Drosophila antennapedia homeodomain fragment[43-58] (penetratin) or NLS based peptides were used. Penetratin and Tat peptides have high positive net charge and interact electrostatically with DNA and cell membranes. TP10 has a lower net charge and different kinetics in cargo delivery compared to these peptides [30]. Since TP10 shows different properties compared to mentioned peptides we aimed to study whether TP10 display similar
features or not when employed as a vector for gene delivery.

Here we evaluated the possible use of TP10, TP10-PEI, and TP10-PNA conjugates in the means of delivering EGFP or luciferase encoding plasmids into three different cell lines. It was evaluated whether TP10 functions as a plasmid delivery vector in the absence of the DNA condensing agent PEI and how it alters the efficiency of existing PEI transfection protocols.

2. Experimental procedures

2.1. Synthesis

TP10 and PNA oligomers were synthesised on Applied Biosystems stepwise synthesiser model 431A or 433A, respectively, t-Boc chemistry and MBHA resin were used in both cases. Synthesis products were purified by reverse phase HPLC C_18 column and theoretical molar weights correlated well to MALDI-TOF mass spectra. The PNA sequence Cys-Gly-Gly-AGGATCTAGGTGAA-Lys-amide (Fig. 1A) was complementary to the 3924-3947 region in the pUC bacterial replication origin of pEGFP-N1 plasmid and is shown previously to invade double stranded DNA [19].

Conjugation of TP10 and PNA via a disulphide bridge was carried out as described elsewhere [31]. Briefly, 3-nitro-2-pyridinesulphonylated TP10 and PNA with a free thiol were stirred overnight in DMSO/dimethylformamide/acetic acid buffer pH 5.5 in a 2/2/1 mixture, and the products were separated in a C_18 reverse-phase HPLC column. Conjugate was identified by absorbance spectra and MALDI-TOF analysis.

2.2. TP10-PEI conjugation

A cysteine was coupled to the Lys\(^7\) side chain of TP10. 8 nmol PEI (MW: 60 kDa, Sigma-Aldrich) in 155 µl 10 mM phosphate buffer pH 6.8 was treated with a bifunctional crosslinker succinimidyl trans-4-(maleimidylmethyl)cyclohexane-1-carboxylate (SMCC) in dimethylsulfoxide for 1 h. SMCC was added in 0, 5, 10, 25, 50, or 100-fold molar excess. Unreacted SMCC was removed by Sephadex G25 Superfine 2.0 ml column. 1.5 ml fractions were retracted further with 100-fold excess of cysteinyalted

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![Diagram](image)

**Fig. 1.** (A) Structure of TP10-PNA conjugate. Amino acids in TP10 sequence are given in single letter code. The cysteine for disulphide bridge is coupled to the side chain of Lys in TP10. (B) Structure of plasmid and TP10-PNA complex. (C) TP10-PEI conjugate. TP10 is given in single letter code, Cys on Lys\(^7\) and SMCC structures are drawn out.
TP10. After 16 h incubation the fractions were dialysed over a 10,000 Da molar weight cut-off filter (SpectraPor, US) overnight. The protocol is a modification from Ref. [32].

The molar extinction coefficient of TP10 was measured from a set of standard solutions and was found to equal EC_{280}=9490 M^{-1}·cm^{-1}. PEI absorbance at this wavelength was defined as the blank value in the standard curve preparation and in a sample analysis. This value was used to estimate the success of TP10 PEI crosslinking reaction 5, 10, 25, 50, and 100-fold molar excess of SMCC over PEI resulted in 1.5, 5, 7.5, 13, and 24 TP10 molecules per PEI, respectively.

2.3. Hybridisation

TP10–PNA was hybridised to pEGFP-N1 (Clontech) or pGL3 Promotor Vector (Promega) plasmid according to the following protocol: 10 µl of TP10–PNA (15–160 nM) or double distilled water for controls and the plasmid (50 µg/ml) were mixed in double distilled water, heated to 90°C, and allowed to cool down to 37°C within 1.5 h. Zhang et al. have previously demonstrated that this particular PNA sequence successfully hybridises to plasmids at 37°C in 1 h [19]. A temperature gradient was used to increase the accessibility of the target site.

2.4. Transfection

10 µl of PEI or TP10–PEI 0.01–0.15 µg/µl and 20 µl plasmid or TP10–PNA treated plasmid (25 µg/ml) were mixed in double distilled water. Prior to applying the cells, the mixture was incubated 15 min at room temperature. Mouse neuroblastoma cells Neuro-2a were grown in minimum essential medium (Eagle) with 2 mM l-glutamine and Earle’s BSS adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum (FBS). HeLa cells and Murine fibroblast C3H 10T1/2 cells were grown in Dulbecco’s Modified Eagle’s Media (DME) with glutamax supplemented with 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Prior experiment the media was replace (1 ml per well in a 24 well plate) and cells were incubated with complexes for 3 h. Thereafter, the transfection reagent was removed and media was changed. All experiments were carried out in full growth media.

2.5. Microscopy

The level of GFP expression was investigated under Leica DM IRE2 fluorescence microscope and photographed with Leica DC 350F camera. The photos were taken on live, non-fixed cells.

2.6. Quantitative assays

Cells transfected with firefly luciferase (pGL3 Promotor Vector, Promega) encoding plasmid were lysed using Passive Lysis Buffer (Promega). Samples were freeze-thawed once and luciferase activity was measured using Dual-Luciferase Reporter Assay System according to manufacturer’s protocol (Promega) on a Victor (Wallac, Finland) instrument. EGFP was quantified on SpectraMax Gemini XS (Molecular Devices, USA).

2.7. Flow cytometry

Flow cytometry analysis was carried out on FACSCalibur equipment and CellGate software. Cells in 24 well plates were trypsinated for 5 min at room temperature and washed 3 times with ice-cold PBS prior analysis. Dead cells and debris were gated out in forward and side scatter spectras. All experiments were carried out at least twice and each time in duplicates. Numerical values given are the mean values from all experiments.

2.8. 2-[^3]H]-Deoxyglucose-6-phosphate leakage

The original protocol [33] was modified to suit 24 well plates. 1 h prior addition of transfection reagents, cells were incubated with 0.25 µCi 2-[^3]H]-Deoxyglucose in 500 µl serum free media. Cells were washed 3 times with PBS to remove extracellular radioactivity and 1 ml fresh serum free media was added. 150 µl aliquots were taken 0.5, 1, and 3 h after treatment with 50 µl transfection agent in PBS upon the cells. After 3 h the cells were treated with 1% TritonX-100 for 5 min and total radioactivity was counted. All time points were correlated to the total radioactivity in cells.
3. Results

3.1. TP10 in PEI mediated gene delivery

Initially, we tested whether TP10 alone is sufficient to interact with pEGFP-N1 plasmid and trigger cellular uptake. No remarkable EGFP expression was observed 24 or 72 h post transfection with pEGFP-N1 pre-incubated with 0.3 nM–10 μM TP10. Since it was obvious that TP10 was on its own and unable to promote uptake of native plasmids, we continued to study pre-condensed plasmids. TP10 was added to PEI condensed plasmid either as free peptide or crosslinked to PEI. To obtain TP10–PEI constructs, a bifunctional crosslinker SMCC first reacted with the amino groups of PEI and then with thiol groups of cysteinylation TP10 (see Experimental procedures; Fig. 1C). 1.5, 5, 7.5, 13, and 24-fold molar excess of TP10 over PEI were tested at N/P (PEI nitrogens per DNA phosphates)=8.

FACS analysis (Fig. 2A,B) and microscopic studies (not shown) demonstrated that addition of TP10 increases the expression of the reporter gene. Surprisingly, the results of free and PEI crosslinked TP10 did not differ at higher TP10 concentrations, 7.5, 13, or 24 TP10 per PEI and yielded in average 8.6±1% of total cell population being transfected (Fig. 2A,B), while standard protocol transfected only 4.8±0.4% of cells. Moreover, the highest tested TP10 concentration had lower effect than moderate concentrations, transfecting only 6.4±1.3% of cells (Fig. 2B, lines 1 and 2). At lower concentrations (25 nM, N/P=8) on the other hand, free TP10 showed no improvement (Fig. 2B, line 3) while TP10–PEI conjugate maintained its activity (not shown).

TP10 mediated improvement was quantified in different cell lines. At N/P=8 and 5 moles of TP10 per PEI the enhancement was 1.9-fold in Neuro-2A cells, 2.7-fold in murine fibroblasts C3H/10T1/2, and 1.5-fold in HeLa cells, indicating that the effects are not cell-line specific.

3.2. TP10–PNA in PEI mediated gene delivery

A single TP10 molecule was anchored to the plasmid according to the abovementioned bioplex technology (Fig. 1A,B). After TP10–PNA hybridisation, the plasmid was condensed and neutralised by PEI. In comparison to PEI standard transfection protocol, TP10–PNA pre-treated plasmid showed a different reporter gene expression pattern. 24 h post application of TP10–PNA treated plasmid, less cells (population 1.9±0.3%) expressed EGFP than the standard protocol (population 3.0±0.7%) (Fig. 3A,B). However, the expression level per cell was
significantly higher (1.9±0.8-fold, p<0.01). 72 h post transfection, the number of transfected cells favoured the modified protocol (transfected populations 4.8±0.4% for standard and 8.6±1% for the TP10–PNA protocol), and the EGFP intensity was still higher in most cells (Fig. 3C,D). This phenomenon was observed in all tested cell lines.

Quantitative luciferase measurements showed that 2 to 5-fold excess of TP10–PNA over plasmid is the most optimal ratio for efficient transfections. Total luciferase expression was up to 3.7 times higher compared to standard protocol at N/P=4 (Fig. 4). Lower PEI concentration favoured lower excess of TP10–PNA. TP10–PNA/plasmid ratios 1, 7, or 10 were not as effective as ratios 2 or 5, but respective transfection efficiencies were still higher compared to the unmodified protocol. 2–5-fold peptide-PNA excess over plasmid equals to 0.3–0.8 nM concentrations in the transfection assay.

It was further analysed whether the observed effect requires TP10–PNA conjugate, or is one of the components or the components in an unconjugated mixture enough to increase cellular uptake. Fig. 5 shows FACS analysis of EGFP expressing cells 72 h after transfection at N/P=8. Neuro-2a cells treated with TP10–PNA conjugates in combination with PEI (line 3) have 8.0±1.2% GFP positive cells compared to PEI treated cells having 4.8±0.6% transfected cells. Unconjugated mixture at 0.3 nM concentration of both components was slightly better (transfected cell population 5.9±1.2%) than standard PEI mediated transfections, and neither of the components alone had any effect on transfection efficiency. Moreover, TP10–PNA conjugate was the only method that gave EGFP expression difference after 24 h. Fig. 6 shows the luciferase levels 72 h post transfection for a set of TP10–PNA and free TP10 concentrations in the presence of PEI. Apparently, free TP10 requires...
about a 100-fold higher concentration than TP10-PNA conjugate to reach the same efficiency.

3.3. Effect of PEI concentration

EGFP and luciferase transfections were performed at various PEI concentrations ranging from 0.25 to 1.0 µg PEI per ml media and 0.5 µg plasmid. This equals N/P ratios from 4 to 16 and theoretical charge ratios (positive/negative) from 1 to 4. At higher N/P ratios reporter gene expression levels were better for all protocols than at lower N/P ratios. Intriguingly, the effects of TP10 and TP10-PNA treatment were more drastic at low PEI concentrations. At N/P=16 the presence of TP10-PNA did not result in a remarkable transfection enhancement compared to N/P=8 or 4 (Figs. 6 and 7). Pictures D, E, and F in Fig. 7 demonstrate the effect of standard PEI protocol at concentrations N/P=4, N/P=8, and N/P=16, respectively. Pictures A, B, and C in the same figure correspond to TP10-PNA-PEI protocol at the same concentrations.

