Delivery of Bioactive Molecules into the Cell: The Trojan Horse Approach

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Running Title: Molecule delivery across biological membranes

Trojan horse

huge, hollow wooden horse constructed by the Greeks to gain entrance into Troy during the Trojan War. The horse was built by Epeius, master carpenter and pugilist… Encyclopædia Britannica

Abstract

In recent years, vast amounts of data on the mechanisms of neural de- and regeneration have accumulated. However, only in disproportionally few cases has this led to efficient therapies for human patients. Part of the problem is to deliver cell death-averting genes or gene products across the blood/brain barrier (BBB) and cellular membranes. The discovery of Antennapedia-mediated transduction of heterologous proteins into cells in 1992 and other "Trojan horse peptides" raised hopes that often-frustrating attempts to deliver proteins would now be history. The demonstration that proteins fused to the Tat protein transduction domain (PTD) are capable of crossing the BBB may revolutionize molecular research and neurobiological therapy. However, it was only recently that PTD-mediated delivery of proteins with therapeutic potential has been achieved in models of neural degeneration in nerve trauma and ischemia. Several groups have published the first positive results using protein transduction domains for the delivery of therapeutic proteins in relevant animal models of human neurological disorders. Here, we give an extensive review of peptide-mediated protein transduction from its early beginnings to new advances, discuss their application, with particular focus on a critical evaluation of the limitations of the method, as well as alternative approaches. Besides applications in neurobiology, a large number of reports using PTD in other systems are included as well. Since each protein

requires an individual purification scheme that yields sufficient quantities of soluble, transducible material, the neurobiologist will benefit from the experiences of other researchers in the growing field of protein transduction.

1 Delivery into Cells and across the BBB – Why Trojan Horse Trickery is in Demand

On the one hand, drugs directed at intracellular target sites need to be sufficiently polar to be easily administered and well distributed in the organism. On the other hand, such substances also need to be hydrophobic enough to transverse the lipid bilayer of the cell. Thus, many drug leads fail to make it into the clinical trials because they do not fulfil those physical properties. To circumvent problems of bioavailability, substances often need to be extensively modified (Rait et al., 2000) or their formulation needs to be

fine-tuned, e.g. for substances that exhibit a low solubility in water (Terwogt et al., 1997). Such problems apply not only to chemically synthesized substances, but for potentially therapeutic proteins as well. Cerebral drug delivery is faced with many additional obstacles due to the characteristics of the BBB, in particular after trauma or stroke (Lo et al., 2001, for review).

Methods like electroporation, chemical transfection, or microinjection can be applied *in vitro*. However, they often damage the cells, and the amount of compound or protein delivered is not easily controlled. Some other methods to gain access into the cell and the brain are briefly discussed in the following paragraphs.

1.1 Naked DNA

Although application of naked plasmid DNA leads to gene expression in mouse muscle (Wolff et al., 1990), liver (Hickman et al., 1994), skin (Hengge et al., 1996) and some other tissues as well as tumors (Vile and Hart, 1993) *in vivo*, there are many problems associated with the method (Byrnes et al., 2001). These include the necessity of repeated injections of large amounts of DNA and low expression efficiency. Nevertheless, significant improvements in formulation and application have been achieved, which could make the method a viable choice for gene therapeutic developments (Herweijer and Wolff, 2003, for a recent review).

Curiously, nucleic acids can not only penetrate cells and tissues, but they can even enhance the uptake of other cargo: Certain polynucleotides increase the delivery of proteins across cellular membranes (Ryser et al., 1975).

1.2 Liposomal delivery

Liposomal cDNA delivery has been achieved in skin (Jeschke et al., 2001) and dopaminergic ventral mesencephalic tissue and Clarke's nucleus to improve cell survival (Bauer et al., 2002) as well as in tumors to inhibit their growth (Tran et al., 2003). Cationic lipids form condensed DNA particles, which are protected against degradation and increase their affinity for cell membranes, enhancing intracellular uptake. Usually, the method lacks efficiency *in vivo*, due to binding to blood proteins or to tissues. Polycationic carriers to deliver DNA are discussed in brief further below.

A combination of liposomes with cell-penetrating peptides has also been tried. In some studies, it did not result in an increased effect of a doxorubicin cargo (Tseng et al., 2002). In other experiments, it further confirmed the versatility of Tat-mediated trafficking (cf. 2.14.2): When multiple Tat peptides are attached to the particle surface, plain and PEG-coated liposomes of 200 nm diameter are delivered into various cell types, where their interior is released within hours (Torchilin and Levchenko, 2003, for a recent review; Torchilin et al., 2001).

Others have efficiently delivered a reporter gene into the primate brain by intravenous delivery (Zhang et al., 2003c). They encapsulated the expression plasmid DNA into liposomes, which were coated with polyethylene glycol (PEG). An insulin-receptor-specific monoclonal antibody was tethered to the PEG, which mediates the transcytosis of the "package" across the BBB and endocytosis across the neuronal plasma membrane. Employing a similar strategy, but using an antibody directed against the transferrin receptor instead of the insulin receptor, a plasmid encoding tyrosine hydroxylase was delivered into the striatum of adult rats, alleviating symptoms of experimental Parkinsonian symptoms induced by 6-hydroxydopamine (Zhang et al.,

2003a). Gene delivery into the eye using the liposome-PEG module was also achieved by coupling it to either the transferrin (Zhu et al., 2002) or the insulin receptor antibody (Zhang et al., 2003b). Methods to ferry proteins across the BBB using transferrin receptor-mediated transcytosis are discussed in 1.3.

1.3 Delivery or Therapeutics by coupling to antibodies and receptor ligands

The transferrin receptor undergoes receptor-mediated transcytosis across the BBB, thereby mediating the transport of its substrate, transferrin (cf. 1.2). Besides transferrin, peptidomimetic monoclonal antibodies can bind to an epitope on the receptor and trigger its transcytosis. The coupling of therapeutics to these peptidomimetics is another promising approach to overcome the BBB (Pardridge, 2002b; Pardridge, 2002c; Pardridge, 2002d, for extensive review). For instance, by coupling bFGF (Song et al., 2002; Wu et al., 2002) or BDNF (Pardridge, 2002a) to a transferrin receptor binding antibody, a significant protection against brain infarct was achieved after intravenous application.

Polyreactive anti-DNA monoclonal antibodies are effective vectors for DNA plasmids, proteins, and other compounds across the cellular membrane (Avrameas et al., 1998). For some of those antibodies, proteoglycans may be involved in the mechanism of their transduction (Avrameas et al., 2001). A 30 amino acid sequence, derived from such an antibody, enhances transfection of DNA when it is linked to a polylysine tail (Avrameas et al., 1999). *In vivo*, fluorescein-linked antibodies caused labeling of 61% of peripheral blood lymphocytes after injection into mice (Ternynck et al., 1998).

Chimeras between a specific receptor ligand and a cytotoxin, like a ribosome inactivating fragment or a bacterial toxin, are internalized into the cell by receptor-

mediated endocytosis (Lappi and Baird, 1990). For example, a recombinant FGF2-saporin fusion protein showed tumor growth and metastasis inhibiting properties *in vivo* (Lappi et al., 1994; Ying et al., 1994). Basic fibroblast growth factor (FGF2), when conjugated to polylysine, also mediates the efficient transfection of plasmid DNA (Sosnowski et al., 1996) or *Pseudomonas* exotoxin (Gawlak et al., 1993) into cultured cells. Of course, this endosomal incorporation only targets cells expressing the FGF receptor. aFGF also penetrates cellular membranes (Wiedlocha et al., 1995). Depending on the experimental conditions, aFGF and bFGF can be used as cargo or as delivery vector. However, the bFGF derived transduction peptide is transduced three times less efficient than penetratin (Waizenegger et al., 2002). (Penetratin is an Antennapedia homeodomain-derived peptide, cf. paragraph 2.7.) Interferon γ, another cytokine with membrane-penetrating properties, has an NLS, and external protein is rapidly transduced into the nucleus *in vitro* (MacDonald et al., 1986). The translocation of bFGF, aFGF and interferon-γ and many other factors has recently been excellently reviewed (Olsnes et al., 2003). Although these proteins have rarely been used to ferry other molecules into cells, the mechanism by which they are internalized is of interest. Some aspects of the transduction mechanism, e.g. by FGF-1, however (Du et al., 1998), is distinct from transduction by PTDs like those derived from the Antp (paragraph 2.7) or the Tat protein (paragraph 2.14) which are discussed below.

1.4 Viral vectors

Virus-mediated gene delivery into neurons has been used successfully *in vitro* and *in vivo*, for instance with adenoviral vectors (Akimoto et al., 1999; DiPolo et al., 1998; Kügler et al., 2003a; Matsuoka et al., 2000; Yukawa et al., 2000), herpes virus

(Antonawich et al., 1999; Lawrence et al., 1997; Linnik et al., 1995; Yamada et al., 1999), lentiviruses (Kordower et al., 2000) or adeno-associated virus (Kügler et al., 2003b; Shimazaki et al., 2000). Unfortunately, murine leukemia virus, one of the most promising viral vectors to treat human diseases for its safety record in clinical trials, can only be transduced into cells that are still dividing, which is rarely the case in the adult CNS. This problem can be partly overcome by applying growth factors like bFGF to stimulate CNS cell division (King et al., 2000). Lentiviruses or adenoviruses do not share this limitation, but often trigger immunological responses. Recombinant adeno-associated viruses are another auspicious system, and have been administered by intravitreal injection into the adult brain with impressive results (Hennig et al., 2003, and others).

While such viral vectors have a wide range of cell targets, serious safety concerns exist regarding their application in gene therapy (Check, 2002; Connolly, 2002; Cornetta et al., 1991; Hansen and Pedersen, 2002; Marshall, 2002), although substantial experience in toxicity mechanisms, vector systems, delivery methods, and clinical protocols has been gained in the last two years (Jooss and Chirmule, 2003; Rainov and Ren, 2003; St George, 2003; Thomas et al., 2003, for recent reviews)

Viruses tend not to disperse well in target tissue and often do not express their gene for long periods (Eck, 1999, for review). In many viruses, the size of the cDNAs that can be introduced is limited. Furthermore, in acute neurological disorders, viral vectors might not express sufficient amounts of the recombinant protein in a short period to be protective, because of a compromised host cell metabolism.

1.5 Peptides that mimic the activity of large factors

Some small peptides can readily enter and exit the brain via the BBB in a passive, temperature and Na^+ independent manner and display CNS effects (Greenberg et al., 1976). Thus, peptides are attractive candidates for future drug development (Gozes, 2001). However, it is unlikely that it will be possible in many cases to design deliverable peptides that retain the activity of the entire protein. Permeability of small proteins is also influenced by age and disease (Kleine et al., 1993), and may trigger an immune response, which further complicates this approach.

1.6 Compounds that mimic peptides

“Peptidomimetics” are oligomers that mimic peptide primary structure through use of amide bond isosteres and/or modifications of the native peptide backbone (Patch and Barron, 2002, for review). Such biomimicry is useful to imitate the activity of antibacterial peptides, viral proteins, or ligands for MHC-II, circumventing the problems of low oral bioavailability, potential immunogenicity and poor metabolic stability of peptides *in vivo*.

1.7 Disruption of the BBB

In addition to overcoming the cellular membrane barrier, a compound that is supposed to exert its action in the brain also must be able to cross the BBB. For many potential pharmaceuticals, such as neurotrophins, the capillary endothelial wall of the BBB is not entirely permeable (Pan et al., 1998; Poduslo and Curran, 1996), and brain uptake that has been measured could, in part, be due to experimental artifacts (Pardridge, 2001). Alternatively, the BBB can be reversibly disrupted by osmotic shock to allow treatment with pharmacological agents (Neuwelt et al., 1980). This treatment, however, bears the high risk of neuronal damage. Another approach, the mechanical

delivery of proteins into the brain, has, in many cases, been hampered by serious drawbacks (Aebischer and Ridet, 2001, for review).

1.8 Transplantation of cells into the brain

Years ago, genetically modified cell grafts were transplanted into the CNS, where they secrete growth factors and survive for periods of up to one year (Nakahara et al., 1996; Tuszynski et al., 1994). Neural stem cells are amenable to gene transfer and survive transplantation into the animal brain (Gage et al., 1995; Sabate et al., 1995; Suhonen et al., 1996). Many recent reviews on the use of stem cells in brain research are available (Bjorklund et al., 2003, and others; Gerlach et al., 2002; Isacson, 2003; Le Belle and Svendsen, 2002; Storch and Schwarz, 2002).

Ex vivo gene therapy using encapsulated cells has also been reviewed (Aebischer and Ridet, 2001). Genetically engineered NIH 3T3 fibroblasts, encapsulated in a biocompatible polymer, have been transferred into the rat vitreous cavity of in a model for retinal dystrophies. Growth factors secreted through the permselective membrane delay photoreceptor degeneration, while the fibroblasts are protected from the attack by the host immune system (Uteza et al., 1999).

2 PTDs – The Application of Trojan Horse Technology

It is known for a long time (Ryser, 1968, for review) that polybasic proteins like poly-ornithine and poly-lysine (Ryser, 1967) and histones (Ryser and Hancock, 1965) highly enhance protein uptake. However, the knowledge and application of the uptake-enhancing properties by smaller basic peptides has developed more recently.

2.1 Protegrin derivatives – SynB peptide vectors, tachyplesins, magainins and dermaseptins

Protegrins are small, 16-18 amino acid long arginine- and cysteine-rich peptides with two intramolecular disulfide bonds. They exhibit a strong antimicrobial activity (Kokryakov et al., 1993). In structure, they are similar to defensins and tachyplesins (Aumelas et al., 1996). Protegrins are capable of forming anion channels in *X. leavis* oocytes (Mangoni et al., 1996) and planar lipid bilayers and liposomes (Sokolov et al., 1999). An analogue called SynB1 does not disrupt the cellular membrane, but is able to deliver doxorubicin (Rousselle et al., 2000; Rousselle et al., 2001) and benzylpenicillin (Rousselle et al., 2002) across the BBB, when applied by *in situ* brain perfusion or, in case of doxorubicin, also through the tail vain. However, in these studies, it was not shown that the concentrations achieved within the brain were sufficient to display any anti-tumor or anti-bacterial activity. *In vitro,* the vectorized doxorubicin did show cytotoxic activity in multi-drug-resistant erythroleukemic cells (Mazel et al., 2001).

Tachyplesins, isolated from hemocytes of horseshoe crabs (Nakamura et al., 1988) and magainins, isolated from Xenopus skin (Zasloff, 1987) can form pores and transduce biologic membranes. Most of the host defense peptides consist of apolar and charged residues arranged in a way that allows the formation of amphiphilic structures (Ojcius and Young, 1991).

Tachyplesins and magainins are mainly produced by plants and animals as an anti-microbial defense system. Thus, they are quite selective for prokaryotic membranes (Matsuzaki, 1999, for a review on the mechanism of peptide/lipid interactions), and their use as trojan horse peptides crossing eukaryotic cell membranes was thought to be quite limited. However, they do cross mammalian membranes and can even take a

cargo with them (Takeshima et al., 2003). Translocation is enhanced by increasing the positive charge of the initially formed pore, which is thereby destabilized (Matsuzaki et al., 1997). Taking advantage of that knowledge, efficient transduction of a modified magainin into cells *in vitro* was achieved (Takeshima et al., 2003). However, cytotoxicity is still a concern.

Dermaseptins are a family of antimicrobial peptides, which are considered to be part of the defense systems of some South American tree frogs. *In vitro*, dermaseptins can lyse microorganisms including bacteria, protozoa, yeast, and filamentous fungi by destabilizing their membranes. Similar to the Tat-derived PTDs (chapter 2.14), there is a strong correlation between their degree of helicity and their biological activity. The helical structure of the peptides organizes the molecules such that they show amphipathic hydrophobicity and charge distribution which seem to be responsible for many aspects of their biological functions (Ghosh et al., 1997). Fluorescently labeled dermaseptin S4 can even penetrate mammalian cells in culture at nontoxic concentrations, (Hariton-Gazal et al., 2002) and accumulates in the cytoplasm. Linking S4 to a nuclear localization signal can confer its import into the nucleus (Hariton-Gazal et al., 2002).

2.2 Bacterial and plant toxins

The translocation domains of diphtheria toxin (Stenmark et al., 1991; Wiedlocha et al., 1994), the anthrax toxin (Ballard et al., 1998; Blanke et al., 1996) and *Pseudomonas* exotoxin A (Prior et al., 1992) are also able to transduce peptides into cells. However, with few exceptions (Liu et al., 1999b), only the delivery of small peptides has been achieved by them. The large family of bacterial toxins that punches holes into eukaryotic cells, like perfringolysin (Rossjohn et al., 1997), are not useful as delivery tools due to their toxicity. The mechanism by which those and a number of other

plant and bacteria-derived toxins penetrate the membrane has been reviewed recently (Olsnes et al., 2003).

2.3 Transportan

Transportan is a synthetic peptide sequence generated by fusing the neuropeptide galanin N-terminal fragment with the wasp venom peptide mastoparan, with an extra lysine inserted between the two peptides (Pooga et al., 1998a). Similar to other PTDs, transportan uptake was reported to take place at 4°C and in the presence of known endocytosis inhibitors. However, before detection of the protein, the cells were fixed in PFA (cf. discussion in paragraph 5.1), which might lead to an overestimation of the actually transduced protein. Keeping that cautionary note in mind, transportan mediates the transduction of GFP and of large protein complexes *in vitro* (Pooga et al., 2001). Transportan and derivatives pass epithelial cell layers more efficiently than the Antennapedia-derived Antp peptide (Lindgren et al., 2003). Other examples of transduction mediated by transportan are given in Table 1.