![Graph of RLU/mg of protein vs DNA: TP10-PNA ratio](image)

Fig. 4. Luciferase expression levels at several DNA: TP10-PNA ratios. Ratio 1:0 indicates the unmodified protocol with no TP10-PNA conjugate added. Black bars have PEI concentration N/P=8, striped bars N/P=16. Results are an average of 4 independent experiments. The number above each column indicates relative change compared to control (i.e. unmodified protocol). Stars indicate statistical significance (*p<0.05, **p<0.01, and ***p<0.001, n=3, independent from experiments presented on Fig. 6).

![Histogram of EGFP expression](image)

Fig. 5. EGFP expressing cells analysed by flow cytometry. PEI concentration everywhere 1 µg/1 µg plasmid (N/P=8). (1) Untreated cells, (2) PEI, (3) TP10-PNA conjugate (0.6 nM), (4) TP10 and PNA unconjugated mixture (both 0.6 nM), (5) PNA (0.6 nM), and (6) TP10 (0.6 nM). Untransfected population of lines 2-6 is removed to enhance clarity.
Fig. 6. Luciferase expression levels 72 h after transfection in Neuro-2a cells. Black bars stand for PEI concentration N/P=8 and striped bars N/P=16. In left, the DNA has been pre-incubated with TP10–PNA in ratios 1:1 and 1:5 and in right, DNA has been pre-incubated with TP10 ratios 1:10 and 1:100. Control stands for standard transfection protocol with not TP10 or TP10–PNA present. Stars indicate statistically significant difference from the control (*p<0.05, **p<0.01, and ***p<0.001, n=3).

Similar tendency was observed for TP10–PEI conjugate and free TP10 (not shown).

3.4. Role of endocytosis in delivery

Originally, CPPs were suggested to enter cells and deliver cargoes in an endocytosis independent manner (see review Ref. [22]). PEI mediated DNA uptake on the other hand, is mainly fluid phase endocytosis and can therefore be regulated by various drugs [34,35]. We evaluated the effects on our modified transfection protocol after following cellular treatments: ATP depletion by 2-deoxy-d-glucose (10 mM) and sodium azide (10 mM), inhibition of acidification of endo-

Fig. 7. EGFP expression in Neuro-2a cells 72 h after transfection. (A–C) With DNA: TP10–PNA ratio 1:5; (D–F) Controls with no TP10–PNA. (A) and (D) N/P=4; (B) and (E) N/P=8; (C) and (F) N/P=16.
cytotic vesicles by chloroquine (50 μM), and microtubule and filament disrupting agent nocodazole (2 μM).

Energy depletion or cytoskeleton disassembly reduced EGFP expression as shown on Fig. 8. No statistically significant differences between standard and modified protocols were seen. Chloroquine, which destabilises endosomes and enhances endosomal release, increases EGFP expression in all cases as expected. Again, presence or absence of TP10 or TP10-PNA did not change this tendency in transfection efficiency.

3.5. Cytotoxicity

2-[3H]Deoxyglucose is taken up by cells via glucose transporters and phosphorylated inside the cells by hexokinase [33]. The product, 2-[3H]Deoxyglucose-6-phosphate, cannot exit the cells as long as the cellular membrane remains intact. At high concentrations, PEI interacts with negative phospholipids of the outer cellular membrane. This interaction causes damage to the membrane and leads to cellular leakage. If 2-[3H]Deoxyglucose-6-phosphate can be detected outside the cell, the intact structure of the cell membrane has been disrupted by the treatment. The highest PEI concentration (N/P=16) was tested together with 5 or 24 TP10 per PEI for 2-[3H]Deoxyglucose-6-phosphate leakage. The peptide concentrations for unconjugated TP10 to PEI-mixture we used were: 190 nM (TP10/PEI=5) and 940 nM (TP10/PEI=24). TP10-PNA conjugate at 2 and 5-fold excess over plasmid was included as well as 2nM PNA and TP10 alone. Data points were gathered 0.5, 1, and 3 h post-treatment with PEI or TP10. Results shown in Fig. 9 demonstrate no statistically significant change in membrane leakage (Student’s t-test, in all cases p>0.05, n=4) and hence no increased toxicity.

No change in protein concentrations, measured in quantitative Bradford assay was observed 72 h post transfection (Figs. 5, 6, and 8). This further confirms

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Fig. 8. EGFP quantification 72 h after transfection with PEI (black), TP10-PEI (striped), TP10-PEI (empty), or TP10-PNA and PEI (gray). (A) N/P=8 and (B) N/P=16. Transfections with no endocytosis modifiers are defined as 100% in each case and the relative change due to chloroquine (50 μM), nocodazole (2 μM), or energy depletion (2-deoxy-d-glucose 10 mM and NaN₃ 10 mM) is presented. The cells were pre-incubated with the uptake modifiers for 30 min and the level was maintained throughout 3 h transfection period. Statistical difference from 100% is calculated using Student’s t-test. (∗p<0.05, ∗∗p<0.01, and ∗∗∗p<0.001, n=3).
low cytotoxicity due to transfection treatment (data not shown).

4. Discussion

A class of peptides identified about 10 years ago, called cell-penetrating peptides (CPPs), has the ability to deliver cargoes across the plasma membrane and into the cytoplasm or the nucleus. This process is attractive since the cargo size could exceed the size of the vector peptide by many times and the mechanism of internalisation was indicated to be energy and temperature independent [25,26]. CPPs still seem to have the ability to facilitate cellular uptake of macromolecules, however, the energy independent pathway by which the uptake is mediated has been questioned and has recently been suggested to mainly depend on endocytosis [37].

Transporters have been shown to deliver 150 kDa proteins [24], gold particles [24], and short oligonucleotides over the cell membrane [38]. Results of this current study, however, demonstrate inability of TP10 to carry 5MDa plasmids across the cell membrane. TP10 does not trigger cellular uptake of plasmids as suggested for other cell-penetrating peptides such as MPG [39] or Tat(47–57) [11]. Neither does a single peptide linked to the plasmid via a PNA oligomer have the ability to transfect cells. However, despite the inability to condense DNA by itself, TP10 enhances the uptake of PEI condensed plasmids. Coating PEI and thereby also the DNA/PEI complex surface with TP10, or presence of at least 10 nM of free TP10, results in increased levels of reporter gene expression. If TP10 is immobilised to the surface of DNA/PEI complexes the concentration can be magnitudes lower than for free peptide (Figs. 2B and 5). As seen in Fig. 2B, higher concentrations of TP10 have a lower transfection efficiency compared to moderate concentrations. Hence, the optimal transfection conditions still have to be evaluated. Although TP10 is not highly charged, the positive charges it contains, could still compete with PEI for DNA phosphates. At TP10/PEI ratio 24, almost every fourth positive charge in the assay comes from TP10. This could be enough to hinder proper formation of PEI/DNA complexes and cells do not as readily take up complexes with loosened structure as properly condensed DNA.

When EGFP expression in a standard PEI transfection protocol was compared to the transfection of TP10–PNA pre-treated plasmids, a highly unexpected difference was observed. 24 h after transfection, the TP10–PNA modified protocol gave a small population of cells expressing high level EGFP, while the unmodified protocol had more transfected cells showing lower EGFP expression per cell. After 2 days the modified protocol had surpassed the standard protocol in both characteristics. The reason for this could be a changed uptake mechanism or different kinetics of reporter gene expression.
Experiments performed with endocytosis modifiers suggest that the TP10/PEI mediated uptake mechanism remains unchanged compared to standard PEI transfection. Additionally, if TP10 would cause a different internalisation route than PEI, TP10–PNA and TP10–PEI conjugate should share the same transfection pattern. Considering that the uptake mechanisms are identical and the 72 h TP10–PNA protocol gives a greater population of EGFP expressing cells, the differences in EGFP expression after 24 h are difficult to explain. Higher EGFP expression levels should mean either higher copy number of plasmids or transcription enhancement by PNA. A lower number of EGFP expressing cells must be a result of either slower nuclear delivery or transcriptional inhibition by PNA. This cannot be true since PNA obviously cannot enhance and inhibit gene expression at the same time. Additionally, unconjugated TP10 and PNA mixture did not show the same profile, thus the effect must require the conjugate. If TP10–PNA complex induces plasmid clustering similarly to Pep-1 mediated aggregation of negatively charged lipid vesicles [40], the observed fact can be explained. This requires two linked interaction sites, therefore unconjugated TP10 and PNA are insufficient to induce aggregation of plasmids. Resulting clusters of two or more plasmids would, due to increased size, reach the nucleus slower but give a higher reporter gene expression.

We cannot exclude possible cell-cycle dependent events, but neither does linear PEI mediated transfection depend on cell cycle [41], nor do TP10 or PNA have any known unspecific effects on cell cycle progression. No unspecific targets were found for the PNA sequence by Blast (http://www.ncbi.nlm.nih.gov/BLAST/) nucleotide search within the mouse genome. By quantification of luciferase expression levels, TP10–PNA and PEI concentrations were optimised while keeping the DNA concentration constant. Not surprisingly, a 2 to 5-fold excess gives better yields than equimolar ratio of TP10–PNA and plasmid. The equilibrium is strongly shifted towards PNA/DNA complex at used concentrations, but kinetics of PNA strand invasion is slow [42]. Higher excess of one component increases the rate and fraction of complex formed at any particular time point. However, ratios 1:7 and 1:10 tended to lose some transfection activity compared to lower ratios. This may be due to PNA self-aggregation.

The pronounced improvements of EGFP expression at lower PEI concentration could be explained by overlapping functions of TP10 and PEI. If TP10 has endosomal proton “sponge” capacity [43,44] at low PEI concentrations the TP10 amino groups could interfere and improve the observed transfection yield. At high concentrations, PEI mediated transfection contributes to the transfection pathway to a higher degree and the peptide mediated route plays a minor role. In fact, buffering endosomal pH could be the major contributor to the observed TP10 effect. At sub-micromolar concentrations, free extracellular peptide has a higher probability to inadvertently be taken up via endosomes. In TP10–PNA or TP10–PEI conjugate form the peptide is most likely internalized through PEI induced endosomes. Therefore, both conjugates result in similar endosomal uptake as a 100-fold higher free extracellular TP10.

In summary, coupling TP10 to PEI or DNA via an appropriate linker or adding free TP10 to PEI mediates an increased population of transfected cells. It is not because of a new internalisation mechanism as previously supposed, but rather by improved endosomal release. This information is valuable for understanding cell-penetrating peptides and their mechanisms of internalisation. The obtained results are of great interest for two reasons. First, the improvement is most significant at low PEI concentrations. Second, TP10–PNA is required at sub-nanomolar concentrations. With no increase in toxicity, such delivery system could be useful in systematic in vivo delivery.

Acknowledgements

This work was supported by grants from European Community (QLK3-CT-2002-01989), Swedish Research Councils (VR-NT and VR-Med), and Estonian Science Foundation (EstSF no. 5137).

References

TP10, a delivery vector for decoy oligonucleotides targeting the Myc protein

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Received 27 May 2005; accepted 13 September 2005
Available online 25 October 2005

Abstract

One approach to investigate gene function, by silencing the activity of certain proteins, is the usage of double stranded decoy oligodeoxynucleotides (ds decoy ODNs). Decoy, in this sense, is ds ODNs bearing the consensus binding sequence for a DNA-binding protein. This can be used in clinical settings to attenuate the effect of overexpressed transcription factors in tumor cells. We here choose to target the oncoprotein Myc. Since oligonucleotides are poorly internalized to cells, a cell-penetrating peptide, TP10, was coupled to the Myc decoy, using two different strategies. Either TP10 was simply mixed with ds decoy ODNs forming complexes through non-covalent electrostatic interactions, or by having a nona-nucleotide overhang in one of the decoy strands, and adding a complementary PNA sequence coupled to an NLS sequence and TP10, which could hybridize to the Myc decoy.

By using these strategies, uptake was significantly enhanced, especially with the co-incubation approach. Interestingly, various endocytosis inhibitors had no effect on the uptake pattern, suggesting that uptake of these complexes is not mediated via endocytosis. Finally, a decreased proliferative capacity was observed when treating the neuroblastoma cell line N2a with TP10-PNA conjugate hybridized to Myc decoy compared to naked Myc decoy and untreated cells. A dose-dependent decrease in proliferation was also observed in MCF-7 cells, when using both strategies. These results suggest an alternative way to efficiently deliver ds ODNs into cells using the cell-penetrating peptide TP10 and prevent tumor growth by targeting the oncoprotein Myc.