The transduction kinetics may in some instances be even faster and higher intracellular peptide concentrations may be reached as compared to Tat and Antp PTDs (Hallbrink et al., 2001; Lindgren et al., 2003). However, the transportan peptide is not inert, as can be expected from the widespread distribution and function of galanin and its receptor in the nervous system (Kask et al., 1997). For instance, it inhibits GTPase activity, although shorter derivatives (Soomets et al., 2000) or those with two amino acid substitutions (Pooga et al., 1998b) with a presumed lesser such enzymatic activity have been designed.

2.4 The hydrophobic (h)-region of signaling sequences

PTDs have been derived from the h-region of the 16 amino acid signal sequence of Kaposi fibroblast growth factor (FGF-4) (Delli Bovi et al., 1987). Different from most other peptides described in this review, these sequences are hydrophobic, rather than positively charged. It is probably the hydrophobic moment, not the amino acid sequence, that contributes to their translocational activity (Ahn et al., 1994).

Carrying a functional cargo, they have been used *in vitro* to inhibit subcellular traffic of NF–κB (Lin et al., 1995; Torgerson et al., 1998), to examine signal transduction pathways (Chang et al., 2000a; Rojas et al., 1998; Rojas et al., 1996) and mechanisms of nuclear localization (Torgerson et al., 1998); as well as for the delivery oligonucleotides (Lo et al., 2001). The method for use of such peptides has been described in detail (Chang et al., 2000b) and their generation recently simplified (Lundberg and Johansson, 2002). Similar to other PTD mediated cellular import, Kaposi FGF-mediated protein transduction was reported to be an energy-independent, non-endocytic process (Dokka et al., 1997). The sequence can be used to ferry large proteins across cellular membranes (Rojas et al., 1998).

A very potent method to examine mammalian gene function was employed by using the FGF-4-translocation sequence to transduce enzymatically active Cre proteins in vitro in a variety of cultured cells and *in vivo* by intraperitoneal injection (Jo et al., 2001). However, Kaposi-FGF derived membrane translocating sequences exhibit a 3 - 4 fold lower import efficiency as compared to derivatives from the Antennapedia homeodomain (Fischer et al., 2002). Others (Kelemen et al., 2002) even reported no transduction of Kaposi FGF linked peptides, while the same peptides linked to the Antennapedia or the Tat PTD were transduced efficiently. Besides the Kaposi FGF

signal sequence, the integrin β_3 precursor (CD61 antigen) signal sequence has also been used to render peptides cell penetrating and to test their function in cell adhesion (Liu et al., 1996). In some cases, however, that sequence appears to interfere with normal protein function in fusion proteins (Kardinal et al., 2000). The Caiman crocodylus Ig(v) light chain, again an apolar peptide, has been used as a delivery vector for oligonucleotides (Chaloin et al., 1998).

2.5 VE-cadherin-derived cell-penetrating peptide

An 18-amino acid peptide derived from the cell adhesion molecule vascular endothelial cadherin, can carry a fluorophore or a 67 kDa protein into endothelial and melanoma cell lines (Elmquist et al., 2001). Interestingly, this transport can occur again at 4°C and thus independent of the endocytotic pathway. Its D-analogue, which is protease resistant, is transduced too, suggesting that receptor binding is not necessary (Elmquist and Langel, 2003). This does not exclude the possibility that the peptide does have a receptor-binding site.

2.6 Loligomers

Loligomers are branched peptides. Each branch is derived from the import signal and the nuclear localization signal of the SV40 large T antigen (Kalderon et al., 1984a; Kalderon et al., 1984b), and the different branches are connected via lysine amino acids using solid phase automated synthesis (Sheldon et al., 1995). Such oligomers are rapidly incorporated into CHO cells *in vitro*, by an adsorptive endocytosis mechanism. Nuclear uptake is energy dependent (Sheldon et al., 1995). Loligomers deliver photoactivated cytotoxic substances (Bisland et al., 1999) and cytotoxic T-lymphocyte epitopes onto the surface of antigen presenting cells (Kawamura et al., 2002). They

have also been used as carriers for a number of reporter gene DNAs in eukaryotic cells (Singh et al., 1999). However, although most cells were transfected, the resulting reporter gene expression was disappointing, a problem that will be discussed below in paragraph 4. Another concern is the toxicity of the peptide.

2.7 Derivatives of the Antennapedia transcription factor and other homeodomain proteins

The first cargo transduction using a PTD was achieved using the homeodomain of Antennapedia (Antp) (Perez et al., 1992). Antp is transduced into many cells, including neurons (Bloch-Gallego et al., 1993; Joliot et al., 1991a; Joliot et al., 1991b; Le Roux et al., 1993). Its third helix, which is a DNA-binding region of the Antp transcription factor, is sufficient to mediate transduction into cells (Derossi et al., 1994; Prochiantz, 1999, for review) and has been named penetratin-1 (Derossi et al., 1998a). Even the C-terminal 7 amino acids transduce into cultured cells, with the basic residues being the most critical for efficient transduction (Fischer et al., 2000).

Penetratin-mediated protein delivery has been used to elucidate mechanisms of cell cycle progression (Lin et al., 2000), to induce apoptosis (Holinger et al., 1999, Herbert, 2000 #1102; Li et al., 2002) or to enhance the efficacy of apoptosis-inducing drugs *in vitro* (Bres et al., 2002). In combination with liposomes it was used to prime cytotoxic T-cells (Chikh et al., 2001). Other studies used it to modulate a variety of cell signaling pathways (Dunican and Doherty, 2001, for review). These and further applications are listed in Table 1. The use of CDK inhibitors, coupled to the Antp PTD, has been excellently reviewed (Fischer and Lane, 2000; McInnes et al., 2003). Even lymphocytes, which are difficult to transfect, are penetrated by the homeobox protein (Fenton et al., 1998).

With regard to applications in neuroscience, penetratin enhanced doxorubicin uptake into mouse brain when applied by *in situ* brain perfusion using serum-free medium (Rousselle et al., 2000). However, when directly injected into the striatum or the ventricles, fluorescein-tagged penetratin triggered large neurotoxic lesions and an inflammatory response (Bolton et al., 2000) and only spread near the lesion site caused by the protein. As internalization of the Antp homeobox domain might be dependent on PSA N-CAM (Joliot et al., 1991b), neurons might be better suited for Antp-mediated transduction rather than fibroblasts (Joliot et al., 1991b, and Table 1) and glial cells (Joliot and Prochiantz, 2004).

In general, applications of Antp fusion proteins *in vivo* have been rare (see Table 1). The Antennapedia PTD works well for fusion proteins less than 100 aa, while for others, the transducibility is hard to predict and seems to depend also on structure (Derossi et al., 1998a; Joliot and Prochiantz, 2004). Linked to dsDNA, Antp looses it transducing abilities (Derossi et al., 1998a). Moreover, at high concentrations (above a few µM) the $Antp_{43-58}$ peptide can induce cell lysis (Drin et al., 2003). However, this toxicity depends on the cell type examined.

Both the Antp as well as the Tat domain (see Chapter 2.14) efficiently enhanced adenoviral transfection *in vitro* and *in vivo* (Gratton et al., 2003). To that end, virus particles were just preincubated with the basic peptides in serum free medium prior to cell infection. Antp peptide has also been used to promote bacteriophage uptake into cells (Joliot and Prochiantz, 2004). Thus, there are instances in which the Antp peptide enhances cellular transduction even of very large particles. Under certain conditions, penetratin-mediated transduction may be even more efficient than transduction mediated by the Tat PTD (Li et al., 2002). Together with the Tat peptide (see Chapter

2.14), penetratin is the PTD that has been used most successfully, which is already suggested by the sheer number of studies using either vector (Table 1). This success may be due to the fact that both Tat and penetratin are natural sequences derived from proteins that transfer between cells, and thus their transducing abilities might have been improved in the course of evolution.

The pancreatic homeoprotein PDX-1, which contains a PTD similar to the Antp PTD, is transduced into pancreatic islands and pancreatic ducts *in vivo* (Noguchi et al., 2003). It enhances insulin production, which may be used for therapeutic purposes without requiring gene transfer. A similar application was employed to increase the expansion of hematopoietic stem cells (Amsellem et al., 2003). The homeoprotein HOXB4 was produced by a mouse stromal cell line, from where it transferred into cocultured human hematopoietic cell populations, favoring their expansion.

It has been suggested that the homeobox transcription factors Engrailed 1 and Engrailed 2 may act as polypeptidic intercellular messengers, as they can be secreted and internalized by live cells by mechanisms distinct from those known for classical transport (Joliot et al., 1998). Interestingly, its intercellular transfer is regulated by phosphorylation of the transduction domain (Maizel et al., 2002), a feature that has, to our knowledge, not been examined for the other transducible proteins described in this review. The homeodomain of Engrailed as well the homeodomain of Fushi-tarazu are both able to transduce a 238 amino acid long green fluorescent protein into cells *in vitro* (Han et al., 2000).

2.8 The herpes simplex virus type 1 (HSV-1) VP22 structural protein

HSV-VP22 forms part of the viral tegument (Murphy and Murphy, 1999, for review). It is secreted from the cells in which it is produced and enters surrounding other

cells, where it reaches the nucleus (Elliott and O'Hare, 1997; Elliott and O'Hare, 1999). A region of 40 amino acids in the C-terminal region of the protein is responsible for this activity. VP22 packages and ferries mRNA from infected cells to uninfected cells, thereby setting up the latter for an attack by the virus (Sciortino et al., 2002).

Even when fused to other peptides and proteins, the VP22 protein retains its ability to cross membranes. It has thus been used to deliver functional protein into the nuclei of other cells even *in vivo* (Dilber et al., 1999). VP22-enhanced protein trafficking has been used to facilitate cell death induced by ganciclovir (Liu et al., 2001a); to examine mechanisms of apoptosis *in vitro* (Morris et al., 2002); to enhance the efficiency of anti-tumor vaccination *in vivo* (Cheng et al., 2002) and in the many examples provided in Table 1.

To enhance the spread of proteins delivered by viral infection, recombinant VP22 fusion proteins have been cloned into adenoviral vectors, which resulted in an increased effect of the expressed protein *in vitro* (Wybranietz et al., 2001), and a better distribution within the retina (Cashman et al., 2002).

Most of the work employing protein delivery by VP22 involves expression in cells, from where it is spread into the nuclei of surrounding cells. However, some investigators did not observe such a spread of the protein (Fang et al., 1998) and found it inefficient to deliver purified fusion proteins (Liu et al., 1999b). When a GFP-VP22 fusion protein was expressed in four different cell lines, it could be readily detected in the transfected cells; however, fluorescence was never observed in live potential recipient cells (Aints et al., 1999). However, GFP could be detected after methanol fixation, which makes it likely, to our mind, that the reported bystander effect observed in some experiments using the VP22 protein ferry might be due to fixation artifacts (see discussion below, 5.1).

Moreover, the VP22 protein approach vitiates many of the advantages of the direct PTD systems, as it requires first to transfect the producer cells with a cDNA, and then to wait for many hours until they express and secrete the protein in sufficient amounts. In addition, the transduced protein is mainly localized in the cell nucleus, which is not desired for many applications. The HSV-VP22 protein production system is commercially available (Voyager™Protein Production Kit, Invitrogen™ life technologies).

2.9 PreS2-domain of the hepatitis-B virus (HBV) surface antigen

The PreS2 protein derived from HBV also displays transducing abilities (Oess and Hildt, 2000). It is not only a structural protein for the viral envelope, but, quite similar to the HIV-Tat protein (see below), also a transcriptional activator. Again, like Tat, the protein domain contains an amphiphatic α-helical protein transduction domain, which transduces the viral activator protein as well as fused proteins into HeLa or HEK293 cells, but also *in vivo* after intravenous application. Interestingly, the PreS PTD has an isoelectric point of 6.01, and thus is not basic like most other known PTDs. In the publication by Oess and Hildt, Western blot analysis and immunochemistry demonstrated transduction. Different from this report, another study shows that PreS2 of the HBV was inefficient to deliver certain peptides into cells, while the same peptides linked to the Antennapedia or the Tat PTD were readily transduced (Kelemen et al., 2002). Further studies are required to clarify that contradiction.

2.10 Pep-1

A 21 amino acid peptide consisting of a hydrophobic and a lysine-rich NLS with an intermittent spacer domain was designed. It internalizes into HS-68 fibroblastic cells and a variety of other cell lines (Morris et al., 2001) and was not toxic on all cells tested.

Surprisingly, it tightly associated with four different proteins and with antibodies by simply mixing and incubation for 30 min, subsequently resulting in intracellular delivery. In another study by the same research group, Pep-1 was used to deliver SDS-PAGE isolated proteins (Taylor and Fernandez-Patron, 2003). Unfortunately, in this study, cell penetration was assessed after methanol fixation, a method prone to artifacts, as discussed below (5.1). The Pep-1 delivery system is commercially available as “Chariot” kit (Active Motif, Carlsbad, CA). To our knowledge, since the inaugural paper, the method has only been applied in a few studies. Note that the Pep-1 described here is not related to the 12-mer HA (hyaluronan)-binding peptide (GAHWQFNALTVR) termed Pep-1 (Mummert et al., 2000).

2.11 Glutathione S-Transferase (GST)

Recently, it was serendipitously found that GST from Schistosoma japonicum also exhibits cell translocating properties (Namiki et al., 2003). This finding still needs to be independently confirmed, and the applicability of the peptide *in vivo* also remains to be tested.

2.12 Other basic peptides

It has been known for a long time that poly-L- and poly D-lysine facilitate transduction of drugs (Ryser and Shen, 1980), oligonucleotides (Wolfert and Seymour, 1996) or proteins (Shen and Ryser, 1978) into cells *in vitro*. Poly-L-lysine (Fisher et al., 2000), or poly-L-lysine linked to various cell-targeting ligands (Midoux et al., 1993; Wagner et al., 1990; Wu and Wu, 1987) facilitate uptake of DNA and fluorescently tagged antisense oligonucleotides (Leonetti et al., 1990) *in vitro*. Uptake of adenoviruses is also enhanced by poly-L-lysine (Mulders et al., 1998).

Other basic peptides have also been successfully tested for their ability to transfect DNA into cells (McKenzie et al., 2000; Wadhwa et al., 1997). D- and L-analogs derived of HIV-Tat, HIV-1 Rev, HTLV-II Rex, BMV Gag, FHV coat proteins, and the DNA-binding segments from c-Fos, c-Jun and GCN4 proteins have been compared in their ability to transduce cellular membranes (Futaki et al., 2001b). All of these peptides were transduced, although they share little homology, apart from a number of arginine residues. Even peptides consisting only of branched chain arginines can be transduced (Futaki et al., 2002). Vpr, an HIV protein which accelerates viral replication, is also able to mediate transduction of β-galactosidase and other cargo (Sherman et al., 2002).

By database searches, Futaki and coworkers (Futaki et al., 2003) have found a large number of peptides that contain arginine clusters. Which of those proteins have membrane-translocating abilities remains to be investigated. Certainly, it is useful to have an eclectic collection of peptides to choose from for specific applications, because a single membrane-penetrating peptide that serves as a solution for all kinds of gene delivery problems does not exist. Rather, the peptide best suited for each case needs to be determined empirically (Wagner, 1999, for review).

2.13 The 70 kDa heat shock protein (Hsp70)

Hsp70 has been shown to deliver proteins into a variety of cells (Fujihara and Nadler, 1999). This protein delivery vector has the advantage that it is of human origin and thus probably less immunogenic than virally derived proteins. The general applicability of the system requires further examination.

2.14 Tat-protein transduction domain (PTD) mediated protein delivery

The protein transduction system discussed in this review should not be mistaken for the **t**win-**a**rginine **t**ranslocation (TAT) pathway, which has been identified and analyzed in many plant chloroplasts (Alder and Theg, 2003) and prokaryotes (Dilks et al., 2003) including archaebacteria (Hutcheon and Bolhuis, 2003, for review). It mediates the secretion of folded proteins (Robinson and Bolhuis, 2001, for review).

2.14.1 The HIV-transactivator of transcription (Tat)

The 101 amino acid transactivator of transcription (Tat) of HIV-1 is necessary for the replication of the virus. It regulates transcription from the HIV-1 long terminal repeat (LTR) (Brigati et al., 2003; Jones and Peterlin, 1994; Karn, 1999, for review) and induces expression of many cellular genes (Demarchi et al., 1996; Pieper et al., 2002, and others). It has therefore been a major focus of HIV-related research for many years (Bres et al., 2002; Goldstein, 1996; Opi et al., 2002; Rusnati and Presta, 2002; Watson and Edwards, 1999). Tat facilitates HIV dissemination within the infected body (Izmailova et al., 2003) and causes the death of uninfected bystander T-cells through TRAIL activation in monocytes (Yang et al., 2003). Thus, the transducibility of Tat appears to be a feature of the molecule that may have given a selective advantage to the virus propagation.

The protein consists of 5 domains. The probably best studied region of Tat is located in domain 4, which contains the basic sequence RKKRRYRRR (Jeang et al., 1999, for review). This short motif is required for the binding of Tat to its activator RNA, TAR. It is essential for the neurotoxic properties of Tat (Chauhan et al., 2003) and, most important for our topic, its transduction: Tat is secreted from virus-infected cells and is

transduced into surrounding cells (Pooga et al., 1998a). The protein is found predominantly in the nucleus, which is dependent on the GRKKR sequence at the N-terminus of the basic motive (Hauber et al., 1989; Ruben et al., 1989).