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Keywords: Cell-penetrating peptide; TP10; Cargo delivery; Decoy; Myc; Endocytosis

1. Introduction

During the past years, oligonucleotide delivery into cells has become a powerful research tool to elucidate gene function and regulation. A major obstacle in the development of therapeutic agents, like oligonucleotides, is the low bioavailability of hydrophilic sub-
stances due to the hydrophobic nature of cellular membranes. Hence, several viral and non-viral vectors have been developed in order to improve uptake of oligonucleotides into cells. The efficiency of oligonucleotide delivery is dependent on several steps: transfection complex adsorption to the cellular surface, cellular uptake, endosomal release, and nuclear translocation of the complex. For virus-based vectors, all these limiting steps are overcome since they express different proteins that facilitate delivery at different levels [1]. However, the viral vectors have limited DNA carrying capacity and are possibly immunogenic in vivo [2].

Several peptide-based vectors have been developed in order to mimic viral entry into cells and escape from endosomes [3]. Also, peptides derived from receptor ligands have been coupled to oligonucleotides in order to trigger endocytic uptake [4,5]. Other groups have conjugated nuclear localization signal (NLS) sequences to enhance the nuclear delivery of oligonucleotides [6,7]. More recently, several short, cationic and amphiphatic peptides named cell-penetrating peptides (CPPs) have gained increasing attention. CPPs are peptides that have the ability to rapidly translocate into most mammalian cells. Interestingly, these peptides have been successfully used to transport various cargoes both in vitro and in vivo, as reviewed in Ref. [8]. CPPs have traditionally been conjugated covalently to cargoes via disulphide bridges [9,10]. Recently, these peptides or CPPs have also been non-covalently linked through electrostatic interactions between negatively charged oligonucleotides and positively charged cargoes [11,12], with high delivery efficiency. Still it is unclear and highly debated what mechanism is responsible for uptake of CPPs and their cargoes. Today it is mainly believed that some peptides are internalized via endocytosis [13,14], while some peptides are claimed to translocate independent of any endocytic component [15,16].

One interesting application of oligonucleotides in basic research and therapy is the usage of ds decoy ODNs to down-regulate the activity of proteins. Decoy, in this sense, is double stranded ODNs bearing the consensus binding sequence for a DNA-binding protein. The decoy inhibits binding of proteins to genes by competing for binding sites. Ds decoy ODNs was first used as a tool for investigating transcription factor

![Diagram](image)

Fig. 1. Sequences and schematic presentation of TP10, TP10–PNA conjugate, Myc decoy, TP10–PNA–Myc decoy and TP10 Myc decoy complex. (A) TP10, unlabeled or labeled with fluoresceinyl or Cys(Npys) for building of TP10–PNA conjugate. (B) TP10–PNA conjugate built of TP10 coupled by a disulphide bridge to a PNA nonamer with an NLS N-terminally. (C) ds decoy ODN, here named Myc decoy, with an overhang for hybridization to TP10–PNA conjugate (D) TP10–PNA–Myc decoy, TP10–PNA conjugate hybridized to Myc decoy. (E) TP10 Myc decoy complex, where n is 8 for a charge ratio of +1 between TP10 and Myc decoy.
activity in cell culture systems [17]. Since then, several transcription factors have been targeted, both in vitro and in vivo using this approach [18–21].

The Myc protein is a basic helix-loop-helix leucine zipper transcription factor involved in cell cycle regulation [22]. Myc binds to a specific sequence, CANNTG (E-box), in promoter regions of different genes leading to up-regulation or down-regulation of a certain gene [23,24]. The Myc protein pushes cells into the S-phase of the cell cycle by up-regulating expression of proliferative proteins. It has been shown that 50–60% of all human tumors overexpress Myc. Since Myc is a well characterized protein involved in tumor development and maintenance, this protein was chosen as a target in an attempt to, for the first time, down-regulate proliferation in neuroblastoma and breast cancer cells by using ds decoy ODNs directed to Myc.

Due to the poor cellular uptake of oligonucleotides we decided to introduce a novel delivery vehicle, recently published by Fisher et al. [25]. By using a ds decoy ODN sequence with one strand containing a flanking nona-nucleotide as compared to the other strand, a 9-mer PNA, complementary to the nine nucleotides, can hybridize to that particular sequence (Fig. 1). To enhance cellular and nuclear uptake, an NLS sequence was directly synthesized at the N-terminus of PNA (not included in the construct used by Fisher et al.) which, in turn, was coupled to the CPP TP10 via a disulphide bridge (Fig. 1B). We have also recently shown that TP10 improves PEI-mediated plasmid delivery when applied in non-covalent complex with the plasmid [26]. Therefore, Myc decoy (i.e. ds decoy ODN targeting the Myc protein) were simply mixed with TP10 to form non-covalent stable complexes (Fig. 1E). Using these strategies, uptake of decoy ODNs was significantly enhanced and a decreased proliferative capacity was observed in two different tumor cell lines.

2. Materials and Methods

2.1. Cell culture

Mouse neuroblastoma cells N2a were grown in Dulbecco’s Modified Eagle’s Media (DMEM) with glutamax supplemented with 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. Human breast cancer cells MCF-7 were grown in RPMI 1640 with glutamax supplemented with 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin.

2.2. Synthesis

The peptides TP10 and NLS-PNA were synthesized on Applied Biosystems stepwise synthesizer’s model 431A or 433A, respectively. A 4-methylbenzyldiamine-polyester resin (MBHA) was used in both cases as solid support giving rise to products with amidated C-terminus. t-Boc chemistry was applied in both cases. Amino acids were purchased from NeoSystem, France and coupled as hydroxybenzotriazole (HOBT) esters while PNA was coupled with 7-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU). After cleavage of peptides and PNA from the resin, the synthesis products were purified by reverse phase HPLC Iomega C18 column and analyzed using MALDI-TOF mass spectra. Conjugation of TP10 and NLS-PNA via a disulphide bridge was carried out as described elsewhere [9]. Briefly, 3-nitro-2-pyrindinesulphenylated TP10 (Fig. 1A) and NLS-PNA with a free thiol (a cysteine in the N-terminus of NLS) were stirred overnight in DMSO/DMF/acetic acid buffer pH 5.5 in a 2/2/1 mixture. Products were separated in a C18 reverse-phase HPLC column and conjugate was identified by absorbance spectra and MALDI-TOF analysis. The sequences of the peptides, PNA and their conjugates are presented in Fig. 1.

2.3. Hybridization

Myc decoy ODNs with different sequences were purchased from CyberGene AB, Sweden:

5′CCC ACC ACG TGG TGC CTT GTG TCC CT
3′ long wt

3′GGG TGG TGC ACC ACG GA 5′ short wt
5′CCC ACC ACG GAG TGC CTT GTG TCC CT
3′ long mutated

3′GGG TGG TGC CTC ACG GA 5′ short mutated

Bold letters represent the E-box to which Myc binds. Underlined letters indicates the mutation in the
E-box. Finally, letters in italic is the complementary sequence to the synthesized PNA. Sequences are taken from Refs. [27,28]. Also, a 5’ modified long wt ODN with 6-carboxyfluorescein-aminohexyl amide (6-FAM) was purchased from CyberGene AB, Sweden.

Complementary ODNs were mixed in a 1:1 molar ratio in water containing 0.9% NaCl and incubated for 1 h at 37 °C while shaking. 0.5 or 1 µM Myc decoy was used in experiments. TP10–PNA conjugate was mixed with Myc decoy in various molar ratios ranging from 0:1 to 5:1. The reaction was carried out for 1 h at 37 °C under slight shaking. TP10 Myc decoy complexes were formed simply by mixing TP10 with ds decoy ODNs (Myc decoy) for 1 h at room temperature. Different concentrations of TP10 were used for co-incubations varying from 1–12 µM depending on ODN concentration, yielding different charge ratios.

2.4. Gel shift- and retardation assays

TP10 was mixed with Myc decoy as previously described. 1 µM Myc decoy (corresponding to 200 ng DNA) was mixed with increasing concentrations of peptides giving rise to a peptide/DNA charge ratio ranging from +1 to +4. Complexes were analyzed by electrophoresis on a 6% agarose gel in TBE buffer, containing ethidium bromide (Sigma, Sweden), for 1 h at 100 V.

TP10–PNA conjugates were hybridized to 1 µM 6-FAM labeled Myc decoy in different molar ratios varying from 0:1 to 1:5. Complexes were analyzed on a 20% polyacrylamide gel at 150 V for 1 h. Pictures were taken in Fujifilm LAS-1000 Intelligent Dark box II using IR LAS-1000 Lite v1.2 software.

2.5. Serum protection assay

Preformed TP10 Myc decoy complexes with different charge ratios were incubated for 5 h in presence of cell culture medium containing 10% FBS before applying the complexes on a 6% agarose gel, containing ethidium bromide, for 1 h.

2.6. Quantitative uptake

10^5 N2a or MCF-7 cells were plated 2 days prior experiment in 12-well plates. After removal of media, cells were incubated with 500 µl of 1 µM 6-FAM labeled Myc decoy with or without TP10 or TP10–PNA conjugate in different concentrations for 1 h in cell culture medium without serum and antibiotics. Following treatment, cells were washed two times with PBS and detached by trypsination for 5 min and centrifuged at 1000 g for 5 min at 4 °C. Cell pellets were lysed in 300 µl 0.1 M NaOH for 1 h and centrifuged at 10,000 g for 10 min at 4 °C. 250 µl of lysate was transferred to a black 96-well plate (Labdesign, Sweden). Fluorescence was measured at 494/518 nm on a Spectra Max Gemini XS fluorometer (Molecular devices, USA) and recalculated to amount of internalized compound by using the linearity of fluorescein and normalized to amount of protein (Lowry BioRad, USA). Fluorescein labeled TP10 and 6-FAM labeled ODNs show a linear correlation between concentration and fluorescence. When analyzing the effect of endocytosis inhibitors, the same protocol was used but cells were pretreated for 30 min with 50 µM chloroquine, 5 µM cytochalasin B, 50 nM wortmannin, 5 µM nocodazole or 10 mM sodium azide and deoxyglucose. All inhibitors were purchased from Sigma, Sweden. As positive controls of endocytosis inhibition 5 µM fluorescein isothiocyanate-dextran (FITC-dextran) (Sigma, Sweden) and 25 µg/ml transferrin tetramethylrhodamine conjugate (Molecular Probes, Eugene, USA) were used.

2.7. Confocal laser scanning microscopy

N2a cells were seeded out on glass cover slips and washed three times with HKR, after which 6-FAM labeled naked Myc decoy or TP10 Myc decoy com-

Fig. 2: Myc Decoy hybridizes to TP10–PNA conjugate(s) and form complex with TP10 that protects against serum degradation. (A) TP10–PNA conjugate hybridize to Myc decoy rendering one or two TP10–PNA conjugates hybridized to each fluorescein labeled Myc decoy, analyzed on a 20%polyacrylamide gel. (B) Myc decoy form complexes with TP10 based on charge interactions. Increasing charge ratio yields large complexes which are retained in the wells. (C) Complexation of Myc decoy with TP10 prevents serum degradation. Complexes were analyzed on a 6% agarose gel.
A
Myc decoy:
TP10-PNA conjugate

2 TP10-PNA : Myc decoy
1 TP10-PNA : Myc decoy
Myc decoy

B
Charge ratio
TP10 Myc decoy complex

Myc Decoy

C
Charge ratio
Serum
TP10 Myc decoy complex
ds Myc Decoy
ss Myc Decoy
Degraded
plex, charge ratio +1.5, were added to a final concentration of 0.5 μM. After 45 min incubation at 37 °C the cells were briefly washed with HKR, mounted in HKR to achieve live cell pictures, and examined using a Leica TCS-SP laser scanning confocal microscope (Leica, Heidelberg, Germany) with a 488 nm laser line from an Argon laser (20 mW).