In 1988, two research groups independently found that the HIV-Tat protein can cross cellular membranes, accumulate in the nucleus and transactivate genes within the cells it penetrated (Frankel and Pabo, 1988; Green and Loewenstein, 1988). Frankel and Pabo demonstrated that activation by Tat was enhanced 7000-fold when 100 µM chloroquine was present. Such chloroquine concentrations are typically used to raise vacuolar pH. The effect by chloroquine was interpreted as preventing the protein from getting degraded. This finding suggests that Tat is taken up via the endocytotic pathway, which is an important detail for the mechanisms of PTD- mediated uptake discussed below (Section 4). However, full-length Tat or a fusion proteins with its basic domain 4 might act differently, as discussed below. In a subsequent study, Mann and Frankel showed that Tat entered cells by adsorptive endocytosis (Mann and Frankel, 1991). No specific receptor was detected, and uptake appeared to involve heparan sulfates. Only 3% of the Tat became cell associated. Uptake was inhibited at 4°C, which is in contrast to findings by some other groups, which used the Tat PTD (see below).

Later it was demonstrated that exogenously provided HIV-Tat linked to dehydrofolate reductase was as efficient in transactivating an LTR-CAT reporter gene as the wild type Tat protein (Bonifaci et al., 1995). Unless the dehydrofolate reductase is cleaved from its carrier, this experiment demonstrates that the Tat protein can deliver an enzyme into the nucleus of HeLa cells. When methotrexate was added, which binds the enzyme and inhibits its unfolding, the protein was not delivered into the nucleus. Other

experiments confirmed that full-length Tat is capable to transduce into cells when linked to other peptides (Demarchi et al., 1996).

The protein transduction mechanism mediated by the Tat basic domain (see chapter 4) might differ from the transduction of the full 101 amino acid protein. As discussed below, many studies suggest that PTD-mediated translocation of cargo proteins does not absolutely require receptor binding. However, it is prudent to keep in mind the identified receptor interactions by extracellular Tat, not only when examining the internalization mechanism. If the PTD is employed to study the function of a certain molecule, receptor binding might also induce cellular reactions that interfere with activities of the linked molecule to be examined and thus lead to wrong conclusions.

Tat binds to the low-density lipoprotein receptor-related protein (LRP), which promotes efficient internalization of Tat into neurons, upon which it is rapidly transferred into the nuclei (Liu et al., 2000). The uptake of the physiological ligands for LRP is thereby substantially inhibited.

Extracellular Tat is also a specific agonist for the chemokine receptor 4 (CXCR 4), thereby inhibiting the replication of certain HIV serotypes (Xiao et al., 2000). Responsible for this virus inhibiting activity are resides 31-35 (Löhr et al., 2003). These residues are located outside the basic domain employed in fusion proteins with cell-transducing abilities.

Tat also specifically binds and activates the Flk-1/KDR, a VEGF receptor tyrosine kinase (Albini et al., 1996), an interaction that is probably responsible for angiogenesis induction by the Tat protein. Moreover, Tat interaction with the VEGFR-1/Flt-1 might be involved in the monocyte activation by HIV infected cells (Mitola et al., 1997). Tat can also bind to certain integrins (Barillari et al., 1993; Vogel et al., 1993) and contains an

RGD cell adhesion motif (Brake et al., 1990). Some researchers doubt whether reentry of Tat into uninfected cells and transactivation of cellular or LTR genes *in vivo* play a substantial role in HIV pathology, but rather claim that “the interactions of Tat with cellular receptors present on cell surfaces induce, or interfere with, a signal cascade” (Noonan and Albini, 2000). Often, when cellular entry of Tat was observed, either high concentrations of Tat were applied, or chloroquine, which perturbs lysosomal activity, was used.

Recently, Hu et al. have shown that Tat induces retinal pigment epithelial cells to express an opioid transport system that is driven by Na^+ or Cl^- gradients (Hu et al., 2003). Moreover, Tat elicits Ca^{2+} -dependent acetylcholine release from cholinergic terminals (Feligioni et al., 2003).

Besides such effects, Tat is also toxic in many instances. High concentrations of HIV-Tat stimulate oxidative stress in the hippocampus, cortex and striatum regions of the mouse brain (Flora et al., 2003). It can cause cell death in the CA3 region and the dentate gyrus (Maragos et al., 2003). Its toxicity for neurons (Haughey and Mattson, 2002; Kim et al., 2003a; Langford and Masliah, 2002; Sabatier et al., 1991) is probably mediated by direct interaction with NMDA receptors (Eugenin et al., 2003; Prendergast et al., 2002; Song et al., 2003), by interfering with the NGF signaling pathway and the Cdk5 activator protein p35 (Peruzzi et al., 2002), by iNOS induction (Liu et al., 2002); and binding to microtubules (Chen et al., 2002a). Tat is thought to contribute to the neuronal abnormalities often observed in HIV infected patients (van de Bovenkamp et al., 2002; Zauli et al., 2000). It can be transported along anatomical pathways to adjacent neuronal populations, causing their demise and neurological abnormalities reminiscent of those seen in HIV patients (Bruce-Keller et al., 2003). Tat is also toxic to

other cells besides neurons. For instance, it induces apoptosis (Kim et al., 2003b) and inflammatory pathways (Toborek et al., 2003a) in brain microvascular endothelial cells and disrupts the blood-brain barrier (Toborek et al., 2003b).

On the other hand, lower concentrations of Tat inhibit apoptosis in Jurkat lymphoblastoid cells when expressed endogenously but also when the protein is added into the culture medium (Borgatti et al., 1997; Gibellini et al., 2001). This effect may in part be mediated by a Tat-induced upregulation of Bcl-2 (Corallini et al., 2002; Zauli and Gibellini, 1996; Zauli et al., 1995) and the PI-3K/Akt pathway (Borgatti et al., 1997). Interestingly, astrocytes stably transfected with proviral HIV-Tat are more resistant to cell injury as compared to control cells (Chauhan et al., 2003). On the other hand, Tat released from those astrocytes caused neuronal cell death. *In vivo*, this toxicity was even observed at sites distant from its production. However, full-length 101 amino acid Tat protein and its short PTD discussed below probably behave quite differently.

Although Tat (48-60) and other basic peptides appear to be not toxic to certain cells even at a concentration of 100 µM (Suzuki et al., 2002; Vives et al., 1997) and few toxic effects have so far been reported for the Tat PTD, it is critical to design appropriate controls when carrying out functional assays with Tat fusion proteins. Although unexpected, the basic domain could activate signaling pathways that affect the outcome of the experiment. The manifold activities of Tat in different cell types are mirrored by its pleiotrophic effects on the cellular transduction machinery. Full length Tat, but also a fragment of amino acids 48-60, can activate the mitogen activated protein kinase Erk in endothelial cells *in vitro* (Rusnati et al., 2001).

2.14.2 Exploiting the protein transduction domain (PTD) of a deadly virus protein

In 1994, Fawell et al. built on the finding of the transducibility of full-length Tat by showing that a variety of cargoes, chemically linked to amino acids 37-72 or 1-72 of HIV-Tat, was delivered into cells *in vitro* and *in vivo* (Fawell et al., 1994). Only 20 min after intravenous injection, the protein was found later in most tissues examined, except in the brain. *In vitro*, chloroquine had little effect on protein delivery, suggesting that the endocytic pathway was not involved.

S. F. Dowdy's group, at that time at Washington University in St. Louis, was the first to generate an in-frame Tat bacterial expression vector and to report the purification of the recombinant proteins (Nagahara et al., 1998). An important aspect of their successful genetic approach was that proteins are less prone to precipitation and are transduced more efficiently when they are isolated under denaturing conditions. The denaturant is subsequently removed in one rapid step, preventing refolding of the protein into its native conformation. The Dowdy group reported the production of an eclectic collection of fusion proteins that were efficiently transduced into cells and showed biological responses *in vitro*. Soon a number of other elegant follow-up studies from this group were published. They designed a Tat fusion protein consisting of a modified Caspase 3 sequence, whose proteolytic activation site would be detected by the HIV protease (Vocero-Akbani et al., 1999). Thus, HIV infected Jurkat T cells are specifically induced to undergo apoptosis, a promising approach for other viral infections as well.

Taking their approach a leap further, in 1999, they reported a novel and powerful approach to deliver proteins throughout the body and even into the brain (Schwarze et al., 1999). Tat PTD, or a recombinant 120 kDa Tat-β-Gal fusion protein, labeled with

fluorescein and injected into the peritoneum (ip), were both rapidly found in blood cells and spleenocytes, as demonstrated by flow cytometry. Most importantly, the Tat-fluorophore conjugate was found in tissues including the brain only 20 min after ip injection, while β-Gal activity was found after 4 h in most tissues and after 8 h in brain and kidney. This appears to be in contrast to the results obtained by Fawell et. al (Fawell et al., 1994); however, in the earlier study the chemically conjugated Tat-β-Gal was intravenously administered, rather than ip, and the brain was examined already 20 min after application of the protein. Although the recombinant Tat-β-Gal used by the Dowdy lab was purified under native conditions from E. *coli*, something in that purification strategy might have rendered the protein in a conformation different from the one used in earlier studies.

The Dowdy lab further reported that they had “used this technology to transduce over 50 proteins to a wide variety of human or murine cell types” (Schwarze et al., 1999). The method was hailed as a means to “combat inherited diseases and other conditions caused by malfunctioning or absent intracellular protein” (Strauss, 1999). A possible drawback of that pioneering publication is that FACS analysis, some of the enzymatic assays and immunoblots, do not prove a transduction into the cell, but could as well be due to an adsorption of the basic domain to the negatively charged plasma membrane. Moreover, analysis by immunocytochemistry can be prone to artifacts, as discussed below (5.1). Also, the prokaryotic β-Gal might be a peculiar case: In most other successful *in vivo* applications, the cargo delivered was much smaller than the 120 kDa β-Gal fusion protein. In contrast to other Tat fusion proteins, Tat-β-Gal was isolated under non-denaturing conditions (Schwarze et al., 2000). Thus, an extensive refolding process of the protein once it entered the interior of the cell was not necessary.

(However, it needs to form a homotetramer to be active (Bayley, 1999).) β-Gal is a highly active protein. Thus, even at a low transduction efficiency of the up to 500 µg of the protein that were injected into each mouse, enzymatic activity might be demonstrated in cells and tissue, in particular when long developing times (16 h in brain sections) for the color reaction are employed.

Other groups did not find a crossing of the BBB mediated by the Tat translocation sequence (Bullok et al., 2002). Moreover, when high amounts of Tat-β-Gal encoding expression plasmids were injected into the liver, soon liver cells began to spill out enzymatically active protein (Hickman et al., 1994, for a description of the technique), which could be readily detected in the heart, for instance. In the brain, however, it only entered the epithelial single cell layer that lines the cerebro-spinal fluid, but could never be found in neurons (J. Reiss, Göttingen, unpublished observations). It seems that the BBB is not permeable for the uptake of Tat fusion proteins in all instances. Nevertheless, the exciting work of S. Dowdy's lab lead to a number of further highly intriguing studies, as discussed below.

2.14.2.1 Delivery of substances and molecules other than proteins

2.14.2.1.1 Delivery of particles and compounds to track cells

One of the most surprising demonstrations of the effectiveness of Tat-PTD mediated internalization was accomplished by the group of R. Weissleder. Their goal was to tag cells magnetically for tracking them by MR imaging. To that end, they used particles with an iron core, a dextrane coating, and covalently linked Tat peptides (Josephson et al., 1999; Lewin et al., 2000; Zhao et al., 2002). Such 45 nm particles are efficiently taken up by human hematopoietic $CD34^+$ and other cells and retained in there for several days.

After intravenous application, the peptide mediates transduction of the beads into the liver parenchyma and other tissues (Wunderbaldinger et al., 2002). MR imaging could visualize labeled cells. Delivery of other contrast-enhancing agents into cells using Tat-derived peptides has also been achieved (Bhorade et al., 2000). Furthermore, Tat peptides have been linked to fluorescent nanobioconjugates and successfully delivered into COS and HeLa cells (Liu et al., 2001b). Linked to ^{99m}Tc, Tat peptides are useful to deliver an agent that can be tracked in the whole body (Bullok et al., 2002; Polyakov et al., 2000). The mechanism of that uptake remains elusive.

Heckl et al. achieved a specific retention of the contrast agent gadolinium in tumor cells *in vitro* and *in vivo* when it was tagged to a c-myc directed peptide nucleic acid and the Antp homeodomain transduction sequence (Heckl et al., 2003). Possibly, this compound might be useful to demonstrate the presence of tumors *in vivo*.

2.14.2.1.2 Delivery of nucleic acids

As demonstrated by a luciferase assay, Tat- and Antp -derived peptide-oligonucleotide conjugates are readily taken up by HeLa cells, even in the presence of serum (Astriab-Fisher et al., 2002; Astriab-Fisher et al., 2000). Options for coupling of oligonucleotides to peptides have been discussed elsewhere (Tung and Stein, 2000). Another possibility besides the covalent coupling is the simple mixing of plasmid DNA with high molecular weight polyarginine, which has been used to transfect nucleic acids into cell line cells (Emi et al., 1997). Other groups have evaluated the feasibility of just mixing shorter peptides with plasmid DNA to achieve transfection. Using that approach, resembling the one used for Pep-1 (cf. 2.10), the Tat peptide delivers DNA into cells (Zhang et al., 2003d). Similar to the Tat PTD, one fraction of thermolysin-digested protamine possesses two basic clusters and is about as efficient as Tat in delivering

plasmid DNA into cells (Park et al., 2003). Other peptides are also efficient in transfecting DNA, even when it is bound by electrostatic forces only, such as branched oligocationic peptides (Plank et al., 1999, here tested on K562 cells), the Tat PTD, the flock house virus (FHV) coat protein and polyarginine peptides with plasmid DNA to transduce COS-7 cells (Futaki et al., 2001a; Niidome et al., 1997). The efficacy was determined by luciferase assay. R_8, Tat, and FHV peptides all yielded a similar luciferase activity, which was, however, two orders of magnitude lower than lipofection (ibid.). An efficiency comparable to lipofection was observed when those arginine-rich peptides were linked to a stearyl group. Improvement of plasmid DNA transfer into COS-7 cells had already been achieved earlier by modifying carrier peptides, using several lipophilic groups, i.e. acyl groups, a dialkylcarbamoyl group and a cholesteryloxycarbonyl group (Niidome et al., 1999). A very efficient delivery of plasmid DNA was also achieved using $(Tat)_2$ and $(Tat)_3$ oligomers (Rudolph et al., 2003) or by interspersing an arginine sequence with histidine and glycine residues (Siprashvili et al., 2003).

Liposomes deliver DNA much more efficiently into various cell types when coated with the Tat-peptide (Torchilin, 2002). These liposome-Tat conjugates can cause expression of GFP *in vitro* and, when directly injected into mouse lung tumors, *in vivo*. The Tat-PTD alone, on the other hand, does not enhance plasmid uptake *in vivo* (Ignatovich et al., 2003). Similar to the Tat peptide, the Antp peptide enhances liposome transfection of reporter plasmids (Ou et al., 2003). PTD-enhanced liposomal delivery has been discussed above (1.2). Another transfection approach employing PTD is to coat lambda phage particles with the Tat domain, thereby using them as vehicles to transfect DNA into eukaryotic cells *in vitro* (Eguchi et al., 2001; Nakanishi et al., 2003).

2.14.2.2 Recombinant fusion proteins

Strategies to chemically link peptides to one another or to oligonucleotides have been thoroughly explained (Fischer et al., 2001) and will not be further discussed here. For most laboratories, generating in-frame fusion proteins using molecular biology methodology is more convenient. As Table 1 lists many examples for *in vitro* applications of PTDs, only a few cases are mentioned in the following paragraph.

2.14.2.2.1 Application of proteins *in vitro*

The intracellular concentration of labeled PTD peptides in cultured cells can rise greatly above the concentration of the surrounding medium, as shown for transportan, Tat and Antp peptides (Hallbrink et al., 2001). This suggests that the cell trafficking of PTD-linked peptides occur preferably inward, which is further supported by other experiments (cf. paragraph 3.2).

Even cells that are very difficult to transfect, for instance osteoblast and osteoclast primary cultures, have been efficiently transduced by functional Tat-HA or Tat-calcineurinα (Dolgilevich et al., 2002). In addition, the Tat-PTD system has been used to examine the role of RhoA in osteoclast function (Chellaiah et al., 2000) and to inhibit osteoclastogenesis by delivering mutant forms of IκB, an inhibitor of NF-κB (Abu-Amer et al., 2001).

Numerous experiments exploiting PTD techniques to inhibit apoptosis have been carried out (Table 1). For instance, an apoptosis repressor with a caspase recruitment domain linked to Tat-PTD decreased cell death in isolated hearts after ischemic insult (Gustafsson et al., 2002). However, instead of inhibiting apoptosis, the induction of apoptosis by cell penetrating peptides can also be desired, e.g. in tumor cells (Schimmer

et al., 2001). The Tat-PTD facilitated transduction of thymidine kinase protein into human embryonic kidney 293 cells from neighboring cells expressing the fusion gene. Thereby, cells were made susceptible to the drug acyclovir, which induced apoptosis in those cells (Tasciotti et al., 2003). The protein was found extracellulary associated with cell surface heparan sulfate proteoglycans, an interaction apparently critical for the transduction of the protein (see paragraph 4.1.2).

To deliver functional antibodies into cells, methods of linking them to the Tat-domain have been reported (Steffen, 2001). An interesting development is the delivery of single chain antibodies derived from a phage display library into cells by linking them to the Tat-PTD (Cohen-Saidon et al., 2003). Using this methodology, the anti-apoptotic protein Bcl-2 was inhibited *in vitro*.

2.14.2.2.2 Applications *in vivo*

It has been claimed that protein transduction domains may be efficient for *in vitro* experiments, for example to transduce antibody fragments, but not for most applications *in vivo* (Niesner et al., 2002). Indeed, there have been many reports on applications of Tat-mediated protein transduction *in vitro*, but reports about successful *in vivo* applications have been rare (see Table 1). In many instances, when such applications have been successful, the peptide cargo was only comprised of a few amino acids, and their site of action was within the vasculature or the liver, which is easily reached upon systemic application. After intravenous application, Tat conjugated proteins are very quickly cleared from circulation (Lee and Pardridge, 2001).

Applications of Tat fusion proteins *ex vivo*, followed by transplantation of the treated cells *in vivo*, have been more promising in several instances. For example, treatment of dendritic cells with an antigenic tumor peptide coupled to the PTD *ex vivo*,

enhancing their antigen presentation, followed by an implantation of the dendritc cells resulted in a protection against tumors *in vivo* (Moy et al., 1996; Shibagaki and Udey, 2002; Wang et al., 2002, see also further examples in Table 1). It should be noted that application of the peptides without the PTD already provided some protection. Thus, the main effect of the PTD might be to facilitate attachment of the proteins to the cellular membrane, which enhances the uptake that would only take place at a low level in the absence of the basic peptide sequence (cf. discussion in chapter 4).

Another approach for tumor treatment was to use a Tat-peptide caspase 3 fusion protein which is specifically stabilized under hypoxic conditions (Harada et al., 2002). It decreased the size of solid mouse tumors *in vivo*. This is an interesting example of targeting the effect of PTD-linked proteins to specific cell populations within the organism. By inhibiting the insulin like growth factor receptor mediated signaling, a Tat fusion peptide partially inhibited renal tumors induced in nude mice (Datta et al., 2001).

Fragments of the hypoxia-inducible factor 1 (HIF-1), linked to the Tat PTD, caused the induction of HIF target genes *in vitro* and the induction of angiogenic markers when applied in a polyurethane sponge that was transplanted subcutaneously in mice (Willam et al., 2002). The *in vivo* effect of the Tat proteins to induce such responses remained local, i.e. the protein apparently did not spread throughout the organism to induce a response in tissues other than those adjacent to the sponge and the injection site.

A subdermal or intra-arterial application of Tat-eGFP only led to very few fluorescent fibers in the muscle periphery or surrounding the blood vessels (Caron et al., 2001). Muscles injected with Tat-eGFP showed intense staining of the ECM, rather than efficient delivery into muscle tissue. In vitro, the same protein was delivered into

myoblasts; however, it was applied at very high concentrations and endogenous GFP fluorescence appeared in the cells not earlier than 14 h after the application of the protein. This might suggest that a rather slow endocytic process mediates its uptake, and that apparent transduction detected by rhodamine-labeled Tat-eGFP could be due to a fixation artifact, as discussed below (5.1). Thus, in spite of the impressive successes of PTD applications described here, the technology may not easily be applicable to all types of cargo.

3 Successful Applications in Neural Cells and the CNS

3.1 Inhibition of cell death

3.1.1 *In vitro* models

3.1.1.1 Potassium deprivation of cerebellar granule neurons

We have shown that a Tat-Bcl-x_L fusion protein was quickly transduced into cerebellar granule neurons *in vitro* and into the retinal ganglion cell layer *in vivo* (Dietz et al., 2002). In cerebellar granule cells, Tat-Bcl-x_L application conferred protection against potassium deprivation-induced apoptosis. The comparatively large number of studies showing activity of PTD linked Bcl-X_L *in vitro* and *in vivo* (cf. 3.1.2.2) suggests that this anti-apoptotic molecule is particularly well suited to cross cell membranes. The arrangement of the alpha-helices in Bcl-X_L is reminiscent of the membrane translocation domain of bacterial toxins, in particular diphtheria toxin and colicins (Muchmore et al., 1996). Thus, although uptake and activity of the protein solely consisting of Bcl-X_L has, to our knowledge, not been observed, intrinsic structural features of Bcl-X_L might aid its passage through membranes. Consistent with this assumption, functional Bcl-X_L can

also be successfully delivered *in vitro* when linked to the diphtheria toxin translocation domain (Liu et al., 1999b).

The ease of transducibility also applies to other members of the Bcl-2 family and their domains (Holinger et al., 1999; Schimmer et al., 2001). A technical problem is that some Bcl-2 family members are toxic for *E.coli*, which is often used for expression.

3.1.1.2 Examples for applications in other neural cells

Besides cerebellar granule neurons, primary cortical neurons are also protected using PTD-delivered molecules. A Tat-HSP70 fusion protein rescued them from peroxinitrite and glutamate-induced cell death, in models for both apoptosis and necrosis (Lai et al., 2002). It should be noted here that Hsp70 alone displays transducing ability (Fujihara and Nadler, 1999).

PC12 cells have also been used to test PTD fusion proteins. Glutamate dehydrogenase (GDH) deficiency is associated with a number of human neurological disorders. Yoon et al. succeeded in delivering a functional Tat-GDH of over 50 kDa into PC12 cells *in vitro* (Yoon et al., 2002). In the same cells, activation of extracellular signal-activated kinase (ERK) was inhibited by a 13 amino acid peptide derived from the binding domain of its activator, MEK, when it was coupled to either Antp or Tat PTD (Kelemen et al., 2002).

3.1.2 *In vivo* models

3.1.2.1 Cell rescue after nerve trauma

A significant portion of retinal ganglion cells, which normally undergo apoptosis when their axon is severed, are rescued from cell death when a Tat-Bcl-X_L fusion protein is injected intraocularly (Dietz et al., 2002). In this well established model for nerve

trauma, the Tat PTD also highly enhanced the neuroprotective effect of GDNF (Kilic et al., 2004). A similar rescue effect had been demonstrated earlier using an anthrax toxin-derived 254 amino acid poly-peptide as a delivery vector, fused to Bcl-X_L (Liu et al., 2001c). In contrast to the Tat-derived vector, delivery mediated by the anthrax toxin requires a specific cell surface receptor. That receptor must at least be present in cerebellar granule neurons, macrophages and retinal ganglion cells, as in all those cells the fusion protein exhibited an apoptosis-inhibiting effect.

3.1.2.2 Ischemia therapy

Intravenously applied Tat-Bcl-x_L entered the brain within 4 h *in vivo* and greatly reduced infarct size after 90 min of medial cerebral artery occlusion, even when the protein was applied after the onset of reperfusion (Kilic et al., 2002). This decrease in structural brain damage was accompanied by a higher neurological performance of the protein-treated mice. In a 30 min cerebral artery occlusion experiment, which results in a disseminated neuronal injury in the striatum, Tat-Bcl-x_L treatment led to a significant reduction in the number of TUNEL and Caspase-3 positive cells. After a 2 h ischemia, Tat-Bcl-x_L was also highly protective (Fig. 2).

In another study, Tat-Bcl-X_L was shown to inhibit staurosporine-induced apoptosis in primary cortical neurons *in vitro* and after a 90 min transient focal ischemia (Cao et al., 2002). By quantitative ELISA, it was shown that IP-injected protein reached concentrations of 4ng/mg cortex tissue. The protein was protective even when applied 45 min after ischemia onset.

Taking the approach of Tat-Bcl-X_L- induced neuronal rescue one step further, the group of S. Ohta modified the anti-apoptotic molecule by site-directed mutagenesis, thereby generating a derivative of Bcl-X_L with a more flexible and mobile pore-forming

domain (Asoh et al., 2002). This protein designated FNK has a higher cytoprotective activity than wild type Bcl-x_L (Asoh et al., 2000). The fusion protein Tat-FNK was quickly transduced into SH-SY5Y neuroblastoma cells and primary neocortical neurons *in vitro*, where it colocalized with a mitochondria marker (Asoh et al., 2002). Upon Tat-FNK application, the SH-SY5Y cells were protected against staurosporine-induced death, while primary neurons were resistant against Glu-induced excitotoxicity. Upon IP injection, the protein was also efficiently delivered across the BBB. In gerbils, it protected the hippocampal CA1 region against a 5 minute ischemic insult. The same protein did not protect against apoptosis induced by a fusion protein between the ligand-binding domain of the estrogen receptor and DeltaFosB (Tahara et al., 2003).

Other models in which PTD-linked Bcl-X_L or domains derived from it have been effective include inhibition of TNF-α-induced cell death in a pancreatic β-cell line *ex vivo* (Embury et al., 2001) and cardioprotection in an *ex vivo* model for heart ischemia (Chen et al., 2002b), which again confirms the suitability of PTD-Bcl-x_L fusion proteins for cellular delivery and apoptosis inhibition (cf. paragraph 3.1.1.1).

Another elegant approach to inhibit ischemic brain damage came from the knowledge that such injury is partly mediated by NMDAR-dependent nNOS activation (Aarts et al., 2002). This activation requires the postsynaptic density protein PSD-95. While blocking the NMDAR is detrimental to humans, inhibiting its interaction with PSD-95 is not. Aarts and coworkers designed two peptides that comprised the amino acids required for the NMADR PSD-95 interaction, thus inhibiting their binding. When those peptides were coupled to the Tat PTD, they were efficiently delivered into neurons *in vitro* and displayed the predicted inhibition of the protein-protein interaction and interference with downstream NO signaling. As a consequence of such protein

application, sensitivity to NMDA excitotoxicity was reduced. *In vivo*, the Tat-linked proteins crossed the BBB when either applied intravenously or intraperitoneally in both rats and mice. Most importantly, the blocking protein strongly alleviated ischemic brain damage in rats, even when applied one hour after onset of brain artery occlusion.

Neurotrophins have a high potential for neuroprotective applications. However, they usually do not readily cross the BBB. Linking of the Tat PTD to GDNF caused this neurotrophin to efficiently penetrate the brain after intravenous application, where it was neuroprotective in a brain infarct model through inhibition of apoptotic processes (Kilic et al., 2003).

The protective effect in models for transient and permanent ischemia was also achieved when a fusion protein between the Tat PTD and the c-Jun N-terminal kinase (JNK) binding motif of JNK-interacting protein-1/islet-brain1 was applied (Borsello et al., 2003). There was a significant reduction in infarct size, even when the protein was applied up to 6 h after the onset of the ischemia. Different from the previously described publications, the middle cerebral artery was occluded for only for 30 min, and the protein was applied by intracerebroventricular injection.

3.1.2.3 Application in striatal excitotoxicity

The application of a cell-permeable peptide (SN-50, (Torgerson et al., 1998)) that inhibits NFκB nuclear translocation, directly administered into the striatum, inhibits quinolinic acid-induced cell death (Qin et al., 1998). The study thus demonstrated that NFκB activation, and possibly activation of other transcription factors inhibited by SN-50, contributes to excitotoxic cell death in striatal neurons.

3.2 Use in Gene therapy

For reasons discussed below (4), the transduction of lysosomal proteins by PTDs may be particularly auspicious. The linkage of the lysosomal enzyme β-glucuronidase to a Tat domain, delivered by an adenoviral vector, leads to a very efficient distribution of the protein in the brain (Xia et al., 2001) and other tissues, and a high alleviation of mucopolysaccharidosis condition in mice (Elliger et al., 2002). Other studies confirmed that linkage to a PTD (HSV-VP22 in this case) leads to an enhanced spreading of a fused protein when adenovirally delivered to the retina and the striatum, as compared to an adenovirus that expressed GFP not linked to a transduction domain (Kretz et al., 2003). However, other researchers did not find evidence of intercellular transport of Tat fusion proteins when they were delivered by an adenovirus vector (Cashman et al., 2003). In the latter experiments, Tat proteins were transduced into bystander cells when released by plasma membrane degradation. This preferential inward transport through the plasma membrane again demonstrates that there are differences between the features of the viral full-length Tat protein in the infected organism and Tat-PTD-mediated trafficking. The full length Tat-protein, like the recombinant protein used by Elliger et al. (which contained the Igκ sequence), may contain a sequence that allows the protein to get secreted from the cells in which it is expressed.

3.3 Study of signal transduction pathways and transmitter release in neurons in vitro

When the third intracellular loop of the angiotensin II type I receptor fused to the Tat PTD was applied on hypothalamus or brainstem neurons, it elicited a response similar to stimulation by angiotensin II (Vazquez et al., 2003), including an increased

firing rate. Thus, PTD technology could be used as a valid alternative to gene transfer technology when studying the structure-function relationship of this G-protein coupled receptor. In another experiment to elucidate a neuronal signal transduction pathway, the FGFR high-affinity binding site for PLCgamma, chemically linked to the Antp transduction domain, inhibited phospholipid hydrolysis and neurite outgrowth in cerebellar granule neurons (Hall et al., 1996; Saffell et al., 1997). As a third example, Tat fusion proteins have been used to elucidate the mechanisms of noradrenaline and dopamine release in PC-12 cells (Fujiwara et al., 2001).

3.4 Long Term Potentiation (LTP)

It was shown that fusion of a peptide inhibitor for PKA to R_{11} and the SV40 NLS resulted in efficient transduction into neuronal nuclei in hippocampal slices (Matsushita et al., 2001). This transduction inhibited long-lasting LTP, but not early LTP, suggesting that the kinase acts in the nucleus to induce LTP. A similar effect was observed using a CPEP-antisense construct, which described in the following paragraph (3.5).

3.5 Delivery of antisense oligonucleotides

The translational regulator CPEP was locally inhibited in one branch of an *Aplysia* sensory-motor neuron coculture by delivering a Tat-linked antisense oligonucleotide (Etkin et al., 2002). Long term facilitation (LTF) in the treated branch was initiated, but not maintained for extended periods (Si et al., 2003). The construct was also delivered into the mouse hippocampus *in vivo* (Etkin et al., 2002). Using a similar approach, an antisense oligonucleotide inhibitor for the β-amyloid precursor protein was used, inhibiting β-APP neosynthesis in primary cortical neurons, which caused an inhibition of neurite and axonal outgrowth (Allinquant et al., 1995).

3.6 Reduction of Nociception

A peptide nucleic acid complementary to the galanin receptor mRNA and linked to transportan or Antp, was able to decrease galanin binding in the dorsal horn *in vivo*, resulting in an inability of galanin to inhibit the C-fiber stimulation-induced facilitation of the rat flexor reflex (Pooga et al., 1998c; Prochiantz, 1998). In addition, an inhibitor of protein kinase C, linked to a Tat-PTD, when delivered inthrathecally, modified pain perception after formalin injection into the rat paw (Sweitzer et al., 2002).

4 Advances towards an Understanding of the Mechanism of Peptide-Mediated Protein Transduction - How do Giant Horses Trespass the cellular Fortification ?

A better understanding of the still unanswered question of the transduction mechanism might not only allow the design of even more efficient transduction domains on paper, but would also lead to a deeper understanding of the infection mechanism by a number of important pathological viruses. Although many studies on the mechanisms of transduction have been made, it has not been fully elucidated yet.

4.1 The Trojan Horse city wall encounter – interaction of proteins with the plasma membrane

A non-specific, electrostatic interaction of the peptide with membrane lipids appears to be crucial (Bellet-Amalric et al., 2000; Ziegler et al., 2003), rather than amphiphilic interactions (Bellet-Amalric et al., 2000). The first step in the transduction mechanism of transportan and the Antp homeodomain, the peptide-lipid interaction, has also been studied by fluorescent techniques. To that end, various types of quenchers were used to study the peptide interaction with phospholipid vesicles of varying charge

density (Magzoub et al., 2003). To show the corresponding secondary structures, CD spectroscopy was employed. Both peptides lie close to the bilayer surface and with their helices usually parallel to the bilayer surface, which may be important for the translocation ability of the two peptides. Control peptides similar in primary structure, but unable to penetrate the membrane, were located more perpendicular to the bilayer. There are clear differences between the Antp and the transportan peptides. While Antp interacts with negatively charged membranes, transportan can interact with the lipids independently of charge (Magzoub et al., 2001). Transportan remains helical when bound, regardless of membrane composition or peptide to lipid ratio. This is in contrast to Antp, which highly depends in its secondary structure on those parameters. At a low lipid to peptide ratio, the dominating structure for Antp is the β-conformation. At this conformation, a strong perturbation of the membrane order is observed (Magzoub et al., 2003), while there is little influence on the membrane structure when the peptide is in a helical conformation (Berlose et al., 1996). In a helical state, peptides lie along the vesicle, while other variants appear to penetrate deeper into the vesicular surface.

4.1.1 Internalization is not dependent on receptor binding

A receptor is not necessary in the type of protein uptake described here. For penetratin, it has been demonstrated that its affinity for lipid membranes is largely governed by nonspecific electrostatic interactions (Persson et al., 2003). One piece of evidence is provided by the finding that the D-forms of both Antp (Brugidou et al., 1995; Derossi et al., 1996) and Tat (Futaki, 2002; Wender et al., 2000) cell penetrating peptides are taken up at least as efficient as the L-forms. Moreover, the Tat-β-peptide is taken up as efficiently as the α-form, both at 4°C and at 37°C. It should be noted, though, that this

transduction was examined in PFA-fixed cells, a problem that is discussed below (5.1), and that in some cases, the reverse sequence of the carrier peptide is *not* transduced (Du et al., 1998).