2.8. Cytotoxicity measurements

10^5 cells were plated in 12-well plates two days before treatment. Cells were treated essentially as described in Ref. [28]. Briefly, cells were loaded with 0.5 μCi of the glucose analogue, 2-deoxy-D-[1-3H]-glucose (Amersham Biosciences, UK) that are taken up by cells and phosphorylated inside cells by hexokinase. Since the phosphorylated product is impermeable over intact cell membranes, efflux of radioactivity is a measure of plasma membrane disturbance. 100 μl extracellular aliquots were taken 5, 15, 30 and 60 min after addition of decoy/peptide complexes. Finally, cells were lysed to get the total radioactivity in each well. All time points were correlated to the total radioactivity in each well. Samples were diluted in 5 ml Emulsifier Safe scintillation liquid (Ferkin Elmer, USA) and measured on β-counter (2500TR Packard, Australia).

2.9. Proliferation

2.9.1. BrdU assay

N2a cells were plated in 24-well plates, 10^3 cells/well, on cover slips. One day after plating, cells were treated with Myc decoy, with and without TP10-PNA conjugate. Two wells remained untreated as positive controls for proliferation. Treatment with Myc decoy was carried out twice under 48 h in 0.5 ml full growth media. Thereafter, cells were pulsed with 30 μM of the thymidine analogue Bromodeoxyuridine (BrdU) (Sigma, Sweden) for 1 h. Cells were washed three times with PBS before fixation with 70% cold ethanol/water. After fixation and washing, cells were permeabilized using 1 M HCl/0.5% TritonX solution for 10 min. Cells were washed three times with 0.5% BSA/PBS and 500 μl primary mouse IgG anti BrdU antibody (Sigma, Sweden), diluted 100× in 0.5% BSA/PBS, was added to wells and incubated for 45 min in room temperature. After washing with 0.5% BSA/PBS, 500 μl secondary FITC-conjugated rabbit anti mouse antibody (Sigma, Sweden), diluted 200× in 0.5% BSA/PBS, was added to cells and incubated in darkness for 45 min. Cells were mounted on objective glasses and analyzed on a fluorescence microscope Leica DM IRE2 (Leica, Germany).

2.9.2. Wst-1 assay

N2a and MCF-7 cells were seeded onto 96-well plates, 10^4 cells/well, one day before treatment. Cells were treated once or twice during 24 or 48 h, respectively with 0.5 or 1 μM wild type or mutated Myc decoy in combination with TP10 or TP10-PNA conjugate in 100 μl full growth media. Cells were then exposed to Wst-1 according to manufacturers protocol (Sigma, Sweden). Absorbance (450–690 nm) was measured on absorbance reader Digiscan (Labvision, Sweden).

3. Results

3.1. TP10–PNA conjugate hybridizes to Myc decoy and TP10 forms complexes with Myc decoy that protects from serum degradation

TP10-PNA conjugate was hybridized in different ratios to 6-FAM labeled Myc decoy and analyzed on a polyacrylamide gel. As expected, ssDNA annealed to form dsDNA within 1 h (Fig. 2A, lane 1–2). However, a 1:1 molar ratio of TP10-PNA conjugate to ds ODNs was not sufficient for effective hybridization (lanes 3–4). At a molar ratio of 1:2 or 1:5, on the contrary, most of the Myc decoy was hybridized to the conjugate (lanes 5–8). Interestingly, at these molar ratios, two TP10–PNA conjugates hybridized to each Myc decoy. Two PNA strands have been observed before to bind to one DNA strand by Hoogsteen and Watson Crick base pairing [29].

Agarose gel analysis of Myc decoy co-incubated with increasing concentration of TP10 clearly shows that TP10 electrostatically interacts with Myc decoy and reduce its ability to move through the gel (Fig. 2B). Already at charge ratio 1, almost every Myc decoy remains in the well whereas complexes are applied (lanes 3–4).

TP10 was co-incubated with Myc decoy in the same manner as previously and then exposed to
10% serum media. According to Fig. 2C, TP10 protects DNA from degradation at all charge ratios, even at charge ratio +1.

3.2. TP10 improves cellular uptake of ds decoy ODNs

Since TP10-PNA conjugates hybridized effectively to the Myc decoy overhang at a molar ratio of 1:2 and 1:5 we decided to analyze the efficiency of the conjugate as a vector for ds decoy ODN delivery. Concomitantly we choose to co-incubate TP10 with Myc decoy using charge ratio +1.5 after interpreting the results from gel-shift and serum degradation assays. The results in Fig. 3A clearly show that TP10 as a non-covalent complex with Myc decoy, i.e. TP10 Myc decoy complex, improves the uptake 100-fold compared to naked Myc decoy. Also, uptake was enhanced 70-fold compared to the commercially available vector, oligofectamine™ (Fig. 3A). The TP10-PNA conjugate, on the other hand, only enhanced uptake slightly at a molar ratio of 5:1 over Myc decoy. Therefore, this ratio was used for further experiments. As a negative control of cellular uptake we used FITC-conjugated dextran and hardly any internalization was observed for the 42 kDa polysaccharide within 1 h. Similar results were obtained on MCF-7 cells (data not shown).

To exclude the possibility that the observed uptake with the TP10 Myc decoy complex was a result of membrane aggregation at the cell surface, we also analyzed the uptake by confocal laser scanning microscopy on live cells. The results in Fig. 4 clearly show that TP10 improves cellular uptake of 6-FAM labeled ds ODNs. Pictures were taken in a focal plane crossing the cell and no membrane aggregation was observed. Interestingly, internalized TP10 Myc decoy complexes

![Graphs showing cellular uptake of TP10 and Myc decoy complexes.](image)

**Fig. 3.** TP10 improves cellular uptake of ds ODNs into N2a cells using both strategies. (A) Delivery of 1 μM TP10 or 6-FAM labeled Myc decoy into cells by complexation with TP10 and hybridization to TP10-PNA conjugate. (B) Endocytosis inhibitors do not influence uptake of Myc decoy complex, charge ratio +1.5, or controls 5 μM FITC-dextran and 25 μg/ml transferrin tetrathylrhodamine conjugate. The values represent the mean of at least three independent experiments each done in triplicate (mean ± SEM, n = 3).
have a diffuse distribution throughout the cytoplasm and partly in vesicles, suggesting that uptake is not exclusively mediated by endocytosis.

3.3. TP10 Myc decoy complexes are readily internalized in the presence of endocytosis inhibitors

Due to the high uptake observed with TP10 in complex with ds decoy ODNs and the distribution of ODNs, we aimed to investigate what internalization route the complex was utilizing. Cells were pretreated with endocytosis inhibitors before applying 0.5 µM of TP10 Myc decoy complex. None of the inhibitors had any effect on internalization, suggesting that uptake may not be mediated exclusively through classical endocytosis (Fig. 3B). The efficiency of the inhibitors was verified by the fact that uptake of FITC-dextran 42 kDa and transferrin tetramethylrhodamine conjugate 80 kDa was decreased about 20–40% for all inhibitors. Surprisingly, chloroquine decreased uptake of transferrin and dextran, which should not be effected by a lysosomotropic agent.

3.4. Decoy ODNs targeting the Myc protein mediate decreased proliferation in a dose-dependent manner

After defining two efficient delivery vectors we studied whether the Myc decoy-sequence had any effect on cellular proliferation and whether our delivery vectors could improve that effect. Results from the WST-1 assay on MCF-7 cells clearly illustrate that the wild type Myc decoy sequence reduces proliferation in comparison to untreated cells or cells treated with a mutated Myc decoy sequence (Fig. 5A). Wild type decoy ODNs without TP10 or TP10–PNA conjugate had no effect on proliferation after one day of treatment but decreased proliferation with approximately 20% after 48 h. TP10 Myc decoy complex on the other hand mediated a decrease in proliferation already after 24 h and a very strong reduction after 48 h. Interestingly, even though uptake studies revealed poor uptake, also the TP10–PNA conjugate hybridized to Myc decoy reduced proliferation to reach the same levels as the positive control (i.e. cells grown in serum free media). Similar results were also obtained on N2a cells (data not shown).
A BrdU assay was also performed to analyze proliferation qualitatively in N2a-cells. Cells were treated twice with ds decoy ODNs with or without TP10–PNA conjugate and analyzed after 48 h for BrdU incorporation. Cells treated with wild type Myc decoy were clearly fewer in number and had less BrdU incorporation per cell (compare Fig. 6A with D, E). The mutated sequence had no effect on proliferation (Fig. 6F).

3.5. Membrane integrity is unaffected by decoy ODNs

In order to confirm that the observed results in uptake and proliferation was not a result of pore formation in cellular membranes, we decided to analyze membrane leakage of deoxyglucose 5,15,30 and 60 min after addition of various Myc decoy complexes. Results in Fig. 7 show that there is no difference in membrane integrity between Myc decoy with or without TP10 or TP10–PNA conjugate, or in relation to untreated cells. Therefore we can conclude that the observed effects are not a result of membrane leakage and concomitant toxicity.

4. Discussion

The major barrier in delivery of oligonucleotides is the cellular plasma membrane. Its hydrophobic nature makes it more or less impermeable for hydrophilic substances. Therefore several delivery vectors have been developed in order to improve uptake of these substances. Other factors that limit the delivery efficiency are poor escape from endosomal compartments and nuclear delivery. Brandén et al. reported improved uptake and nuclear delivery of oligonucleotides when coupling an NLS sequence to them [6]. Other groups have designed peptides that mimic viral endosomal escape in order to enhance the effect of oligonucleotides and proteins inside cells [3,13].

In recent years, a growing number of CPPs have been designed and utilized in delivery of hydrophilic macromolecules, for reviews see Refs. [8,30]. These peptides are internalized rapidly into cells and were for many years believed to translocate in a receptor- and endocytosis independent manner. However, recent studies in this field implicate involvement of proteoglycans on cell surfaces and concomitant endo-

**Fig. 5.** Myc decoy decrease proliferation in MCF-7 cells. (A) Delivery of Myc decoy by TP10-PNA conjugate or TP10 decrease proliferation over time. Cells were treated with Myc decoy complexed with TP10 or hybridized to TP10–PNA conjugate 24 and 48 h after seeding and assayed for proliferation by WST-1 after 48 or 72 h. (B) Myc decoy inhibits proliferation in a concentration dependent manner. Cells were treated after 24 and 48 h with 0.5 or 1.0 μM Myc decoy, TP10/TP10–PNA conjugate. W1 = wild type, Mut ≡ mutated, SF = serum free media. The values represent the mean of three independent experiments each done in duplicate (mean ± SEM, n = 2).

When comparing the effect of Myc decoy after 48 h at two different concentrations, 0.5 μM and 1 μM, proliferation is reduced in a dose-dependent manner (Fig. 5B).
Fig. 6. TP10-PNA-Myc decoy decrease BrdU incorporation in N2a cells. Cells were grown on cover slips and treated with 1 μM Myc decoy twice in 48 h followed by BrdU and Hoechst staining. (A) Untreated cells. (B) 30% serum. (C) Untreated, no BrdU. (D) Wild type Myc decoy. (E) Wild type Myc decoy with TP10-PNA conjugate, 1:2 ratio. (F) Mutated Myc decoy with TP10-PNA conjugate, 1:2 ratio.

Fig. 7. Cell membrane integrity is unaffected in N2a cells when exposed to 1 μM Myc decoy alone or in complex with TP10 (charge ratio >1.5), hybridized to TP10-PNA conjugate (1:5 ratio) or transfected with oligofectamine. Cells were loaded with 0.5 μCi of 2-deoxy-3-3Hglucose 1 h before exposure and samples were normalized to total radioactivity in each well.

cytotoxic uptake [12]. Still there are discrepancies regarding what endocytotic pathway is utilized. Some groups claim that uptake occurs via lipid-raft dependent caveolae-mediated endocytosis [31] while others claim that macropinocytosis is the main route for internalization [13]. CPPs like MPG and Pep-1 are still thought to be internalized via an endocytosis independent pathway when co-incubated with oligonucleotides or proteins, respectively [16,32]. Nevertheless, independent of uptake mechanisms, CPPs have been proven useful in delivery of oligonucleotides and plasmids of various sizes both in vitro and in vivo.