In further agreement with receptor-independence is the observation that PTD-linked compounds are transduced into most cells examined so far, although some studies did find that efficiency varied with the type of cell under investigation (Koppelhus et al., 2002). The Antp peptide also transduced into artificial giant unilamellar vesicles (Thorén et al., 2000), which strongly supports a receptor independent transduction mechanism.

4.1.2 The role of heparan sulfate (HS)

Cell surface heparan sulfate proteoglycans (HSPG) appear to play a role in the transduction of Tat proteins. HS are present in almost all cells; which is consistent with the observation that most cells so far tested *in vitro* could be transduced by PTDs. It was shown that the sulfated glycosaminoglycans heparin and HS bind full-length Tat protein, an affinity that is abolished when heparin/HS are desulfated (Rusnati et al., 1997). Cells that are defective in HS expression are impaired in Tat-GFP internalization (Tyagi et al., 2001). Soluble heparin and enzymes that degrade HS added to the culture also inhibited uptake of the fusion protein. To facilitate Tat uptake, in that study chloroquine was added to the culture medium, which changes the endolysosomal pH. As discussed below, contrary to earlier assumptions, endosomes appear to play a role in PTD mediated internalization. When the Tat heparin interaction is inhibited by heparin-mimicking compounds, extracellular full-length Tat no longer displays transactivating activity (Rusnati et al., 1998). Heparan inhibits neurotoxicity of full-length HIV-Tat protein *in vitro* (Chauhan et al., 2003).

Tat PTD fusion proteins also bind heparin (Hakansson and Caffrey, 2003). When the interaction of heparin with the Tat PTD is blocked by heparin-mimicking compounds, transduction is inhibited (Console et al., 2003). Tat- and other basic peptide-mediated protein transduction is more efficient into cells that express glycosaminoglycans and heparan sulfate, or when dextran sulfate is provided (Console et al., 2003; Mai et al., 2002). Tat fusion proteins attach to the heparinase III soluble fraction derived from the cell surface (Tasciotti et al., 2003), i.e. the fraction that contains the HSPG.

Heparin, a soluble analogue of heparan sulfate glycosaminoglycans (GAGs), inhibits uptake of a Tat-thymidine kinase fusion protein (Tasciotti et al., 2003). Heparin sulfates do not only influence Tat-mediated protein trafficking, but also the uptake of other basic peptides. Transduction of Antp peptides is inhibited by the presence of heparin sulfate proteoglycans (Console et al., 2003) and poly-lysine mediated drug delivery is inhibited by low concentrations of heparin (Shen and Ryser, 1981). Heparin also induces conformational changes in VP22 (Kueltzo et al., 2000). However, it has been found that the Tat PTD can also be transduced into cells by a route that does not involve the HS proteoglycans (Silhol et al., 2002). The Antp PTD can transduce artificial pure lipid bilayers (Thorén et al., 2000). On the other hand, in some experimental conditions, epithelial cells that form tight junctions, even when they express heparan sulfate, cannot be transduced (Violini et al., 2002). As the lipid and protein composition of the apical and the baso-lateral plasma membrane domains are different, the translocation barrier may not apply to both cellular surfaces.

Thus, the role of heparin sulfates is not fully clear yet. Seemingly existing contradictions may be due to differences among the transduction of full length viral Tat, the short Tat-PTD peptide, other basic peptides and varying cargoes examined.

4.2 The stuff Trojan Horses are made of – structural requirements for cell penetrating peptides

4.2.1 Primary structure: The basic character of most PTDs

As described above, many peptides have the ability to penetrate into cells, for example Antp, Tat, HIV-1 Rev, FHV coat protein and the transcription factors c-Fos, c-Jun and GCN4 (Futaki et al., 2001b). Strangely, those peptides apparently do not share common motifs, apart from 4 to 8 arginines. Their transduction appears to occur at 4°C, i.e. it seems not to depend on the classical endosomal pathway. Consistent with the assumption that PTD-mediated protein delivery is independent from a specific receptor, even the D-amino acid analogues of Tat- derived and poly Arginine peptides do get transduced into cells (Bonny et al., 2001; Futaki et al., 2001b; Wender et al., 2000). The basic character of a peptide alone is not sufficient for the translocation, as in some studies peptides rich in lysine get transduced with much less efficiency than the arginine-rich peptides (Futaki et al., 2001b). Arginine-rich histone also facilitates protein uptake, while lysine-rich histone does not (Ryser and Hancock, 1965) or to a much lesser degree (Buschle et al., 1997). On the other hand, for some cargo, the transduction efficiency seems to be more efficient for polylysine (Mai et al., 2002; Park et al., 2002a).

Other experiments are in agreement with the concept that charge alone is not the sole driving force. For instance, substituting two tryptophanes in the penetratin peptide with phenylalanines, strongly diminishes translocation (Derossi et al., 1994). These amino acid exchanges alter the peptide-membrane interaction (Lindberg et al., 2003). On the other hand, the membrane must be negatively charged to interact with penetratin and to allow its deep insertion into the membrane (Christiaens et al., 2002). For

penetration to occur, the peptide has to initially bind the cell surface, by electrostatic interaction with lipids, followed by a presumed membrane destabilization to allow translocation (Dom et al., 2003). Increasing the number of clustered positively charged amino acids enhances attachment of a protein to the membrane, but prevents its transport to the nucleus (Futaki et al., 2001b). An arginine octamer appears to present the optimal length for an efficient transduction. Moreover, polymers of ornithine, lysine and histidine are not transduced, indicating that the guanidine headgroup of the arginine is a critical structural component responsible for the biological activity (Mitchell et al., 2000; Umezawa et al., 2002). Futaki suggested that maybe hydrogen bonding between the highly basic arginine guanidino groups and the phospholipids in the membrane bilayer is involved in the translocation mechanism (Futaki, 2002). Such a scenario would be in agreement with the mechanism of translocation suggested for a number of anti-microbial membrane-perturbing peptides (Saberwal and Nagaraj, 1994).

4.2.2 Secondary structure: Are amphiphilic α-helices required?

In general, cationic amphiphilic α-helical peptides, which display a hydrophilic and, on the opposing side, a hydrophobic face, are efficient transducers of DNA into cells (Murphy et al., 1998; Niidome et al., 1997). The transducibility of peptides consisting exclusively of tryptophan and arginine residues (Nur-E-Kamal et al., 1999; Williams et al., 1997), which generate an amphiphatic molecule, suggests that such a structure promotes membrane transfer.

Mastoparan and transportan adopt an α-helical structure when interacting with artificial micelles (Lindberg et al., 2001). For the peptides melittin (Gazit et al., 1995) and mangainin (Chen et al., 1988), an increase in membrane permeability has been found,

which correlates with an increase in α-helical structure. From experiments with synthetic amphiphatic peptides and lipid bilayers, it is known that cationic peptide side chains bind to anionic lipid bilayers (Dathe et al., 1996). Such experiments support the idea that the structural requirements for cell-penetrating peptides may vary with the membrane they are to penetrate: Decreasing the anionic charge of a membrane decreases binding affinity, while making it more susceptible to the disturbing effect of cationic peptides. Less helical peptides display a reduced bilayer disturbing activity. In membranes with a low anionic charge, the dependence on peptide helicity for peptide/bilayer interaction increases and is critical to induce membrane permeability.

Although additional studies seem to suggest the necessity of an α-helical structure of the PTD (Scheller et al., 1999), others are at odds with this finding (Derossi et al., 1996; Futaki, 2002). Penetratin variants that are mutated at their tryptophan residues do not transduce well, albeit their helical content is increased by the mutation (Czajlik et al., 2002). Drin et al. found that the Antp peptide is not sufficiently helically amphiphatic to form a pore and cross membranes (Drin et al., 2001). This may not apply to all experimental conditions, as penetratin, “when residing at the surface of a membrane, is chameleon-like in terms of its induced structure” (Magzoub et al., 2002), i.e. it can assume different conformations, which has also been demonstrated in other experimental setups (Persson et al., 2001). Further complicating the goal to make clear rules for the primary and secondary structure of efficient PTD, modifications on the amino acid side chains of a PTD suggest that the flexibility of the molecule is also important for transduction characteristics (Wender et al., 2000). Moreover, the same peptide may be able to be internalized by several internalization pathways, and the cargo attached to it can influence the predominantly used mode.

4.3 Does it take extra horses to pull a trojan horse in? – The apparent energy independence of cell penetrating peptide mediated internalization

Protein transduction appears to readily occur at 4°C (Derossi et al., 1994; Futaki et al., 2001b; Mai et al., 2002; Vives et al., 1997; Wender et al., 2000), suggesting that currently known endocytosis pathways do not play a crucial role in the translocation mechanism. Consistent with this conclusion, penetratin fusion protein delivery was thought to be energy- and receptor-independent (Derossi et al., 1996). Depolarization of membrane potential, as well as inhibition of microtubule function and vesicular trafficking showed no inhibitory effect (Polyakov et al., 2000). Sodium azide and iodoacteamide, or low temperature, also had little influence on Tat-modified liposome transduction, again suggesting an energy-independent character of that transduction (Torchilin et al., 2001). However, some-energy dependent component for the delivery of free Tat peptide and Tat-peptide IgG conjugate was found in that study, possibly due to a concurrent pinocytosis not mediated by the PTD . Endocytic and non-endocytic pathways of protein transduction into cells or tissues are obviously not mutually exclusive. To complicate the matter, protein transduction mediated by an arginine peptide is inhibited by the presence of the metabolic inhibitor sodium azide, which suggests that this transduction is energy dependent (Wender et al., 2000), while other studies did not find any effect on protein transduction with the endocytosis inhibitors (Suzuki et al., 2002). As discussed below (paragraphs 4.4.3 and 5.1), at least some of the findings that seemed to prove the miraculous energy-independence of transduction, are caused by experimental artifacts. We will come back to the energy issue when discussing cell penetration by endocytosis.

4.4 Models of cell penetrating peptide-mediated protein transduction

Several lines of evidence suggest that the transduction mechanism mediated by various different arginine-rich peptides is similar (Futaki, 2002; Suzuki et al., 2002). Many features of the transduction are related, and different peptides can competitively inhibit each other's internalization. On the other hand, Transportan and Penetratin appear to penetrate membranes by different mechanisms, as studies with fluorescently labeled proteins and molecular modeling suggests (Lindgren et al., 2000). Other studies indicate that the transduction mechanism and requirement depends on the size and nature of the cargo (Lindsay, 2002; Silhol et al., 2002).

There is no evidence that the penetratin or Tat derived peptides can aggregate, form a channel and pull their cargo through the pore, similar to the observations made for protegrin, magainins, or tachyplesins (cf. paragraph 2.1). Moreover, this is not a likely scenario, as such a channel would need to accommodate such a high variety of different cargoes. Furthermore, an information we can glean from the transduction of liposomes by the Tat peptide comes from the fact that at least one hour after intracellular delivery, the liposomes maintain their integrity. Thus, the liposomes do not fuse with cellular membranes to be transduced, nor do they form a pore that would allow cellular proteins to leak out (Torchilin et al., 2001).

4.4.1 Direct membrane penetration

Stephen Dowdy's group suggested that Tat fusion proteins directly penetrated cellular membranes: being in a denatured, high energy state, energy to drive transduction could be released upon cell entry when the protein refolded with the aid of chaperones (Nagahara et al., 1998), or transduction would be driven by the momentum

of the molecule after it had contacted the membrane (Schwarze et al., 2000). Therefore, the Dowdy group (Becker-Hapak et al., 2001; Nagahara et al., 1998) and others (Bonifaci et al., 1995; Jin et al., 2001; Kwon et al., 2000) have suggested that transduction of proteins is most efficient when they are isolated in a high energetic state. Other experiments by Bonifaci et al., as mentioned above (2.14.1), further suggested that transduction of HIV-Tat required an unfolding step (Bonifaci et al., 1995). However, the denaturation of proteins is not an absolute requirement for transduction. For instance, in the first publications that showed the proof-of-principle of Tat mediated protein transduction (Frankel and Pabo, 1988; Green and Loewenstein, 1988; Schwarze et al., 1999) and in later publications (Klekotka et al., 2001), non-denatured proteins were used. An absolute requirement for using denatured proteins would be surprising, as the full length HIV-Tat protein, when synthesized in infected T-cells, is thought to be in a native confirmation, but still can be secreted and taken up by noninfected cells. A molten-globule-like state during the transduction would mean that the majority of the protein tertiary structure is disrupted, while most of the secondary structure is preserved, thereby exposing the interior, apolar structure of the protein (Kueltzo and Middaugh, 2000).

4.4.2 Inverted micelle formation

For the Antp peptide, others favor a model in which a crucial step in the transduction is the formation of an inverted micelle in the membrane (Berlose et al., 1996; Derossi et al., 1998a; Joliot and Prochiantz, 2004). The interaction of the protein with the membrane would cause a disruption of the bilayer, allowing the protein to move into the bilayer, covered by an inverted micelle. The micelle would then release the protein to the opposite side of the bilayer (Prochiantz, 1999; Prochiantz, 2000).

From isothermal titration calorimetry on unilamellar vesicles, Binder and Lindblom recently concluded that “asymmetrical distribution of the peptide between the outer and inner surfaces of the charged bilayer causes a transmembrane electrical field, which alters the lateral and the curvature stress acting within the membrane. At a threshold value these effects induce internalization of penetratin presumably via inversely curved transient structures” (Binder and Lindblom, 2003). NMR and other measurements, on the other hand, do not provide evidence for a non-bilayer structure during Tat-membrane interaction (Ziegler et al., 2003).

Measurements of plasmon-waveguide resonance and impedance spectra on penetratin with artificial lipid bilayer membranes show that penetratin does not penetrate into the hydrophobic core of the lipid membrane causing measurable disruption of the bilayer structure. It also does not form a pore or channel, or even an α-helical structure (Salamon et al., 2003). These experimental findings are not compatible with the concept of a formation of inverted micelles, because the formation of such micelles will change the structure of the bilayer in such a way as to alter both the lipid packing density and the refractive index anisotropy. Since PWR spectra are sensitive to both of these quantities, one would anticipate that this would be observable. G. Tollin and coworkers did not see such spectral effects, and thus concluded that such structures did not form. However, they caution that in their experiments solid-supported lipid bilayers were used, and in an actual biological membrane, or even in a vesicle, it is possible that such structures could be formed (Salamon et al., 2003, and G. Tollin, personal communication).

4.4.3 Penetration by endocytosis?

Recent studies have challenged the statement of the energy- and endosomal pathway-independence of cationic peptide translocation into eukaryotic cells (Drin et al., 2003; Koppelhus et al., 2002). By confocal microscopy, fluorescently labeled cell penetrating peptides displayed a punctuate pattern in the perinuclear space after their transduction, suggesting an accumulation within endosomal vesicles. Moreover, the translocation was strongly inhibited by energy depletion of the cells, low temperature, and known inhibitors of endocytosis. Internalized peptides were demonstrated to be in the acidic compartment and Inhibition of endosome acidification produced a marked decrease in peptide internalization (Drin et al., 2003). From those studies, the authors concluded that SynB and pAntp43-58 peptides penetrate into cells by an adsorptive-mediated endocytosis process rather than by temperature-independent translocation.

The group of M. Belting (Sandgren et al., 2002) has found that DNA and glycosaminoglycans linked to the Tat PTD accumulated in large, acidic, de-novo formed, cytoplasmic vesicles and were later transferred to the nuclear compartment. The endocytic mechanism of Tat-plasmid complex uptake was confirmed by additional studies (Ignatovich et al., 2003). By microscopy on living cells, is was also observed that the internalization of full length Tat and the Tat PTD, fused to GFP, are internalized by a slow, caveolae-mediated mechanism (Ferrari et al., 2003). As such, it is temperature dependent. Note, however, that monitoring of transduction by linking GFP to the transduction domain can be misleading, as refolding of GFP after transduction into its active conformation is not always efficient (Sacchetti et al., 2001).

For Tat-mediated cell penetration, Mauro Giacca's group has demonstrated an endocytic path by additional experiments (Fittipaldi et al., 2003). Looking at the

transduction mechanism in even more detail, they provided manifold evidence that Tat proteins are internalized by an active endocytic pathway, originating from cell membrane lipid rafts and involving caveolar endocytosis. First, Tat-GFP peptides were localized in discrete vesicles. Second, they neither colocalized with transferrin, known to be internalized from clathrin-coated invaginations from the plasma membrane, or with the early endosome antigen 1, known to be associated with early endosomes, or a marker for lysosomes. Thus, internalization is not dependent on endocytosis by clathrin-coated vesicles. Third, after internalization, the Tat proteins colocalized with cholera toxin, known to be internalized from clathrin-independent lipid rafts. Fourth, the Tat proteins colocalized with caveolin-1, a protein known to be associated with lipid raft caveolae. Fifth, the Tat-GFP containing vesicles were resistant against Triton X–100 solubilization, a feature characteristic for lipid rafts. Drugs that disrupt lipid rafts or caveolar trafficking inhibit Tat internalization, while drugs interfering with microtubules had no effect. Sixth, they did not observe internalization at 4°C. Seventh, the transduction kinetics is very slow, which would be in agreement with the caveolar pathway (Thomsen et al., 2002). In the light of these findings, aspects of the homeoprotein secretion process of might be noteworthy: Part of the cell-penetrating Engrailed-2 colocalizes with caveolin-1 and is associated with cholesterol- and glycosphingolipid-rich caveolae (Joliot et al., 1997). This is the case in COS7 cells as well as embryonic rat brains. While the largest amount of protein is associated with the outside of the caveolar membranes, a small fraction of the protein is inside the vesicles, suggesting that Engrailed-2 can translocate them (Joliot et al., 1997). Thus, cell penetrating proteins taken up by vesicular transport could later be released into the cytoplasm.