In the present work we aimed to investigate the potential role of TP10 as a delivery vehicle for double stranded decoy ODNs targeting the oncogenic transcription factor Myc. TP10 has recently been shown effective in delivery of decoy ODNs targeting NF-B, using PNA as an anchor to couple the CPP to the double stranded decoy [25]. Furthermore, we have very recently shown improved PEI-mediated plasmid delivery by co-incubating TP10 with plasmid/PEI complexes [26]. Therefore we applied both strategies in this work. The results in Fig. 3A clearly illustrate that TP10 in a non-covalent complex with a 16 kDa ds
ODN significantly increase the uptake of the cargo at a charge ratio of +1.5 (corresponding to 12 μM TP10 to 1 μM ds ODN). Results also indicate that complexes are formed rapidly (Fig. 2B) and are insensitive to serum exposure (Fig. 2C). Since the uptake of ODNs co-incubated with TP10 was significantly higher than that of corresponding hybridized TP10-PNA conjugate, we wanted to elucidate the internalization mechanism. None of the endocytosis inhibitors or energy depletion agents had any pronounced effect on internalization (Fig. 3B). Cytochalasin B is an actin depolymerizing agent that prevents formation of macropinocytosis essential for macropinocytosis [33], supporting an uptake mechanism other than macropinocytosis or that it constitutes a very small component. Chloroquine that is a weak base that inhibits maturation and transport of vesicles into late endosomes and neutralizes the pH of the latter [34] and Wortmannin which inhibit macropinocytosis and early endosomal fusion by blocking PI-3K [35], did not have any effect on internalization, indicating that our complexes partly bypass the endosomal compartment. The inhibitor of tubulin polymerisation, nocodazole, which bind tubulin [36] and thereby block intracellular transport, did also not have any effect on uptake. Also, nystatin, a lipid-raft disrupting agent did not influence the uptake (data not shown). Finally, ATP depletion by 2-deoxy-xyloose and sodium azide [37] did not affect internalization, further emphasizing that uptake of TP10 Myc decoy complexes is not energy dependent. The uptake of the controls, transferrin and dextran, which indicate clathrin mediated endocytosis and macropinocytosis respectively decreased with 20–40% in the presence of inhibitors. Increased inhibition was not possible to achieve due to toxicity of the inhibitors at higher concentrations. Confocal microscopy co-localization studies (data not shown) displayed partial co-localization between TP10 decoy complex and dextran and also with transferrin. This suggests that there still might be an endocytosis component involved, but sufficiently small, not to be detected by the uptake assay by the more sensitive confocal microscopy. These findings are in line with previous observations on another CPP, MPG [16] that promote gene delivery independently of the endosomal pathway. On the other hand, delivery of alydin by transportan decreased after energy depletion [38] suggesting different uptake mechanisms depending on cargo properties.

Finally we aimed to investigate the biological significance of these findings. We chose to target the Myc protein since it is well characterized regarding DNA-binding [39,40] and the expression of the protein is frequently deregulated in various tumors. Although, the TP10-PNA Myc decoy only increased uptake into cells slightly it had a strong effect on cellular proliferation (Fig. 6). Interestingly, when comparing uptake (Fig. 3A) and proliferation (Fig. 5A), the effect of TP10-PNA Myc decoy is higher than with TP10 Myc decoy complex. However, non-covalent complexes have a stronger effect overall on proliferation in MCF-7 cells.

Quantification of the number of Myc decoy that are internalized per cell reveals that there are approximately 400,000 Myc decoy molecules per cell when delivered by a 5 times excess of TP10-PNA conjugate. The uptake for TP10 Myc decoy complex gives 20 million Myc decoy per cell. This should be compared with an average of 61,000 Myc proteins per cell for MCF-7 cells in the cytoplasm and nucleus [41]. Also all internalized Myc decoy may not be accessible for the Myc protein in the nucleus and cytoplasm due to the strong interactions between TP10 and DNA. The rapid turnover of Myc (t½=30 min) could also decrease the effect of internalized Myc decoy speeding up degradation and transport from the nucleus.

In conclusion, the delivery efficiency of TP10 and strong biological effect of Myc decoy in combination with TP10, suggest a novel strategy to down-regulate proliferation in tumor cells. Finally, the results clearly illustrate that TP10 is a suitable vector for ODN delivery that by-passes the endosomal compartment.

Acknowledgements
This work was funded by Swedish Science Foundation (VR-NT) and European Community (QLRT-2001-01989).

References


Paper III
Cargo-dependent cytotoxicity and delivery efficacy of cell-penetrating peptides: a comparative study

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The use of CPPs (cell-penetrating peptides) as delivery vectors for bioactive molecules has been an emerging field since 1994 when the first CPP, penetratin, was discovered. Since then, several CPPs, including the widely used Tat (transactivator of transcription) peptide, have been developed and utilized to translocate a wide range of compounds across the plasma membrane of cells both in vivo and in vitro. Although the field has emerged as a possible future candidate for drug delivery, little attention has been given to the potential toxic side effects that these peptides might exhibit in cargo delivery. Also, no comprehensive study has been performed to evaluate the relative efficacy of single CPPs to convey different cargos. Therefore we selected three of the major CPPs, penetratin, Tat and transportan 10, and evaluated their ability to deliver commonly used cargos, including fluorescein moiety, double-stranded DNA and proteins (i.e. avidin and streptavidin), and studied their effect on membrane integrity and cell viability. Our results demonstrate the unfeasibility to use the translocation efficacy of fluorescein moiety as a gauge for CPP efficiency, since the delivery properties are dependent on the cargo used. Furthermore, and no less importantly, the toxic effect of CPPs depends heavily on peptide concentration, cargo molecule and coupling strategy.

Key words: cell-penetrating peptide, cytotoxicity, delivery vector, penetratin, transactivator of transcription (Tat), transportan.

INTRODUCTION

The ability to cross the lipid bilayer of cells and access the cell interior is still one of the major obstacles to overcome in order to progress current drug development. Various techniques have therefore been developed in order to improve cellular uptake of bioactive agents [1]. CPPs (cell-penetrating peptides) have, since their discovery in 1994 [2], been widely used to deliver a wide range of bioactive compounds across cellular membranes of several cell types both in vivo and in vitro (reviewed in [3,4]). Delivery seems to be independent of cell type and can be directed to diverse compartments inside the cell [5]. No proper definition of CPPs has been formulated, but most, if not all, carry a net positive charge, are less than 30 amino acids long and have the ability to rapidly translocate large molecules into cells. Still it is unclear which mechanism is responsible for uptake of CPPs and their cargos. Originally it was believed that CPPs translocated cell membranes in a receptor- and energy-independent manner. However, more recent studies suggest that the uptake for most CPPs is an energy-dependent process with initial binding of peptides to proteoglycans on the cell surface and concomitant endocytosis of peptides [6-8]. However, there are still reports claiming that membrane translocation is independent of endosome formation and that uptake, in line with early reports, occurs directly through the outer cellular membrane [9,10].

There is a constantly growing number of CPPs introduced in the literature with different chemical properties and abilities to ferry various cargos across cellular membranes. However, studies on CPP-mediated cargo delivery performed so far have focused on achieving high delivery yields, and few studies have focused on the toxicity that these peptides might exhibit. Lindsay and co-workers analysed the toxicity of CPP-mediated peptide delivery in 2005 [11], and Saar et al. [12] studied the toxicity of free CPP uptake. Yet, the results are divergent regarding uptake efficacy and toxicity of single CPPs and different cargo molecules, making comparisons between one study and another very difficult. Furthermore, different cells, cell passages, incubation times, concentrations etc. have been used, making comparisons between studies all the more complicated. Since no comprehensive study has been performed to evaluate the relative efficacy of single CPPs to deliver different cargos, and whether the cargo type might influence the toxicity of peptides, a study using the same conditions would offer a more accurate comparison concerning these aspects.

In the present study, we aimed to evaluate the delivery efficiency and cytotoxicity of three well characterised CPPs, Tat (transactivator of transcription), TP10 (transportan 10) and penetratin [2,13,14] (Table 1), using different cargos. These cargo molecules include carboxyfluorescein that is used on a routine basis to assess the cellular uptake of peptides, dsDNA (double-stranded DNA) that can serve as model for deoxy oligonucleotides or possibly siRNAs (short interfering RNAs) and two model proteins: streptavidin and avidin. Proteins have been transported into cells by CPPs and have been shown to be biologically active [3,4], and, in the present study, streptavidin and avidin were utilized as model proteins, as they have been widely studied and have very high affinity for biotinylated peptides, making conjugation less cumbersome. In addition, since different proteins have different pl values, choosing these two proteins as models is appropriate as their pl values are significantly different.

Abbreviations used: CHO, Chinese-hamster ovary; CPP, cell-penetrating peptide; DMF, dimethylformamide; dsDNA, double-strand DNA; FBS, fetal bovine serum; HAT, Hammarsten-Ahrén-Ranger; HOB, 1-hydroxybutylbenzotriazole; LDH, lactate dehydrogenase; siRNA, small interfering RNA; Tat, transactivator of transcription; T helper, t-lymphocyte, TP10, transportan 10; WST-1, water-soluble tetrazolium salt 1.

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(streptavidin, pl 5.5, and avidin, pl 10.5). The present study does not consider the biological activity of the delivered cargos (see [15] for a thorough review), but rather the cargo-dependent toxicity that the CPP translocation exhibits.

We show that the cellular uptake of CPPs is cargo-dependent, and our results also imply that the cytotoxicity of these peptides is cargo-dependent. Furthermore, we show that the cytotoxicity and internalization level of TP10 varies significantly depending on the cargo-coupling position within the peptide. Taken together, we believe that these results could be useful in future experiments utilizing CPPs to facilitate the choice of peptide depending on cargo and choice of conjugation strategy.

**EXPERIMENTAL**

**Peptide synthesis and purification**

The peptides (Table 1) were synthesized in a stepwise manner on a 0.1 mmol scale on an automated peptide synthesizer (Applied Biosystems, Model 433A) using a t-Boc (t-butoxycarbonyl) solid-phase peptide synthesis strategy; t-Boc amino acids were coupled as HOBr (1-hydroxybenzotriazole) esters to a p-methyl benzhydrylamine resin (amino acids and resin purchased from Neosystem) to obtain C-terminally amidated peptides. Deprotection of the formyl protecting group on tryptophan was carried out in 20% piperidine in DMF (dimethylformamide) for 1 h. Peptides used for uptake studies were N-terminally labelled and TP10 was also labelled orthogonally on Lys4 with carboxyfluorescein using 5 mol of 5,6-carboxyfluorescein, 5 mol of 1,3-di-isopropylcarbodiimide, 5 mol of HOBr and 20 mol of N,N-di-isopropylethylamine dissolved in 1:1 (v/v) DMSO/DMF overnight [16]. Biotin (Sigma) was coupled as a HOBr ester to the N-terminus of Tat and penetratin or to the ϵ-amino group of Lys4 of TP10.

The peptide was finally cleaved from the resin using liquid HF at 0°C for 1 h in the presence of p-cresol (1:1). Peptides were purified using reverse-phase HPLC on a C18 column, 20–100% acetonitrile/0.1% TFA (trifluoroacetic acid) gradient, and the molecular mass was determined by MALDI-TOF (matrix-assisted laser-desorption ionization-time-of-flight) MS using a PerkinElmer/Foster Multimode MALDI-TOF mass spectrometer. The peptide purity was > 90% as determined by analytical HPLC.

**Cell culture**

HeLa cells were grown in MEM (Dulbecco’s modified Eagle’s medium) with GlutaMAX™ supplemented with 0.1 mM non-essential amino acids, 10 mM sodium pyruvate, 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. CHO (Chinese-hamster ovary) cells were grown in MEM (minimal essential medium) with GlutaMAX™ supplemented with 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were grown at 37°C in a 5% CO2 atmosphere. All media and chemicals were purchased from Invitrogen.