Other results confirmed an energy-dependent, endosomal pathway for Tat- and Arg_9-mediated protein delivery (Richard et al., 2003). A lack of internalization of Tat and Antp peptides, linked to oligonucleotides, at 4°C, and accumulation of the conjugate in cytoplasmic vesicles has also been observed (Astriab-Fisher et al., 2002). Uncharged antisense molecules linked to the Tat PTD and a fluorophore were also found in vesicles after their transduction as well, when examined in unfixed or mildly fixed cells (Moulton et al., 2003).

For other cell-penetrating α-helical amphipathic peptides, both temperature-sensitive as well as insensitive components of the translocation have been found (Scheller et al., 1999). By a combination of HPLC and confocal laser-scanning microscopy, the penetration of amphiphatic peptides seems to occur by both endocytic and non-endocytic mechanisms (Scheller et al., 2000). Other studies suggest that temperature-independent protein translocation is a fixation artifact, an issue that will be discussed below (paragraph 5.1). A combination of both endocytic and other mechanisms of cell penetration is also suggested by cell fractionation experiments, in which the cytosolic compartment was separated from the endocytic compartment (Zaro and Shen, 2003). The Tat peptide as well as YGR_8 was found primarily in the cytosolic compartment, while YGK_9 was mainly located in the endocytic fraction (Zaro and Shen, 2003). In these studies, only the short cell penetrating peptides, without a cargo, were examined; thus, for larger proteins, the mode and efficiency of cell transduction may be different. FGF-4 h-region mediated transduction, on the other hand, has also been demonstrated on T-lymphocytes (Torgerson et al., 1998), which do not contain caveolae (Fra et al., 1994). Different from most other PTDs discussed here, the h-region is not positively charged (cf. paragraph 2.4); thus, a different mechanism may apply in that

case. As some of the experimental results mentioned here appear to be contradictory to one another, it would be intuitive to conclude that different mechanisms may apply to different types of cell penetrating peptides and their cargo.

A simple scenario for basic-peptide mediated protein delivery was already suggested by Ryser in 1982 (Ryser et al., 1982). He observed that poly-lysine association of horseradish peroxidase (HRP) increased the rate of its uptake by three orders of magnitude and EM studies showed that the uptake of HRP alone and basic-peptide conjugated HRP occurred along the same endocytic pathway. The protein transduction facilitation by basic peptides might only lie in the strong attachment of the cationic peptide side chains to the membrane, possibly mediated by HSPGs, from which the molecules would be taken up by constitutive endocytosis. This would also take into account the fact that the viral PTDs can be replaced by a row of arginines. Such an explanation would not exclude that, at the same time, “trojan horse peptides” make their way through cellular membranes via additional pathways. From the recent evidence, it might turn out that their transduction into cells and tissues is not such a mystery after all, but rather, that it follows multiple pathways of internalization that are textbook knowledge.

5 Limitations of Tat-mediated protein transduction

Considering the potential ability of unlimited delivery into cells, the method has led to fewer publications since its incipience than might have been expected. One reason could be that once the proof of principle has been accomplished in a few high-profile journals, just the application of the methodology will not gain much further acclaim.

On the technical side, for various reasons, not all proteins can be expressed in *E.coli* sufficiently well, and eukaryotic proteins expressed in prokaryotes will lack many post-translational modifications vital for their proper function. Liposomal gene delivery methods, etc., are commercially available and may be faster for some applications.

Furthermore, the fast and equal distribution of PTD-linked molecules in the organism, tissue and cells, may be an advantage in some cases, but a serious disadvantage in others. A medication that should act locally might have side effects if it cannot be restricted to a certain compartment. Proteins that are supposed to be targeted to a cellular membrane and thus include a respective localization signal, may not be targeted to the desired site when a PTD signal is present in the same molecule. As discussed below, PTD-linked molecules that display a specific distribution have been designed.

5.1 Pitfalls when evaluating results gained using PTDs

Not all publications examining protein transduction into cells have actually proven that the protein does get transduced into the cell interior. FACS analysis, as often described in that context, might only demonstrate that the protein attaches to the cell surface, unless the cells have been extensively treated with protease. Such attachment is not surprising, as the PTD is highly basic and thus will undergo electrostatic interactions with negatively charged moieties on the cell membrane. In addition to the Tat domain, many PTD-fusion proteins also include a His_6 domain, which further increases the number of basic side chains at the N-terminus. Even if transduction is shown, it might have occurred after cells have been removed from the culture dish or after the tissue has been disrupted for analysis, as such basic proteins also have an affinity for the cell nucleus.

5.1.1 Examples for inefficient cell penetration

A number of research groups did observe an attachment of their chosen substance to the cell surface, but not a transduction into the cell. An example is the Tat PTD-.mediated attachment of diphtheria toxin A-fragment to the outside of the cell. The molecule was not cytotoxic, indicating that it did not get transduced by the Tat domain (Falnes et al., 2001). The size of the fragment is only about 21 kDa, i.e. it is much smaller than other proteins reported to be transduced. Moreover, the A-fragment is efficiently delivered into cells when associated with the toxin B-fragment, demonstrating that the A-fragment can overcome the membrane barrier when linked to the right carrier domain.

Other reports confirm that some Tat-linked compounds do attach well to the cellular membrane, but that their transduction is poor (Liu et al., 2001b). Some researchers have even stated that the Tat PTD is not able to transport an aromatic carboxylic acid, neither into artificial liposomes nor into intact MDCK cells (Krämer and Wunderli-Allenspach, 2003), and that earlier contradictory results have been due to experimental artifacts.

Leifert et al. showed that the Tat domain, when expressed in eukaryotic cells in fusion with a full length nucleoprotein or eGFP, did not enhance the secretion and subsequent uptake of the protein by bystander cells (Leifert et al., 2002). Moreover, they confirmed that methanol fixation of tissue can lead to artifacts, simulating protein transduction, when in fact none has occurred. It should be noted, however, that proteins applied in other studies were first expressed in *E. coli* and often isolated under denaturing conditions. They are likely to have a different conformation when compared

to the same proteins directly expressed in and released from eukaryotic cells. Moreover, Tat might stimulate the uptake, but not the secretion of a fused protein.

Using the Tat-PTD, a relatively short fluorescently labeled peptide, acting as an inhibitor of the protozoan parasite *Leishmania* gp63 protein, did get transduced into the parasite as well as into bone marrow macrophages (Corradin et al., 2002). However, even at concentrations as high as 5 µM, the intensity of the fluorescence in macrophages, as evaluated using confocal microscopy, remained fairly low. In *Leishmania*, the protein preferably accumulated in the nucleus and in the kinetoplast. Thus, it seems that PTD-mediated protein transduction, even of small peptides, may at times be not very efficient even in the culture dish.

In many reports, high extracellular concentrations, in the order of 10µM of PTD-peptide conjugates, are needed to observe a biologic effect (Fahraeus et al., 1998; Lindsay, 2002, for instance), which suggests that the uptake is not always as efficient as earlier studies had suggested.

Even if uptake can be demonstrated, the protein may not be active in the cell if it remains within the endocytic compartment. The Tat-Calpastatin fusion protein, for instance, is taken up by primary cortical neurons, but does not inhibit calpain-mediated spectrin breakdown because it is not released from the vesicles (Sengoku et al., 2003).

Consistent with the above concerns regarding the initial models for PTD-mediated protein transduction, it was found that Tat labeled ^{99m}TC could not pass through confluent layers of tight junctions forming epithelial cells lines, nor could it even enter the cells (Violini et al., 2002). Epithelial cells that are known not to form tight junctions, could be transduced. Moreover, provided that the ^{99m}TC remained bound to the transduction domain, it could not pass the epithelium lining the urinary bladder *in vivo*.

Some apparent protein transduction might be due to delivery across impaired membrane barrier function in damaged cells. To exclude such artifacts, assays have been designed that allow to evaluate whether proteins are delivered into functional cells, capable of protein expression (Ye et al., 2002). It was found that commercially available supramolecular delivery agents were effective as protein carriers. The Tat PTD was effective, too, while the HSV-VP22 was not.

An elegant approach to show that proteins indeed cross cellular membranes is to label the C-terminal end of the protein with a farnesylation signal (Wiedlocha et al., 1995). Enzymes required for farnesylation are only present in the cytoplasm and nucleus; thus, protein modified in this way must have crossed cellular membranes.

5.1.2 Artifacts due to cell fixation or FACS analysis with insufficient protease treatment of cells

Melan and Sluder warned already over a decade ago that “misleading apparent localizations of soluble proteins can result from their redistribution and/or differential extraction during the preparation of cells for primary antibody application” (Melan and Sluder, 1992). Thus, it is known for some time that the fixation step can have a profound effect on the result of histochemical analysis, for instance when looking at the localization of antisense oligonucleotides (Pichon et al., 1999). The use of strong fixatives is prone to lead to an overestimation of the transduced material and will result in a more diffuse rather than a vesicular staining (Moulton et al., 2003).

The theory that basic peptides initially bind to the membrane and are then translocated to the cell’s interior during later experimental processing was further investigated by performing immunocytochemistry without or with a prior fixation step (Richard et al., 2003). In the former case, Tat and Arg_9 peptides colocalized with an

endocytosis marker, while after formalin fixation, the peptides were detected in the nuclei. As described above, basic peptides possibly attach to the cell surface or are located within endosomes. Upon fixation, external and internal membrane barriers might get damaged, and the proteins would relocate to acidic structures within the cell, such as DNA. In light of such possible artifacts, some of the earlier published studies have to be reevaluated (Vives et al., 2003). Similar artifacts, erroneously suggesting delivery to the cytoplasm and nucleus, have been reported after methanol and acetone fixation of HSV-VP22 mediated protein delivery (Lundberg and Johansson, 2001; Lundberg and Johansson, 2002) as well as Tat-, R_8-, K_8-mediated delivery (Lundberg et al., 2003). Such artifacts would be in agreement with the fact that apparent transduction was observed with all cell types, at ambient temperatures, and for the retro-inverso peptides, as described above. The possibility of artifactual results was also shown for the $Antp_{43-58}$ and the SynB peptides. At 4°C, they attach to the cell surface, and then diffuse to the cell's interior during the fixation step (Drin et al., 2003).

Trypsin digestion reduces to 10-20% the amount of fluorescence registered by FACS analysis in cells that have been treated with a fluorophore linked to the Tat PTD (Richard et al., 2003). This means that much of the protein remains attached to the outside of the membrane – even after several washing steps - and does not enter the cells. A similar conclusion was reached when the Antp PTD domain was examined. Thus, protease treatment should be performed to obtain an accurate FACS quantification of transduced cell penetrating peptides (Vives et al., 2003).

On the other hand, many others have observed transduction of PTD-bound proteins when cells had not been fixed (Futaki et al., 2001b, for instance; Joliot et al., 1991a; Nori et al., 2003b; Vives et al., 1997; Waizenegger et al., 2002). In a recent

study, in which the authors were well aware of possible artifacts, transduction of the Antp, Tat and heptaarginine peptides was observed (Thoren et al., 2003). For Antp, they described an endocytic pathway, while for R_7, uptake appeared to be energy- and endocytosis–independent.

There are many studies, as discussed in this review, that have shown a biologic effect of PTD mediated protein delivery. As has already been discussed (Lundberg et al., 2003), *some* of those effects might be explained by interaction of the compounds with cell membrane receptors, function within the caveolar endosomes (Hjalm et al., 2001, for instance) or partial release of the proteins from lysosomes into the cytoplasm and nucleus.

5.2 Safety concerns

As PTD-linked compounds transduce cellular membranes, and readily penetrate the epidermis and dermis (Jin et al., 2001; Park et al., 2002a; Rothbard et al., 2000), contamination of the skin or of mucous membranes or the uptake of aerosol into the lungs is a potential hazard. Therefore, the S. F. Dowdy lab, which has been a major force in developing the method, recommends to handle Tat fusion proteins as if they were a 35S compound or a low Biological Safety level 2 infectious agent (Becker-Hapak et al., 2001).

6 Methodological considerations

6.1 Choice of constructs

Moving the transduction epitope to a different position within the sequence may result in a more efficient transduction (Li et al., 2002), which needs to be empirically determined for each specific protein.

As the His_6 tag makes proteins prone to sequestration into inclusion bodies during the expression process in *E. coli*, it may be advantageous to design expression vectors that use a different affinity tag. Alternatively, Tat fusion proteins can be purified using heparin affinity chromatography (Hakansson et al., 2001), which takes advantage of the strong binding of the basic domain to heparin-like molecules.

6.2 Protein purification

Detailed protocols for the purification of Tat fusion proteins have been worked out by the Dowdy group (Becker-Hapak et al., 2001; Vocero-Akbani et al., 2001; Vocero-Akbani et al., 2000). The problem of protein precipitation when removing the denaturant is addressed there as well. The low solubility when trying to isolate transducible fusion proteins is a common problem. This may often be due to the neutralization of the PTD with its cargo (Derossi et al., 1998a), and may be alleviated by avoiding the use of phosphate buffer, increasing ionic strength or adding a small percentage of DMSO. Solubility of recombinant proteins is a problem not specific to PTD fused proteins and thus valuable hints are given in some more general publications (Kumar et al., 1998; Samuel et al., 2000; Samuel et al., 1997; Schein, 1990, for instance).

The chemical coupling of cargo and vector via disulfide bonds, in contrast to fusion proteins synthesis in expression systems, has the advantage that polypeptides as well as oligonucleotides can be coupled to the protein transduction domain, and that the cargo is released from its vector after its transduction into the cytoplasm (Derossi et al., 1998a). The latter minimizes unwanted interactions with the cargo, and also keeps it trapped inside the cell.

6.3 Application of the PTD-linked fusion protein

Dying cells often release (negatively charged) DNA, to which the basic PTD can attach, thus decreasing transduction efficiency. In *in vitro* experiments, this problem can be diminished by concurrent DNAse treatment of the cells. In some cases, transduction of protein is more efficient in serum-free medium *in vitro*.

To examine whether a protein is just attached to cell surface heparan sulfates, rather than transduced, cultured cells may be treated with heparinase III, and supernatants, associated with the cell surface fraction, may be tested for recombinant protein by western blotting (Tasciotti et al., 2003).

The IP application of PTD-linked proteins may be enhanced by adding SDS to the vehicle (Schutze-Redelmeier et al., 1996). The detergent increases solubility of the protein, which is, as mentioned above (paragraph 6.2), often a concern when isolating proteins expressed in *E. coli*. It will also mask the positive charges on the protein surface, thus delaying uptake of the protein into the abdominal cavity and possibly enhancing its delivery across the BBB.

In some instances, when enzymes are to be transduced, it may be necessary to preincubate them with the cofactors required for their activity (Eum et al., 2002).

It is critical to know that many PTD, including Tat, Antp and R7, tightly bind to glass and plastic surfaces (Chico et al., 2003). This binding is reduced on siliconized surfaces. Surface adherence is of concern not only because of protein loss on microfuge tubes, etc. during protein isolation and application, but also because PTD that have been added to cultured cells, for instance, may mistakenly thought to be associated with the cell layer, while in fact the binding is provided by plastic-basic peptide interaction. An even more effective procedure against adsorption of basic peptides to glass and plastic

is achieved by coating the surface with poly(ethylenimine) (Brink et al., 1992; Persson et al., 2003).

7 Outlook: Improvements of the method

7.1 Improvement of transduction domains and constructs - Designing high-tech trojan horses

7.1.1 Improvement of transducibility

Artificial peptides with an α-helical, amphiphilic structure can be designed, which exhibit similar uptake characteristics like virus- or homeodomain-derived peptides (Oehlke et al., 1998). In some cases, however, such peptides turn out to be toxic (Kardinal et al., 2000).

Arguing that the capability of the HIV-Tat protein to transverse biological membranes might be a coincidence, rather than a viral feature that has been exposed to evolutionary selective pressures, the Dowdy group has undertaken efforts to synthetically optimize the protein transduction potential of the Tat PTD (Ho et al., 2001). The Tat PTD has an α-helical structure with basic amino acids on one face and hydrophobic residues on the opposite one. Dowdy and coworkers designed an “ideal” peptide that enhanced these structural motives and indeed showed that those peptides exhibited an improvement of protein transduction potential *in vitro* and *in vivo*. However, it should be noted that protein transduction was examined by FACS or fluorescent microscopy on fixed cells, which does not clearly distinguish between attachment of the peptide to the cell surface and its transduction into its interior (cf. 5.1). Whether the enhanced fluorescence observed in blood cells *in vivo* is accompanied by a higher delivery into tissues including the brain, remains to be determined.

Using a similar line of argument, an “ideal” peptide was also derived from the Antp sequence (Williams et al., 1997). It has a predicted α-helical structure, consisting solely of arginine amino acids on one face of the helix and tryptophane on the other, providing a positive charge on the former and a hydrophobic face on the latter. It was an efficient ferry for a Grb2-binding peptide (Williams et al., 1997). Another modified version of the Tat and Antp PTDs was designed to enhance the uptake and efficiency of the C3 bacterial Rho GTPase inhibitor (Bertrand and McKerracher, 2003; Winton et al., 2003). Unfortunately, the sequence of that new PTD is kept secret.

Fischer *et al.* found that the 7 C-terminal amino acids of the penetratin sequence are already sufficient for transduction *in vitro* (Fischer et al., 2000). Moreover, amino acid substitutions in the peptide led to an either preferred localization in the nucleus or in the cytoplasm. This system is successfully used to deliver peptides into cells (Schaschke et al., 2002, for instance)

The translocation of a poly-lysine or polyarginine peptide was reported to be as efficient as the transduction of the Tat peptide (Park et al., 2002b). Other cationic peptides have been shown to be efficient transducers as well (Mi et al., 2000). However, in such studies, which evaluate fluorescent microscopic pictures or FACS analysis, need to be carefully reevaluated for artifacts, as discussed above (5.1).

For use in a mouse macrophage cell line, transduction seems to be optimal for a peptide with 8 arginines in a row (Futaki et al., 2001b), a molecule that can readily be synthesized (Wender et al., 2001). Longer arginine stretches cause the protein to attach to the membrane, with very little transduced into the nucleus. In Jurkat cells, the optimal length for efficient protein transduction seems to be 9 consecutive arginines. This result was later confirmed when comparing 42 different ^{99m}Tc labeled peptides (Gammon et

al., 2003), although that study did not clearly distinguish between attachment of the peptide to the membrane and penetration into the cell's interior. Derivatives of heptaarginine, interspersed by other amino acids, are also efficient transduction domains (Rothbard et al., 2002).

In chemically synthesized peptides, some studies suggest that the use of D-amino acid transduction domains is to be preferred over the corresponding L-isomers, as the naturally not occurring D-peptides display a higher stability, e.g. in serum medium (Wender et al., 2000). However, decreased degradation does not account for all of the increase in cellular accumulation of the D-enantiomere (Gammon et al., 2003). On the other hand, some studies suggest a *decreased* stability of D-peptides in serum medium (S. Feller, Univ. of Oxford, UK, pers. communication). These properties also seem to depend on the type of PTD and cargo.

If PTD delivery occurs by the endosomal pathway, both efficacy and the range of proteins and cell types that can be targeted could be increased through strategies that promote release from the vesicles, as has been suggested for DNA transfection by loligomers (Singh et al., 1999) and for protein delivery (Green et al., 2003).

7.1.2 Specific delivery using PTDs

PTD-linked molecules distribute evenly throughout tissues and cells. This can be desired, but often, one would like to have a means to direct a molecule to a specific compartment. Using different branched amino acids, it may be possible to design a variety of peptides that direct themselves, including a cargo, to a desired organelle (Futaki et al., 2002).

The recently confirmed role of glycosaminoglycans in PTD mediated uptake may allow to direct proteins to specific target tissues (Console et al., 2003).

Mi et al. have screened an M13 phage display peptide library and identified a PTD specific for synovial cells (Mi et al., 2003); a procedure that might be applicable to identify peptides specific for other cell types and tissues.

Another strategy is to cleave off the transduction domain by including an organelle-specific cleavage recognition site. Thus, the PTD-linked cargo would accumulate in the respective compartment. This elegant strategy has been achieved using a mitochondria-specific signal sequence (Del Gaizo and Mark Payne, 2003). Thereby the cargo molecule was enriched in mitochondria *in vitro* and, after IP injection, *in vivo* in the heart, kidney, liver and brain. It is also possible to design peptides that specifically act in tumor cells, while not affecting normal cells (Chen et al., 1999).

7.2 Using different expression systems

To allow efficient expression, cloning vectors containing the Tat domain using codons preferred by *E. coli*, the HIS tag optionally fused to the N- or C-term and with polylinkers very convenient for cloning the respective fragment, both in eukaryotic and prokaryotic systems have been designed (Yang et al., 2002b). An additional glycine residue to aid free bond rotation between the transduction domain and the fusion protein might also enhance transduction.

It is noteworthy that the application of Tat fusion proteins expressed in eukaryotic cells by transient transfection apparently leads to an accumulation of the gene products in the nuclei, in particular in the nucleolus, rather than in the cytoplasm. This is different from the exogenous application of *E.coli*-produced proteins on cultured cells (Yang et al., 2002b). Tat fusion proteins reextracted from transfected eukaryotic cells were unable to transfect cells *in vitro*. Possibly, this is due to the fact that the HIV-Tat RNA-binding domain at lysine residue 50, which is also part of the PTD, is acetylated when expressed

in eukaryotic cells (Dormeyer et al., 2003) or the consequence of another posttranslational modification.

7.3 High throughput screening of peptide function

Methods to synthesize peptides consisting of a transduction domain and a peptide to be functionally tested with high purity and in large numbers are available (Awasthi and Nielsen, 2002; Zhang et al., 1998). Thus, we now have the tools in our hands to screen short peptides for their function within the cell.

8 Concluding remarks

We have attempted here to give an extensive overview about different aspects of PTD-mediated substance delivery and to list many publications of this growing field of research. In spite of the critical aspects of the technique mentioned, we believe that it is in the realm of possibility to improve tailor-made PTD peptides, so that they can be targeted to specific cells and organelles. PTD of the future may be smaller in size and thus show less interference with the molecule to be examined, allow easier expression, purification, and efficient delivery even of large molecules *in vivo*. PTD-mediated molecule delivery will play an increasing role in therapeutics and functional analysis.

Table 1: Protein Transduction Domain (PTD)-mediated Transport

General Topic	Cargo	Effect	*In vivo* ?	PTD	Literature
βAPP function	15 or 25 nt antisense βAPP	decrease in axon and dendrite outgrowth in primary cortical neurons	n	Antp	(Allinquant et al., 1995)
5HT receptor signal transduction	5HT R – G-protein interacting site, PLCβ and Phosducin like prot. derived peptides	inhibition of phosphatidylinositide hydrolysis and other signal transduction cascades	n	FGF-4	(Chang et al., 2000a)
Adhesion receptors	integrinβ1 and β3 peptide fragments	functional analysis in erythroleukemia and endothelial cells and fibroblasts	n	h region of hu integrinβ3	(Liu et al., 1996)
Angiogenesis	PAK proline-rich region	inhibition of endothelial cell migration and contractility	n	Tat	(Kiosses et al., 2002).

antibiotics delivery	benzylpenicillin	drug delivery across BBB by *in situ* brain perfusion	y	SynB1 (protegrin derivative)	(Rousselle et al., 2002)
Antibody delivery	anti-tumor antibody FAB fragments	delivery into breast carcinoma cell line	n	HIV-Tat aa 37-62	(Anderson et al., 1993)
Antibody delivery	IgG2a, IgG1 IgG	Delivery into 3T3 fibroblasts	n	Kaposi FGF	(Zhao et al., 2001)
Anticancer drug delivery	Bcl-2 BH-3 domain	enhanced apoptosis in ovarian carcinoma cells after DOX treatment	n	Antp	(Minko et al., 2003)
Anticancer drug delivery	E2F DNA binding inhibiting peptides	induction of apoptosis, inhibition of proliferation in tumor cells	n	Tat	(Montigiani et al., 2003)
Anti-cancer treatment	cytosine deaminase suicide gene	enhanced cytotoxicity in melanoma cells	n	HSV-VP22, adeno viral delivery	(Wybranietz et al., 2001)

anti-cancer treatment	p21(WAF1) derived peptides	growth inhibition in ovarian cancer cell lines	n	Antp	(Bonfanti et al., 1997)
Anti-cancer treatment	p53-derived peptide	binding to mdm-2, specific cytotoxicity in tumor cell lines	n	Antp	(Kanovsky et al., 2001)
Anti-cancer treatment	PNA against telomerase	telomerase inhibition in melanoma cell line	n	Antp	(Villa et al., 2000)
Anti-cancer treatment	short Purα-derived peptides	inhibition of melanoma proliferation	n	Tat	(Darbinian et al., 2001)
Anti-cancer treatment	SoS Grb2SH3 binding derived peptide	inhibition of proliferation of a CML cell line	n	Antp	(Kardinal et al., 2001)
Anti-cancer treatment	SoS1 CRKLSH3 binding derived peptide	inhibition of proliferation of primary CML blast cells	n	Antp	(Kardinal et al., 2000)
Anti-cancer treatment	thymidine kinase, EGFP	enhanced cytotoxicity of gancyclovir	n	HSV-VP22	(Liu et al., 2001a)
Antigen	472 aa of	transduction of dendritic cells *in*	y	Tat	(Shibagaki

introduction into dendritic cells	tyrosinase-related protein 2	*vitro*, protection against melanoma *in vivo*			and Udey, 2003)
Antigen introduction into dendritic cells	influenza matrix protein epitope, melanoma antigen gp100 epitope	induction of antigen-specific cell types	n	Tat	(Tanaka et al., 2003)
Antigen presentation	tumor-associated antigen OVA	antigen presentation to CD4 T-cells, tumor regression in mice	ex vivo	Tat	(Shibagaki and Udey, 2002)
Anti-HIV therapy	TAR-targeted PNA	inhibition of Tat mediated transactivation, inhibition of HIV production in infected H9 cells	n	transportan	(Kaushik et al., 2002)
anti-neoplastic drug delivery	20 aa of CDKN2/INK4A	inhibition of S-phase entry in HaCaT cells	n	AntpHD	(Fahraeus et al., 1996)
anti-neoplastic drug delivery	cdk2 inhibitory peptide	apoptosis induction in transformed cells	n	pAntp	(Chen et al., 1999)
anti-	doxorubicin	cytotoxicity in multidrug-resistant	y	SynB1	(Mazel et al.,

neoplastic drug delivery		tumor cells in vitro, delivery across BBB by *in situ* brain perfusion			2001)
anti-neoplastic drug delivery	doxorubicin	drug delivery across BBB by *in situ* brain perfusion and intravenous application	y	SynB1, Penetratin	(Rousselle et al., 2000; Rousselle et al., 2001)
anti-neoplastic drug delivery	doxorubicin, FITC	delivery into ovarian carcinoma cell line	n	HPMA-Tat	(Nori et al., 2003a; Nori et al., 2003b)
anti-neoplastic drug delivery	HSV thymidine kinase	cell death by ganciclovir in neuroblastoma cell line, tumor regression in mice	y	HSV-1 VP22	(Dilber et al., 1999)
anti-neoplastic drug delivery	p53	apoptosis induction in osteosarcoma cells	n	HSV-1 VP22	(Phelan et al., 1998)
anti-neoplastic drug delivery	phosphorothioate oligonucleotide targeted to *MDR1* gene	inhibition of cell surface expression of P-glycoprotein	n	Tat and pAntp	(Astriab-Fisher et al., 2000)
anti-neoplastic	Pseudomonas exotoxin	cytotoxic, anti-angiogenic, anti-migratory activity on	n	aFGF	(Merwin et al., 1992)

drug delivery		microvascular endothelia			
anti-neoplastic drug delivery	Pseudomonas exotoxin	growth inhibition of mouse carcinoma xenografts	y	aFGF	(Siegall et al., 1994)
anti-neoplastic drug delivery	repressor protein E2 of HPV-16	E2 dep. reporter gene expression in COS7 cells	n	Tat	(Pepinsky et al., 1994)
anti-neoplastic drug delivery	saporin	tumor growth inhibition, anti-metastatic activity in a melanoma model	y	FGF2	(Lappi et al., 1994; Ying et al., 1994)
Anti-neoplastic drug delivery	Smac/Diabolo	enhancement of apoptosis and anti-proliferative effect in breast cancer cells	n	Antp	(Arnt et al., 2002)
Antisense delivery	17 mer anti-myc PNA	inhibition of myc expression, apoptosis, cell cycle in Burkitt's lymphoma cells	n	NLS	(Cutrona et al., 2000)
Anti-tumor drug delivery	Oxygen-dependent degradation-β-Gal and Caspase 3	solid tumor size reduction	y	Tat	(Harada et al., 2002)
Anti-tumor therapy	p53	apoptosis induction in breast cancer cells	n	Tat, Antp	(Li et al., 2002)

Anti-tumor therapy	T-cell peptide TRP2	delivery into dendritic cells, tumor; inhibition of B16 tumor and lung metastasis in mice	y	Tat	(Wang et al., 2002)
Anti-tumor therapy	thymidine kinase	increased apoptosis HEK293 cells by aciclovir treatment	n	Tat	(Tasciotti et al., 2003)
Anti-tumor treatment	papilloma virus antigen	increased CD8+-mediated anti-tumor response	n	HSV-VP22	(Cheng et al., 2002)
Apoptosis	11 amino acid peptide	inhibition of NFκB activation, apoptosis acceleration in polymorphonuclear neutrophils	n	Tat	(Choi et al., 2003b)
Apoptosis	6aa ICE inhibitor	Rescue of PC12 cells from apoptosis induction	n	pAntp	(Troy et al., 1996b)
Apoptosis	BAD BH3 domain	Apoptosis induction in 32D cells	n	Antp	(Schimmer et al., 2001)
Apoptosis	Bak BH3 domain peptide	death induction of HeLa cells	n	Antennapedia	(Holinger et al., 1999)
Apoptosis	Bcl-X_L	apoptosis inhibition in cerebellar granule neurons and macrophages and axotomized retinal ganglion cells	y	254 aa anthrax toxin derivative	(Liu et al., 2001c)
Apoptosis	Bcl-X_L	staurosporine, poliovirus and	n	Diphth	(Liu et al.,

		radiation induced apoptosis inhibition in different cell lines		eria toxin R binding domai n	1999b)
Apoptosis	Bcl-x_L	protection in various models of cerebral infarct	y	Tat	(Kilic et al., 2002)
Apoptosis	Bcl-x_L	protection from cerebral infarct	y	Tat	(Cao et al., 2002)
Apoptosis	Bcl-x_L	Rescue of cerebellar granule cells after K^+ and serum deprivation; protection in nerve trauma model	y	Tat	(Dietz et al., 2002)
Apoptosis	Bcl-X_L, β-Gal, PEA-15	Inhibition of TNF-α-induced apoptosis in a pancreatic β-cell line	n	Tat	(Embury et al., 2001)
Apoptosis	calpastatin	inhibition of calpain activation in neurons	n	R_{11}	(Wu et al., 2003a)
Apoptosis	Cathepsin B inhibitor	Delivery into MCS-7 cells	n	Antp heptap eptide	(Schaschke et al., 2002)
Apoptosis	dn H-Ras	inhibition of IL-5 induced survival of blood eosinophils	n	Tat	(Hall et al., 2001)

Apoptosis	E2F-1; p73 DN and inactive; p53 DN; GFP	inhibition of T-cell receptor mediated apoptosis in T-cells;	n	Tat	(Lissy et al., 2000)
Apoptosis	eIF4E-binding protein and eIF4G derived peptides	Apoptosis induction in MRC5 cells	n	Antp	(Herbert et al., 2000)
Apoptosis	FLIP	resistance to Fas-mediated apoptosis in primary T-cells	n	Tat	(Algeciras-Schimnich et al., 1999)
Apoptosis	IκBα dominant negative	B-cell Ag receptor signaling block in primary murine B-cells	n	Tat	(Schram and Rothstein, 2003)
Apoptosis	modified Caspase-3 protein	Death of HIV infected Jurkat T cells	n	Tat	(Vocero-Akbani et al., 1999)
Apoptosis	neuroamidase	Apoptosis induction in HeLa cells	n	HSV-VP22	(Morris et al., 2002)
Apoptosis	NLS of NF-κB (SN50	inhibition of dsRNA-induced apoptosis in β-cells	n	h-region	(Robbins et al., 2003)

	peptide)			of FGF-4	
Apoptosis	p16, HPV-E7	CDK4/6 inhibition, phosphorylation of pRB, sequestering of pRB from E2F-1	n	Tat	(Lissy et al., 1998)
Apoptosis	p53 suppressor	Inhibition of apoptosis	n	Antp	(Mittelman and Gudkov, 1999)
Apoptosis	single chain antibody against BH1 domain	Bcl-2 inhibition in mast cells and a breast cancer cell line	n	Tat	(Cohen-Saidon et al., 2003)
Apoptosis inhibition	FNK, a Bcl-x_L derivative	protection of neurons and neuroblastoma against staurosporine or Glutamate *in vitro*, reduction in cerebral ischemic damage	y	Tat	(Asoh et al., 2002)
Apoptosis inhibition	inhibitory domain of calpastatin	inhibition of calpain activation; Apoptosis inhibition in LCLC 103H cells	n	pAntp	(Gil-Parrado et al., 2003)
Apoptosis, HIV pathology,	(FITC-tagged) β-galactosidase	fluorescence in 293T and Jurkat T cells, G2 cell cycle arrest, apoptosis induction	n	HIV sVpr full	(Sherman et al., 2002)

cell cycle	, full-length sVPR			length protein	
Atherosclerosis	APOBEC-1	increase in VLDL secretion in primary hepatocytes by apoB mRNA editing	n	Tat	(Yang et al., 2002a)
Axon guidance	PKC pseudosubstrate	neural growth cone collapse	n	pAntp	(Theodore et al., 1995)
Axon regeneration	C3 bacterial Rho GTPase inhibitor	enhanced axon outgrowth	y	secret synthetic sequence	(Bertrand and McKerracher, 2003; Winton et al., 2003)
Bone development	hemagglutinin, calcineurinα	osteoblast differentiation, inhibition of osteoclast resorption	n	Tat	(Dolgilevich et al., 2002)
Bone development and turnover	calcineurin Aα	attenuation of osteoclastic bone resorption *in vitro*	n	Tat	(Sun et al., 2003)
Bone development and turnover	const. active and dom. neg. Rho	stress fiber formation or block of podosome formation	n	Tat	(Chellaiah et al., 2000)
Bone	IκBα mutant	Inhibition of osteoclastogenesis	n	Tat	(Abu-Amer et