**Quantitative cellular uptake**

A total of 2 × 105 HeLa or CHO cells were seeded 24 h before experiments in 12-well plates to reach 70% confluence. In the uptake studies on free peptide, cells were treated with 1 or 5 μM fluorescence-labelled peptides in 50 μl of serum-free medium. In transfection studies of dsDNA, peptides in various concentrations were co-incubated for 30 min in 50 μl of 0.9% NaCl solution. Cells were then treated with 0.2 μM fluorescein-labelled dsDNA and CPPs in 500 μl of serum-free medium. In the protein transfection experiments, streptavidin or avidin was either complexed with 1 μM biotinylated peptides in serum-free medium or co-incubated with free peptides at various molar ratios in 0.9% NaCl solution to a final volume of 50 μl. Cells were then treated with 0.2 μM protein and CPPs in 500 μl of serum-free medium. At 1 h after treatment or 90 min after treatment with proteins, cells were washed twice in HKR (Heps–Krebs–Ringer) buffer before trypsinization. The trypsination step is crucial in order to remove membrane-associated peptides and/or cargo [17]. Cells were centrifuged at 1000 g for 5 min at 4°C, and cell pellets were lysed with 300 μl of 0.1 M NaOH for 60 min, after which 250 μl of lysis was transferred to a black 96-well plate. Fluorescence was measured at 494/518 nm on a Spectra Max Gemini XS fluorimeter (Molecular Devices) and recalculated to the amount of internalized compound and normalized to the amount of total protein (Lowry assay; Bio-Rad). Fluorescein-labelled peptides, dsDNA and proteins displayed a linear correlation between concentration and fluorescence. DNA was purchased from CyberGene AB, and proteins (streptavidin and avidin) were from Invitrogen. Single-stranded DNA, fluorescein-labelled at the 3’ end of the antisense strand, was hybridized to the sense strand in Milli-Q water containing 0.9% NaCl for 1 h at 37°C to create dsDNA with two nucleotides overhanging at the 3’ end. The DNA sequences are presented in Table 1.

**LDH (lactate dehydrogenase) leakage assay**

Membrane integrity was measured using the Promega CytoTox™ assay (Promega). In brief, 105 HeLa or CHO were seeded in 96-well plates 2 days before treatment with 100 μl of the above-mentioned compounds at different concentrations in serum-free medium. After 30 min, 80 μl of medium was transferred to a black fluorescence plate and incubated for 10 min with 80 μl of CytoToxOne™ reagent followed by 40 μl of stop solution. Fluorescence was measured at 560/590 nm. Untreated cells were defined as no leakage and 100% leakage was defined as total LDH release by lysing cells in 0.18% Triton X-100 in HKR buffer.

**WST-1 (water-soluble tetrazolium salt 1) assay**

HeLa or CHO cells were seeded on to 96-well plates, 105 cells/ well, 2 days before treatment. Cells were treated according to the same procedure as in the LDH leakage assay, but for 24 h. Cells were then exposed to WST-1, according to the manufacturer’s protocol (Sigma). Absorbance (450–690 nm) was measured on a Digiscan absorbance reader (Labvision). Untreated cells were defined as 100% viable. Although the WST-1 assay measures...
Table 2  Long-term toxic effects in HeLa and CHO cells after treatment with CPPs

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RESULTS

TP10 induces membrane leakage and reduces proliferation dose-dependently

To get a general view of the toxic profile displayed by the selected CPPs, the cytotoxic properties of free (unlabelled) peptides were assessed in HeLa and CHO cells. In order to get a comprehensive analysis of the cytotoxicity, both membrane disturbance and mitochondrial activity was studied. An LDH leakage assay was used to measure the acute membrane disturbance caused by the peptides, and a WST-1 assay was employed to study the more long-term change in proliferation (i.e. mitochondrial dehydrogenase activity). As seen in Table 2, neither penetratin nor Tat affected proliferation at concentrations up to 50 μM. Conversely, TP10 displayed long-term toxic side effects in HeLa and CHO cells at 20 μM. Overall, CHO cells appear to be more resistant to peptide treatment than HeLa cells. Penetratin and Tat have no effect on membrane integrity (Figure 1c, and results not shown), whereas...

Microscopy

A total of 2 x 10⁵ HeLa cells/well were seeded 1 day before exposure with the above-mentioned compounds in 12-well plates. Cells were treated for 1 h and analysed with an Olympus 1 x 70 microscope (with a DP50 camera) using the Viewfinder Lite V1.0 software.

Statistical analyses

All results are means ± S.E.M. for at least three independent experiments performed in quadruplicate, and statistics were calculated using ANOVA, with Dunnett’s post-hoc test. **"P < 0.01; *P < 0.05.

**Figure 1**

Both TP10 and penetratin are dose-dependently internalized into CHO (a) and HeLa (b) cells, whereas the uptake of Tat is negligible. Introducing a fluoroscence moiety N-termilnally on Tat decreases the membrane disturbance (d), but increases the long-term toxicity (d). In (a) and (b), 2 x 10⁵ cells/well were seeded 1 day before the experiment in 12-well plates, treated for 1 h with 1 or 5 μM peptide and analysed after washing, hypotonication and centrifugation. Uptake is presented as the amount of internalized peptide (pmol/mg protein). In (c) and (d), cells were treated with the same concentrations as in (a) and (b), but were analysed after different time points. LDH leakage was measured 50 min after treatment (d) and WST-1 activity was assayed 24 h after treatment (d).
Q1 Figure 2 ??????

TP10 labelled orthogonally with carboxyfluorescein displays several-fold higher uptake and significantly lower toxicity than N-terminally labelled peptide. (a) Uptake study of TP10, labelled at two different positions, in CHO and HeLa cells was conducted as described in Figure 1. (b) LDH leakage in HeLa cells after 30 min of treatment with TP10, FmOC-TP10 or FmOC-TP10 at different concentrations in serum-free medium. (c) WST-1 activity in HeLa cells 24 h after treatment with TP10, FmOC-TP10 or FmOC-TP10 at different concentrations in serum-free medium.

TP10 induces LDH leakage (approx. 20%) at 10 μM in HeLa cells (Figure 2b). The same was observed in CHO cells (results not shown).

Uptake of fluorescein-labelled CPPs: cargo attachment changes the cytotoxic properties of CPPs

When fluorescein-labelled, the selected peptide have vastly different cell-penetrating properties. From Figure 1, the following order of uptake yields was observed: TP10 > penetratin > Tat, where the uptake of Tat is nearly undetectable (Figures 1a and 1b). The yield of internalized peptide is overall slightly higher in CHO cells (Figures 1a and 1b). To see whether the fluorescein moiety alters the toxic properties of the peptides, the membrane integrity and proliferation was compared between free or fluorescein-labelled peptides. Penetratin demonstrated no signs of toxicity, either as a free peptide or when fluoresceinylated in any of the two assays (results not shown). Tat, in contrast, displayed slightly higher membrane leakage as a free peptide (Figure 1c), but interestingly, proliferation was reduced significantly at 20 μM with the labelled peptide (Figure 1d), but not as a free peptide. Since the fluorescein moiety apparently influences the cell viability, it is highly possible that it also alters the uptake of some peptides. If this is true, many of the uptake studies performed on CPPs might only show the uptake of the fluorescein-labelled peptide, which do not inevitably correlate with the uptake of the free CPP.

Internalization and cytotoxicity of TP10 is dependent on the cargo-coupling position within the peptide

On a routine basis, TP10 has been orthogonally conjugated with cargo molecules. Although orthogonally coupled TP10 has been shown to efficiently deliver various cargos [19], we wanted to elucidate whether coupling carboxyfluorescein N-terminally to TP10 would change the uptake pattern and cytotoxic profile of the peptide. Surprisingly, internalization of N-terminally labelled TP10 is decreased 4-fold compared with the orthogonally labelled peptide (Figure 2a), suggesting that the cargo-coupling position is an important aspect to consider when designing conjugates, at least in case of TP10. This observation also supports further the hypothesis that uptake of free peptides might deviate significantly from that of fluorescein-labelled ones. Furthermore, orthogonally labelled TP10 displays significantly lower membrane perturbation and long-term toxicity compared with free TP10 or TP10 labelled in the N-terminus (Figures 2b and 2c). Additionally, N-terminally labelled peptide exhibited both higher membrane leakage and long-term toxicity compared with free peptide (Figures 2b and 2c). As seen in Figure 3, both free and N-terminally labelled TP10 slightly altered the morphology and proliferation of HeLa cells treated with 10 μM peptide, while the orthogonally labelled peptide had a negligible effect on cell morphology.

CPPs promote internalization of dsDNA in a relatively non-toxic fashion

As seen in Figure 4a, all three peptides dose-dependently promoted cellular internalization of dsDNA with the following efficiency order: TP10 > penetratin > Tat. This pattern is in agreement with the one observed for fluorescein-labelled peptides (Figures 1a and 1b), with the exception of Tat, which seems to be more efficient in translocating dsDNA than when used as a free peptide (Figures 1a, 1b and 4a). The same fluorescein-labelled dsDNA was used further in complex with increasing amounts of CPPs to examine the cytotoxicity of peptides in non-covalent complexes with dsDNA. Both penetratin and Tat are, together with dsDNA, non-toxic at concentrations of up to 50 μM (Figures 4b and 4c). There is a tendency that Tat in complex with dsDNA decreases the WST-1 activity at concentrations above 20 μM, although this decrease is not significant (Figure 4c). Intriguingly, it appears that the cargo decreases the toxic side effects of TP10, as no sign of toxicity was observed at 10 μM TP10 together with dsDNA (Figures 4b and 4c). This is compared with free TP10, which displays approx. 20% LDH leakage (Figure 2b) and approx. 11% decrease in WST-1 activity at 10 μM (Table 2).

Protein uptake varies depending on the CPP and the delivery strategy used

The selected CPPs were studied in two different protein delivery strategies with two dissimilar proteins (streptavidin and avidin).
First, the peptides were co-incubated with the proteins and, secondly, the peptides were conjugated to the proteins through a biotin linker.

When comparing data from co-incubation studies on streptavidin, the translocation efficacy is penetratin > TP10 > Tat (Figure 5a). No significant difference in uptake was observed with avidin with or without peptide co-incubation, most probably since avidin has a cationic nature that prohibits electrostatic interactions with positively charged CPPs (Figure 5b). Again, penetratin and Tat showed no toxicity as determined by LDH leakage (Figure 5d). Also, the toxic side effects of TP10 seemed to decrease together with protein compared to free peptide, suggesting that peptide–protein interactions somewhat shield the cellular membrane from perturbation (Figure 5d). However, when measuring the mitochondrial activity of cells 24 h after streptavidin treatment, we observed a strong reduction of approx. 75% in cell viability, concluding that streptavidin is toxic if applied for longer periods of time (Figure 5e). Interestingly, when co-incubating streptavidin with increasing concentrations of TP10, the long-term toxicity clearly decreases, suggesting that the peptide might form stable complexes with the protein that remain unaltered inside cells and thereby shield the cytotoxic epitopes of the protein (Figure 5c).

This pattern was not observed for Tat or penetratin, indicating that these peptides form less stable complexes with streptavidin (results not shown).

The CPP efficacy pattern changes completely when using biotinylated CPPs that form nearly irreversible interactions with streptavidin. Then the observed uptake is as follows: TP10 > Tat > penetratin (Figure 5c). The same pattern was observed with avidin, but, overall, the level of uptake was higher, most probably since avidin is more cationic, allowing interactions with negatively charged cell surfaces (Figure 5e). Strikingly, Tat efficiently internalizes to cells when conjugated via biotin to proteins, but is poorly internalized as a fluorescein-coupled peptide compared with TP10 and penetratin (Figures 1a, 1b and 5c).

**DISCUSSION**

In the present study, we compared three of the major CPPs by means of their delivery yield and toxic side effects. We selected the two first discovered CPPs, penetratin and Tat, where the latter has been most extensively used (reviewed in [201]). Both peptides are highly cationic and they have a low amphipathic moment.

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The striking differences in uptake and toxicity observed between N-terminally and orthogonally labelled TP10 (Figure 1) might be assigned to the hydrophobic property of fluorescein. When coupled N-terminally, it increases the already hydrophobic nature of the peptide tail, resulting in increased membrane destabilization and elevated cytotoxicity. When coupled orthogonally, fluorescein is attached to the cationic part of the peptide, possibly reducing the amphiphilicity of the peptide and hence the cytotoxicity. This is just speculation, but it has been shown previously that there might be a correlation between amphiphilicity and toxicity of CPPs [12]. This observation also supports further the hypothesis that uptake of free peptides might deviate significantly from that of fluorescein-labelled ones.

The routine use of carboxyfluorescein as a CPP cargo molecule provides a valuable tool for measurements of cellular uptake; however, it is not a biologically relevant cargo. Therefore, to investigate further the potential of using CPPs to facilitate uptake of biologically relevant molecules, dsDNA was utilized as a model cargo for decoy-DNA or siRNA. All CPPs in the present study can promote dsDNA uptake in a dose-dependent manner. Both penetratin and Tat are virtually non-toxic together with dsDNA at concentrations up to 50 μM. The presence of dsDNA seems to decrease the cytotoxic side effects caused by TP10, most probably due to electrostatic and hydrophobic interactions between the peptide and the DNA, which makes cellular membranes less exposed to the peptide.

Introducing proteins to cells offer a great therapeutic potential as many diseases are caused by deficient protein expression. The major obstacle in protein delivery to date is the poor bioavailability of these molecules. Unlike oligonucleotides, where several transfection reagents have been developed to facilitate the cellular uptake, few delivery vectors exist for proteins. CPPs have been utilized in numerous studies to convey bioactive proteins inside cells [3]. In most cases, peptides have been recombinantly expressed from plasmids as a fusion with the protein, resulting in a conjugate of one peptide per protein [22,23]. Some protein transduction experiments have been conducted with CPPs using the same co-incubation strategy as for oligonucleotides, resulting in non-covalent complexes of several peptides per protein [24,25]. Both of these strategies have been successfully used to transport various proteins inside cells both in vitro and in vivo.

To get a comprehensive study of protein translocation by CPPs, two diverse methods to promote protein uptake were applied together with two proteins (streptavidin and avidin). Streptavidin and avidin display, as mentioned in the introduction, similar and dissimilar properties, making them suitable as models for whichever protein is desired. Either the CPPs were co-incubated with proteins or biotinylated CPPs were pre-incubated with proteins to form stable CPP-protein complexes. TP10 and penetratin was found to be the most potent vectors for protein delivery when co-incubated with the proteins (Figure 5a). This is not unexpected, since they both have several hydrophobic residues that can contribute to protein complex formation. Tat, on the other hand, does not promote any protein uptake when utilizing this co-incubation strategy. Since the Tat peptide is a highly cationic with few hydrophobic residues, it might be less prone to forming CPP complexes. Tat is, on the other hand, a potent vector for protein uptake when conjugated to the protein through a stable linker. Then Tat is an efficient as TP10 to promote uptake of both avidin and streptavidin (Figure 5c). These results are in line with several other studies implying that uptake of fluoresceinylated Tat is nearly negligible [26], whereas it is readily internalized when conjugated to a protein [23]. One plausible explanation for this behaviour could be that Tat utilizes different internalization routes depending on coupled cargo. Fluoresceinylated Tat might

**Figure 4**

Q1: The authors mention the importance of measuring both acute membrane toxicity, as well as long-term toxic effects when studying CPPs, since the two do not necessarily correlate.

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**Citations:**

1. El-Andaloussi and others

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**Notes:**

- Figure 4: Q1
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The ability of CPPs to deliver proteins through the plasma membrane, as suggested recently [27], but the uptake is not high enough to be visualized. In contrast, when a large cargo has been attached to the peptide, the uptake mechanism is changed to endocytosis, as suggested previously [8,23,28]. Furthermore, it seems that conjugating peptides to proteins is overall more effective than co-incubating peptides with proteins, at least when using the above-mentioned proteins.

On the whole, TP10 presents itself as the most efficient CPP of the tested peptides to deliver different cargos. As with many other delivery vectors, it seems to be a correlation between efficacy and toxicity at higher concentrations. Therefore, although TP10 is an effective transporter, it cannot be administered to cells at as high concentrations as Tat or penetratin.

Penetratin displays virtually no membrane perturbation or long-term toxicity up to 50 μM concentration, and the uptake yield is average compared with Tat and TP10 for all tested compounds. Tat coupled to fluorescein is, as reported previously [29], poorly taken up by the cell lines tested compared with penetratin and TP10. However, when conjugated to protein, Tat translocation is increased dramatically. Consequently, our results emphasize that it is unfeasible to compare the uptake of fluorescein-labelled CPPs and we can use these results as a gauge for translocation efficacy for various cargos. Furthermore, certain CPPs may be practical for use with one cargo, but be insufficient to use as a vector for another. Cargos, such as dsDNA and proteins, seem to decrease both the acute and long-term toxicity of CPPs. However, this is not true for all cargo molecules, since the fluorescein moiety increases the toxicity of Tat (Figure 1d), and also TP10 displays completely different cytotoxic properties when conjugated to fluorescein (Figures 2b and 2c).

There are many impediments to overcome before CPPs can be used as efficient and safe pharmaceutical vectors. Obviously, the mechanism of entry must be resolved, but, additionally, it is
important to use appropriate peptide vectors for desired cargos. We have shown that different peptides are suitable for different cargos and that the uptake yield of one cargo does not assure that the same CPP is effective in delivery for all bioactive molecules. In addition, we have also shown that the cytotoxicity of peptides is highly dependent on the cargo used and the cargo-coupling position within the peptide. In conclusion, we believe that the results presented in this paper can serve as guidelines to select appropriate CPPs for specific cargos.

We thank the Swedish Research Council (VR-N, VR-Med) for supporting this work.

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Paper IV
Co-Transduction of Sleeping Beauty Transposase and donor Plasmid via a Cell-penetrating Peptide. A simple one step Method

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Abstract
Transposable elements have emerged as a promising candidate for human non-viral gene-therapy. The Tcl/mariner transposon Sleeping Beauty is to date one of the most efficient transposons in mammals. Sleeping Beauty transposase has so far mostly been delivered to cells via a DNA source. This might cause spontaneous integration of the transposase gene and cause fatal damage to the affected cell. Hence, it would be advantageous to employ a non genetic source for the transposase.

We here show that a novel Cell-penetrating peptide, M918, has the ability to facilitate cellular delivery of both the transposase Sleeping Beauty as a protein and a transposon donor-plasmid carrying an antibiotic resistance gene in vitro. The technique is a simple and straightforward one-step method that might render a safe and efficient delivery platform for Sleeping Beauty mediated gene therapy.

Introduction
Human gene-therapy has great potential both as a tool for increased understanding of genetic mechanisms, and as a cure for various genetic diseases. However, there are still several impediments to overcome and safety aspects must be considered before applying gene-therapy as an option to treat human diseases. The technology to insert genes into cells and tissues to treat diseases, and hereditary diseases in particular is in its infancy. Currently, the most common delivery vector is altered viruses. Still, viruses are pathogens and therefore numerous problems exist that hamper viral gene-therapy (Dobbelstein, 2003, Manchello-Corvo and Martin-Duque, 2006). Non-viral delivery methods suffer from disadvantages such as low transfection efficiency and non-integrated chromosomal gene expression. On the other hand, non-viral delivery presents certain advantages over viral methods such as simple large-scale production and low host immunogenicity, and recent advances in vector technology have yielded molecules and techniques with increased transfection efficiency (Li and Huang, 2006).

Transposons, also referred to as transposable elements, can move genetic elements directly from one position to another within the genome. Class II transposases proteins employ a cut and paste mechanism of genetic material which allows for stable genomic integration, long-term expression and possible circumvention of an immunogenic response. These features make transposable elements an excellent candidate for efficient non-viral gene therapy (Ivics and Izsak, 2006). Sleeping Beauty (SB) (Ivics et al., 1997) is a member of the Tcl/mariner superfamily of Class II transposable elements and it is active in a wide range of vertebrate cells, from fish to human (Plasterk, 1996, Izsak et al., 2000).

To date, most studies carried out on SB have been performed by simultaneous transfection of a plasmid expressing the transposase together with a transposon donor plasmid (Hollis et al., 2006, Liu et al., 2006). Although efficient, this technique provides a possibility for spontaneous integration of the transposase gene into the host genome, rendering critical risk of chromosomal rearrangements in the affected cell (Wilber et al., 2006), and to circumvent this risk it would be desirable to avoid the transposase DNA source. This has been achieved by delivery of Sleeping beauty transposase (SB-tpase) mRNA (Wilber et al., 2006, Wilber et al., 2007), but in this study our aims were to deliver the transposase protein into cells in vitro.

However, the ability to ferry full length proteins and plasmids across the lipid bi-layer still remains limited by the poor permeability and the selectivity of the cell membrane for large
molecules. Extensive progress has been made in the development of vectors for large molecules, and one promising technique is peptide-mediated translocation by Cell-penetrating peptides (CPPs) (Jarver and Langel, 2006). No suitable definition of CPPs has been formulated, but most, if not all, carry a net positive charge, are less than 30 amino acids long and have the ability to rapidly translocate large molecules into cells. CPPs have been shown to efficiently carry a wide variety of bioactive compounds across lipid bilayers both in vivo and in vitro (EL-Andaloussi et al., 2005). Still, it is unclear which mechanism is responsible for translocation of CPPs and their cargos. Recent studies suggest that the uptake for most CPPs is an energy dependent process with initial binding of peptides to proteolipid on the cell surface followed by endocytosis of peptides (Richard et al., 2003), but there are some reports stating that CPPs translocate into cells in a receptor-and energy independent manner (Tunnemann et al., 2006).

We have evaluated two CPPs, Tat (GRKKRRQPRRPPQ-NH2) (Vives et al., 1997) and M918 (MVTVLFRRLRRACGPPRVRV-NH2) (EL-Andaloussi et al., 2007) for their ability to translocate the SB-tpase protein either alone, or together with a transposon carrying donor plasmid (pT/Neo) across the plasma membrane. The transposon donor plasmid carries a Neomycin resistant gene flanked by two terminal inverted repeats (IRs) designed by Ivics et al. (Ivics et al., 1997). These flanking SB IRs are required for SB transposase mediated transposition (Ivics et al., 1997). Further, we designed and expressed a fusion protein between Tat and SB-tpase in order to obtain a transposon with cell-penetrating properties, Tat- SB-tpase.

We show here uptake of SB-tpase alone and, more imperative, co-uptake of both the transposase and a transposon donor plasmid mediated by the CPP M918. The procedure is a simple one-step method where the protein, plasmid, and peptide are co-incubated prior to cell treatment. Cells are subsequently exposed to the transfection mixture and both the transposase and the plasmid are efficiently translocating the plasma membrane (for proposed scheme see Fig 1).

Material and methods
Peptide synthesis and purification
Peptides were synthesized in a stepwise manner in a 0.1 mmol scale on an automated peptide synthesizer (Applied Biosystems, U.S.A., Model 433A) using t-Boc solid-phase peptide synthesis strategy. tert-Butyloxy carbonyl amino acids (Neosystem, France) were coupled as hydroxybenzotriazole (HOBT) esters to a p-methylbenzhydrylamine resin (Neosystem, France) to obtain C-terminally amidated peptides. The peptides were cleaved from the resin using liquid HF at 0°C for 1 h in the presence of p-cresol and thio-creosol. Peptides were purified using reversed phase HPLC (Gynkotek, Germany) and molecular weight was determined by MALDI-TOF mass spectrometry using Perkin Elmer proTOF™ 2000 MALDI O-TOF Mass Spectrometer (Perkin Elmer, U.S.A.).

Cell culture
CHO cells were grown in D-MEM : F12-glutamax media, and HeLa cells were grown in DMEM-glutamax. Both media were supplemented with 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10 % FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown at 37 °C in 5 % CO2 atmosphere. Media and chemicals were purchased from Invitrogen (Sweden).

Plasmid construction
We used plasmid pET-15b carrying Lac- operone and His-tag sequence, designed for straightforward protein expression and purification (pET-15b with SB-tpase insert was a kind gift from Dr. Zsuzsanna Iszvak, Max Delbrück Center for Molecular Medicine, Berlin, Germany). pET-15b-Sleeping beauty transposase (pET-15b- SB-tpase) was transformed into XL-1 Blue E. coli through heat-shock and plated on to LB-agar plates containing 100μg/ml Ampicillin.

Tat-SB-tpase fusion protein was obtained using forward primer 5’-TTATTTCCCATATGAGGAGAACCGGAGA CAGCGACGGAAGATGGGAAAAATCAAAG AAAT-3’ and reverse primer 5’- GCGGATCCTTCGAGCTATTTTGTAGC ATTTGCTTTAAAT-3’ PCR SB-tpase product was cleaved with restriction enzymes XhoI and Ndel (Invitrogen, Sweden), and ligated into pET-15b, using T4 DNA ligase (Invitrogen, Sweden). Transposon donor plasmid, pT/Neo(Ivics et al., 1997), was also a kind gift from Dr. Zsuzsanna Iszvak, Max Delbrück Center for Molecular Medicine, Berlin, Germany. Plasmids were purified using QIAGEN (Sweden) Plasmid Maxi Kit.
Protein expression and purification

Plasmid pET-15b- SB-tapase was transformed into Rosetta (DE3)pLysoS™ E. coli (Novagen) through heat-shock. Cells were grown in LB-media supplemented with 1 % glucose to OD600 approximately 0.6 at 37 °C. Cells were induced with 0.4mM IPTG for 3 h at ambient temperature.

Cells were lysed in 50mM Na2PO4, 2mM β-Mercaptoethanol 0.1 mg/ml lysozyme, and sonicated. 0.5 M NaCl, 2 mM imidazol was added to the cell-lysate and centrifuged for 15 min at 1,000 g. Supernatant was incubated with Ni-NTA resin (QIAGEN, Sweden) for 1 h at 4°C. SB-tapase protein was eluted using 50 mM Na2PO4, 2 mM β-Mercaptoethanol with increased concentration of imidazol (up to 200mM) and eluate was diluted with equal volumes of 2xPBS to prevent protein from precipitating. Eluates containing SB-tapase was dialyzed against PBS at 4°C over night and protein concentration was determined by Bio-Rad (U.S.A) protein assay kit. By this method, we were able to purify 30 mg SB-tapase from 1 l E. coli culture. The SB-tapase was stable for at least one week when stored at 4°C (analyzed by coomassie stained 10 % standard SDS gel).

Peptide transfection for Western blot and MTT-staining

6x10⁴ CHO cell were seeded in 24 well plates 48 h prior to treatment. Transposase, or transposase and plasmid were incubated for 25 min at ambient temperature in 100 μl PBS at desired concentrations. CPPs were added to the transposase, transposase/plasmid mixture and incubated for an additional 25 min. Cells were treated with the transfection mixture in 200 μl of serum free media for 90 min at 37 °C. 200 μl serum containing media was added to the cells and incubated for an additional 90 min at 37 °C. Cells were then either: i) washed twice with PBS, tryspinized for 15 min and analyzed by Western blot, or ii) left to grow for 24 h and then trypsinized and transferred to 60 mm Petri dishes and were left to grow for 14 days in the presence of 1000 μg/ml G418 (media was changed every 4th day). After 14 days, the cells were stained by MTT.

Western Blot detection of Sleeping Beauty

After induction of protein expression, cells were lysed by freeze-thaw. 10 μg of total protein content was separated on a 10% SDS gel and blotted to a Hybond-N™ membrane (Amersham, Sweden). Membrane was hybridized with anti-SB-tapase antibody (R&D Systems, U.S.A) diluted 1:1000, for 12 h at 4 °C. Secondary antibody, anti-Mouse HRP (Invitrogen, Sweden) was diluted 1:1000 and incubated at ambient temperature for 1 h. Membrane was developed using Enhanced Chemiluminescence substrate (Perkin Elmer, U.S.A). Gel quantification was carried out using Image Gauge V3.46 (Fuji Film). All uptake quantifications are normalized against total amount of Tubulin in the corresponding sample.

Deoxyglucose leakage

50,000 CHO cells were seeded in 24-well plates two days before treatment. Cells were loaded with 0.5 μCi of the glucose analogue, 2-deoxy-D-[1-3H]-glucose (Amersham Biosciences, UK). The glucose analogue is taken up by cells and phosphorylated inside cells by hexokinase. Since the phosphorylated product is unable to pass over intact cell membranes, efflux of radioactivity from the cells is a measure of plasma membrane perturbation. Cells were treated in 300 μl serum free media and 100 μl of the extra-cellular media were analyzed 30 and 60 min after addition of the peptide/protein/plasmid mixture. To get the total radioactivity in each well, cells were finally lysed with Trion X and this lysate was regarded as 100% membrane disturbance. All samples were diluted in 5 ml Emulsifier Safe scintillation liquid (Perkin Elmer, USA) and measured on β-counter (2500TR Packard, Australia). Leakage of untreated cells was defined as 0 % membrane disturbance (mean ±SEM, n=3).

MTT-staining

50 μg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was dissolved in serum free media. The MTT containing media was added to the cells, and cells were then incubated for 60 min at 37°C. Cell colonies that had formed in the Petri dishes were photographed on a light table.

Results and Discussion

We aimed to develop a peptide based delivery method to translocate the SB transposase as a protein together with a transposon donor plasmid into cells in vitro. This would be desirable in order to circumvent the use of DNA as a source for SB-tapase in transposon mediated gene therapy. Here, we have evaluated two different CPPs, Tat and M918, for their
Figure 1: Schematic proposal for cellular uptake of SB and donor plasmid by M918 through complex formation. M918, SB, and pTPNeo are incubated prior treatment to form CPP-protein-plasmid complexes. The cells are exposed to the mixture and both SB and pTPNeo are rapidly translocated across the plasma membrane either as a complex formed outside the cell or by a mutual or two separate pathways.

Figure 2: A) Efficient purification of SB-tetrase from Rosetta E.coli on Ni-NTA resin. Eluted in 50 mM Na2PO4, 2 mM β-Mercaptoethanol, with increasing concentrations of imidazol. 1) Molecular weight marker (Invitrogen). FT Flow through from column, 1) 2 mM imidazol, wash step, 2) 10 mM imidazol, 3) 20 mM imidazol, 4) 100 mM imidazol. 5) 1000 mM imidazol, containing pure Sleeping Beauty transposase. Separation on standard 10% SDS gel. B) Uptake of 1 µg SB-tetrase protein in CHO cells by M918. 6x10⁶ CHO cell seeded in 24 well plates 48 h prior to treatment. Peptide concentration unit in molar and also as ration protein to peptide. Lanes 3-6 are loaded in replicates. 1) Cells harvested without trypsinization, 2) Only Sleeping Beauty transposase 3) 40 µM / 1:500 M918 4) 20 µM / 1:250 M918 5) 10 µM / 1:125 M918 6) 5 µM / 1:62.5 M918. Beneath, the amount of Tubulin (Tub) in every sample is shown. All of the protein uptake yields have been normalized against amount of Tub in the sample. C) Quantification of M918 mediated uptake of SB-tetrase displayed as fold increase compared to only protein. Uptake quantified from one representative Western blot.

ability to deliver the SB-tetrase together with a transposon donor plasmid into cells. Further, the Tat amino acid sequence was introduced N-terminally to SB-tetrase with the purpose to generate a transposase with cell-penetrating properties, Tat-SB-tetrase. Tat is to date the most well studied CPP and it has been extensively used as a vector for numerous of bioactive compounds and Tat fusion proteins has been utilized to facilitate cellular uptake of proteins in vivo and in vitro (Nagahara et al., 1998). M918 is a novel CPP which has been shown to have great potential as a peptide vector for a wide range of compounds, including DNA, siRNA and proteins (EL-Andaloussi et al., 2006).

A His-tag was introduced to the N-terminal of SB-tetrase by introduction of the SB-tetrase cDNA into the pET-15b plasmid, and the protein was purified on a Ni-NTA column. To get high levels of purified protein (Fig. 2A), the expression was performed at ambient temperature for 3-4 h using 0.4 mM IPTG. If higher temperature or longer expression time was employed, the purified protein yields decreased dramatically.

Tat-SB-tetrase fusion protein showed no signs of translocation in CHO cells. This result is unexpected, since Tat fusion proteins, as previously mentioned, have been reported to readily translocate into cells both in vivo and in vitro. When co-incubation of Tat, SB-tetrase, and donor plasmid was performed, no uptake of SB-tetrase or the transposon plasmid could be detected (Fig 3B, 4B). The low protein uptake is possibly due to electrostatic repulsion between
the highly cationic Tat (for sequence, see introduction) and SB-tpase (theoretical pl 10.6).

Still, Tat-mediated cellular uptake was monitored by western blot and low yields of the transposase might have translocated the membrane, but not in sufficient amounts to be detected. A small number of cell colonies could be detected using MTT staining 14 days post TAT-delivery (Fig 4B). However, being so few, this colony formation might be an effect of spontaneous donor plasmid uptake and not a result of CFP mediated translocation. M918 conversely displays efficient uptake of SB-tpase in both CHO and HeLa cells (Fig 2B and data not shown). Although the peptide is cationic like Tat, M918 has more hydrophobic amino acid residues that can contribute to protein interactions (for sequence, see introduction), facilitating complex formation between the peptide and the SB-tpase.

Surprisingly, translocation by M918 seems to be more efficient at lower CPP concentrations. Uptake is increased about 4.5-fold at 1:125-250 ratios (10-20 μM peptide concentration) compared to protein alone, and decreases with and higher CPP concentrations (Fig 2B, C).

Further, higher CPP concentrations cause slight cytotoxic side effects, Fig 3D. As seen in figure 2B:1 and 3A:1, trypsinization is very important in order to degrade extra cellular proteins and thereby avoid false positive protein uptake. SB-tpase seems to be taken up by the cells alone (Fig 2B:2 and 3A:2). This is probably an effect from interactions between the negatively charged plasma membrane and the positively charged transposase, and does not necessarily indicate cellular uptake.

When performing M918 mediated co-uptake with both the SB-tpase and the transposon donor plasmid, the transposase was taken up only by cells. Similar concentrations and ratios as in sole protein uptake seem to be most efficient. Again, lower concentrations appear to translocate more protein than higher concentrations. Although uptake of the transposase is most efficient at a 1:125 ratio (SB-tpase:CPP), colony formation 14 days post transfection is most apparent at 1:500 ratio (40 μM peptide concentration) (Fig 4C). It is important to bear in mind that two entities with highly divergent properties are transported into the cells simultaneously, and that one entity is taken up does not consequently imply that the
other is as efficiently introduced to the cell. It is also apparent that M918 together with SB-tpsa and pT/Neo donor plasmid causes a slight membrane perturbation (about 20 % deoxyglucose leakage) after 60 min at 40 μM, which possibly contributes to the efficient plasmid delivery and, consequently Neomycin (G418) resistance, 14 days post transfection, Fig 4C. This is not unexpected, since uptakes of macromolecules with the size of plasmids are expected to create some membrane disturbance when translocated across lipid bi-layers.

Neither Tat nor M918 were by themselves able to transport the pT/Neo donor plasmid into cells at concentrations up to 40μM (data not shown). Nevertheless, it is evident that M918 is capable to deliver both SB transposase and pT/Neo donor plasmid simultaneously into cell cultures in vivo.

Concluding Remarks

This is the first study where two so vastly divergent molecules as a cationic protein and a plasmid, are simultaneously introduced into cells by a peptide vector. As discussed previously, to deliver SB transposase protein and transposon donor plasmid concurrently via CPP mediated delivery have several advantages over available methods. The most obvious being the use of protein as a source for the transposase instead of DNA. This modification eliminates the risk of spontaneous integration of the transposase gene into the affected cells genome, which could have devastating effects in vivo. In this study we have shown that M918 is a potent vector for co-uptake of both SB-tpsa and transposon donor plasmid in vivo. The simple one-stop peptide mediated uptake might be a suitable method for safe and efficient non-viral gene therapy.

Acknowledgements

This work was supported by grants from European Community: LSHB-CT-2005-018961, and Swedish Research Council: VR-NT

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