development and turnover	protein				al., 2001)
Cancer vaccine	fluorescently labeled peptides	delivery of peptides to antigen presenting cells	n	fluorescence microscopy in fixed cells, FACS w/o trp	(Buschle et al., 1997)
Cardioprotection	ψεRACK	ischemia reduction in isolated hearts	n	Arg_7	(Chen et al., 2001b)
Cardioprotection	εPKC derived peptide	Reduction of ischemia	y	Arg_7	(Chen et al., 2001a)
Cardioprotection	δPKC inhibitor and εPKC activator	synergistic ischemia Cardioprotection by both peptides	n	Tat	(Inagaki et al., 2003)
Cardioprotection	BH4 domain of Bcl-x_L	decrease in damage after ischemia on excised hearts	n	Tat	(Chen et al., 2002b)
Ca-sensing receptor signaling	filamin derived peptide,	inhibition of ERK1/2MAPK activation in HEK	n	Tat	(Hjalm et al., 2001)

	interacting with CaR				
Cell cycle	Cdk-2	re-entry into S-phase in transfected cell line	n	Tat	(Klekotka et al., 2001)
Cell cycle	cdk4/6 inhibitor	p21-/- complementation in oligodendrocyte precursor cells	n	Tat	(Zezula et al., 2001)
Cell cycle	Cyclin E, Cyclin D3	restoration of proliferation response in c-Rel$^{-/-}$ B cells by Cyclin E	n	Tat	(Hsia et al., 2002)
Cell cycle	INK4A derived peptide	inhibition of pRB phosphorylation; G1 arrest in pancreatic cancer cell lines	n	Antp	(Fujimoto et al., 2000)
Cell cycle	key cell cycle regulator p21	Cell cycle arrest, differentiation in NKNKT-3 hepatocyte cells	n	Tat	(Kunieda et al., 2002)
Cell cycle	p14^{INK4a} protein (cdk4/6 inhib.)	G_1 arrest in primary fibroblasts	n	Antenn apedia	(Kato et al., 1998)
Cell cycle	p16(INK4A)	inhibition of CDK4/6/cyclinD and G0 to G1 transition in T-cells	n	Tat	(Lea et al., 2003b)
Cell cycle	p16^{INK4a} protein (cdk4/6	prevention of pRB hypophosphorylation, G_1 arrest	n	Tat	(Ezhevsky et al., 2001; Ezhevsky et

	inhib.) dom-neg Cdk2, E1A				al., 1997; Gius et al., 1999)
Cell cycle	peptide derivatives of INK4a tumor suppressor	block of S-phase entry in HaCaT cells	n	Antp	(Fahraeus et al., 1998)
Cell cycle	PI3 K SH2 binding domain of PDGFRβ	stimulation of S-phase entry in C2 muscle cell line	n	Antp	(Derossi et al., 1998b)
Cell cycle	SSeCKS cyclin binding motif and derivatives	Cyclin D 1 release	n	Antp	(Lin et al., 2000)
Cell cycle, cell migration	$p27^{KIP1}$ protein (CDK2 inhibitor)	G_1 arrest in Jurkat T cells migration in HepG2 carcinoma cells	n	Tat	(Nagahara et al., 1998)
Cell cycle, Ras signal transduction	SOS-derived peptidimer	block of Grb-2-SOS binding and MAPK phosphorylation in fibroblasts and NGF-induced PC-12 differentiation, colony	n	Antp	(Cussac et al., 1999)

		inhibition in transformed cells			
Cell cycle, signal transduction	Pro-rich domain of PAK-1	block of PAK1-Grb2 association, decreases EGF-induced extension of membrane lamellae in HaCaT cells	n	Tat	(Puto et al., 2003)
cell cycle/anti-cancer treatment	c-myc helix1-derived peptide	inhibition myc signaling and of proliferation, apoptosis induction in breast cancer cells	n	Antp	(Giorello et al., 1998)
cell cycle/anti-cancer treatment	waf1/cip derived peptides	CyclinD1/Cdk4, CyclinE/Cdk2 inhibition, necrosis induction in lymphoma cells	n	Antp	(Mutoh et al., 1999)
Cell survival	PI3 K SH2 binding domain of PDGFRβ	protection of cereb. gran. cells from serum deprivation	n	Antp	(Williams and Doherty, 1999)
Cell transfection	reporter plasmid DNA	luciferase expression in COS-7 and bronchoepithelial cells	n	$(Tat)_2$ and $(Tat)_3$ oligomers	(Rudolph et al., 2003)
Conditional	Cre	Lox-P mediated recombination	y	FGF-4	(Jo et al.,

knockouts	Recombinase	in different cell lines and in mouse tissue after IP injection			2001)
Conditional knockouts	Cre recombinase	Lox-P mediated recombination in a leukemia reporter cell line, primary mouse fibroblasts, ES and B-cells	n	Tat	(Hashimoto et al., 2002; Joshi et al., 2002)
Conditional knockouts	Cre recombinase	Lox-P mediated recombination and repression of luciferase reporter by siRNA	n	Tat-NLS	(Kasim et al., 2003)
Conditional mutagenesis	Cre recombinase	lox-P recombination in fibroblast and ES cells, primary spleenocytes	n	Tat	(Peitz et al., 2002)
CTL activation	HLA-Cw3 CTL epitope	CTL priming in mice	y	Antp	(Schutze-Redelmeier et al., 1996)
Cytoprotection	HSF70	in HSF-/- cells protection against thermal stress and hyperoxia	n	Tat	(Wheeler et al., 2003)
Decidual cell differentiation	NLS of NF-κB (SN50 peptide)	induction of stromelysin-1 expression in stromal fibroblasts	n	h-region of FGF-4	(Strakova et al., 2003)
Delivery into the retina	GFP	delivery into ocular cell lines and retinal layers *in vivo*	y	HSV-VP22,	(Cashman et al., 2002)

				adeno viral deliver y	
DNA transfection	GFP encoding plasmid	GFP expression in NIH/3T3 fibroblasts and rat H9C2 cardiomyocytes *in vitro* and in Lewis lung carcinoma tumor *in vivo*	y	Tat-liposo me associ ation	(Torchilin et al., 2003)
DNA transfection	GFP reporter plasmid	Enhanced lipofectamine transfection of endothelial cells	n	Antp	(Ou et al., 2003)
DNA Transfection	plasmid DNA	Reporter gene expression in CHO cells	n	loligom er 4	(Singh et al., 1999)
DNA transfection	reporter plasmid	β-gal expression in COS-1, 3T3, BHK, and endothelial cells	n	poly-lysine-conjug ated bFGF	(Sosnowski et al., 1996)
Drug delivery	HPMA copolymers	transduction into cytoplasm and nucleus of Hep G2 and A2780 cells	n	Tat	(Jensen et al., 2003)
Drug resistance	methotrexate	growth inhibition of Chinese hamster ovary cells	n	poly-lysine	(Ryser and Shen, 1980)

EGF signal transduction	Grb2SH2	inhibition of EGF-induced EGFR-Grb2 association and MAPK activation	n	12 aa from h signal sequence of Kaposi FGF	(Rojas et al., 1998)
EGF, PDGF signal transduction	EGFR Grb2 binding site	inhibition of EGF and PDGF stimulated MAPK phosphorylation in myoblasts	n	arginine/tryptophane 16 aa peptide	(Williams et al., 1997)
EGFR signaling	EGFR autophospory lation site	reduced Ras and MAPK activation in NIH-3T3 cells overexpressing EGFR	n	12 aa from h signal sequence of Kaposi FGF	(Rojas et al., 1996)
Endothelial cell migration	Rho, Rac, Cdc42	VEGF-induced migration of endothelial cells	n	Tat	(Soga et al., 2001)

eNOS regulation	Caveolin-1	inhibition of hydraulic conductivity in venular microvessels	n	Antp	(Zhu et al., 2003)
Eosinophil migration	RhoA	Detachment of Eosinophils in the presence of chemoattractant	n	Tat	(Alblas et al., 2001)
Ethanol effects	NLS of NF-κB (SN50 peptide)	inhibition of carbachol-induced astrocytoma proliferation and p65 nuclear translocation	n	h-region of FGF-4	(Guizzetti et al., 2003)
Ethanol effects	RACK-1 and derived peptides, receptor for activated C kinase	c-fos and PAC-1 expression in C6 glioma cells	n	Tat	(He et al., 2002)
FGF-1 signal transduction	FGF-1 NLS	DNA synthesis in NIH-3T3 cells	n	12 aa from h signal sequence of Kaposi FGF	(Lin et al., 1996)
FGFR signal	FGFR	inhibition of FGF stimulated PI	n	Antp	(Hall et al.,

transduction	binding site for PLCγ	hydrolysis and neurite outgrowth in cerebellar neurons			1996)
FGFR1 signal transduction	9aa phosphopeptide PLCγ inhibitor	inhibition of neurite outgrowth	n	Antp	(Saffell et al., 1997)
Fructose-Diphosphate metabolism	14-3-3 9 aa phosphopeptide	block of phosphofructokinase-2 to 14-3-3 protein in HeLa cells	n	Antp	(Pozuelo Rubio et al., 2003)
galanin signal transduction	PNA complementary to the galanin receptor	Block of galanin receptor expression in Bowes cells, suppression of galanin signaling *in vivo*	y	pAntp, transportan	(Pooga et al., 1998c)
GDH disorders	50 kDa glutamate dehydrogenase	activity in PC-12 cells	n	Tat	(Yoon et al., 2002)
Gene delivery into brain	luciferase and β-Gal	reporter gene expression in primate brain after intravenous application	y	liposome with human insulin	(Zhang et al., 2003c)

				recept or antibo dy	
Gene delivery into the eye	β-Gal expression vector	reporter gene expression in the mouse eye after intravenous application	y	liposo me with an antibo dy against the transfe rrin recept or	(Zhu et al., 2002)
Gene silencing	oligonucleotid e	delivery of fluorescently labeled ODN	n	MPG (HIV-gp41/S V40 T-ag NLS)	(Morris et al., 1997)
Gene silencing	uncharged antisense	translocation of a fluorescein-PMO conjugate, suppression of	n	Tat, Pep-1,	(Moulton et al., 2003)

	molecules	myc-regulated luciferase, correction of aberrant splicing into HeLa cells		NLS	
G-protein signaling	G-protein βγ subunit binding peptides	block of G-protein interaction in primary arterial smooth muscle cells; kinase 1/2 activation, MAPK, Jun phosphorylation, PLPC activity; release of Ca^{2+} from internal stores	n	Tat	(Goubaeva et al., 2003)
GTPase signal transduction	dominant negative Rac-1	block of Tiam-1 induced membrane ruffling and lamellipodia	n	Tat	(Wennerberg et al., 2002)
Hematopoietic cell transduction	GFP	fast transduction into different terminally differentiated hematopoietic cells and myeloblasts *in vitro*	n	modified Tat	(Lea et al., 2003a)
HIV-Tat function	full length Tat	LTR activation	n	full-length Tat	(Marzio et al., 1998)
Hormone-induced steroidogenesis	benzodiazepine receptor (outer mitoch. membrane)	Inhibition of receptor function and of steroid production in mouse tumor Leydig cells	n	Tat	(Gazouli et al., 2002)

	binding peptides				
Hyaluronan signaling	CD-44 derived peptides	blocking of CD-44 mediated cell migration	n	Antp	(Peck and Isacke, 1998)
Hydrogen Peroxide Signal transduction	catalase	catalase activity in rat lung L2 cells, inhibition of ELK and ERK phosphorylation	n	Tat	(Watanabe et al., 2003)
Inflammation	gp91^{phox} docking sequence=9a a NAD(P)H oxidase inhibitor	decreased angiotensin II-induced leukocyte infiltration in the rat aorta	y	Tat	(Liu et al., 2003)
Inflammation	ICAM-1 peptides	inhibition of transendothelial lymphocyte migration	n	Antp	(Greenwood et al., 2003)
Inflammation	IKKβ C-term. 11 aa peptide	Inhibition of NFκB activation	y	Antp	(May et al., 2000)
Inflammation	IKKβ-C-term. peptide	inhibition of TNFα-induced NF–κB activation in HeLa cells and inhibition of inflammation *in vivo*	y	Antp	(May et al., 2000)
Insulin	syntaxin H3	decrease in insulin release in	n	Tat	(Ohara-

release	domain	pancreatic MIN6β cells			Imaizumi et al., 2002)
Insulin secretion	phosphotyrosine phosphatase antisense PNA	increase in glucose-induced insulin secretion in mutant rat pancreatic islets	n	transportan	(Ostenson et al., 2002)
Ischemia	9 amino acid peptide from the NR2B receptor, PSD-95 PDZ 1,2 domains	block of NMDAR-PSD-95 interaction, decrease in ischemic brain damage	y	Tat	(Aarts et al., 2002)
Ischemia	Bcl-x_L	protection in various models of cerebral infarct	y	Tat	(Kilic et al., 2002)
Ischemia	BDNF	protection in various models of brain infarct	y	transferrin antibody	(Pardridge, 2002a)
Ischemia	GDNF	neuroprotection after systemic application in a brain infarct model	y	Tat	(Kilic et al., 2003)
Ischemia	Hypoxia-	induction of HIF-dependent	n	Tat	(Willam et al.,

	inducible factor 1	genes in endothelial cells, angiogenic response			2002)
Ischemia, Blood brain barrier	bFGF	2/3 reduction in brain infarct size after intravenous injection	y	transferrin receptor	(Song et al., 2002)
Ischemia	20 aa c-Jun N-terminal kinase binding motif	neuroprotection when applied after transient and permanent MCAO occlusion	y	Tat	(Borsello et al., 2003)
Ischemic preconditioning	PKC derived peptides	inhibition of RACK function, PCK signaling and preconditioning protection in cardiomyocytes	n	Antp	(Liu et al., 1999a)
Kinase inhibition	cGMP-dependent protein kinase Iα	inhibition of NO-induced cerebral artery dilation	n	Tat, Antp	(Dostmann et al., 2000)
Leishmaniasis	MacMARCKS effector domain (from gp63)	transduction into parasites and macroph., leishmanolysin inhibition in the parasites	n	Tat	(Corradin et al., 2002)
LTP	EGFP, PKA inhibitory	inhibition of CREB and Ser845 phosphorylation, block of L-LTP	n	R8 + SV40	(Matsushita et al., 2001)

	peptide	in hippocampal slice culture		NLS	
Lysosomal storage disease	β-glucuronidase	gene therapy in mucopolysaccharidosis type VII mice	y	adeno virally delivered Tat	(Elliger et al., 2002)
Lysosomal storage disease	β-glucuronidase	efficient distribution in the mouse brain	y	Tat and inverse Tat, protein expressed in adeno virus	(Xia et al., 2001)
Malaria treatment	spectrin-binding domain of ankyrin	inhibition of trophozoites and schizont maturation in red blood cells	n	Antp	(Dhawan et al., 2003)
Malignant transformation	minimum CDC-42 binding domain of ACK1	growth inhibition of ras-transformed NIH-3T3 fibroblasts	n	WR peptide	(Nur-E-Kamal et al., 1999)

Mitochondrial targeting	mitochondrial malate dehydrogenase signal sequence-eGFP	NIH-3T3 and PC12 cells, and after IP application *in vivo* in liver, heart, kidney, brain mitochondria	y	Tat-mMDH	(Del Gaizo and Mark Payne, 2003)
Mitogen signal transduction	Grb10 SH2 and SH3 domain peptides	Inhibition of mitogen-induced DNA Synthesis and proliferation in 3T3 fibroblasts	n	Antp	(Wang et al., 1999)
Mitogen signal transduction	PSM-SH3 domain	inhibition of growth factor-induced DNA synthesis in fibroblast cell lines	n	Antp	(Riedel et al., 2000)
Molecular imaging	^{99m}Tc	Quick accumulation in Jurkat cells, distribution in the body after intravenous application, useful imaging agent	y	Tat	(Bullok et al., 2002; Polyakov et al., 2000)
MRI contrast enhancement in tumor cells	c-myc-spec. PNA-Gd^{3+} complex	increase in signal intensity HeLa cells and rat prostate adenocarcinoma	y	Antp	(Heckl et al., 2003)
Myocardial infarction	apoptosis repressor	protection of H9C2 cells against H_2O_2, protection of isolated heart	n	Tat	(Gustafsson et al., 2002)

	with caspase recruitment domain	against ischemia			
Nerve trauma	GDNF	apoptosis inhibition in retinal ganglion cells after axotomy	y	Tat	(Kilic et al., 2004)
Neuronal Degeneration	Calpastatin	Inhibition of spectrin in a cell-free system, but NOT in primary neurons	n	Tat	(Sengoku et al., 2003)
Neuronal differentiation	Antp 60 aa homeodomain	differentiation in neuronal culture	n	pAntp	(Joliot et al., 1991a)
Neuronal excitotoxicity	NLS of NF-κB (SN50 peptide)	reduction in quinolinic acid-induced cell death in the striatum	y	h-region of FGF-4	(Qin et al., 1998)
Neuronal plasticity	PKA inhibitory peptide	Inhibition of long-lasting LTP in brain slices	n	11R	(Matsushita et al., 2001)
Neutrophil activation	C-term of tyrosine kinase Pyk-2	inhibition of adherent neutrophil activation by TNF	n	Tat	(Han et al., 2003)
Neutrophil migration	β_3 integrin cytoplasmic	inhibition of transmatrix migration of neutrophils	n	Tat	(Bruyninckx et al., 2001)

	tail peptides				
NFκB signal transduction	superrepress or IκBα	inhibition of NFκB activation in HeLa cells	n	Tat	(Kabouridis et al., 2002)
NF-κB signal transduction	NLS of NF-κB (SN50 peptide)	inhibition of integrin α2 induction by collagen in 3T3 cells	n	h-region of FGF-4	(Xu et al., 1998)
NF-κB signal transduction	NLS of NF-κB (SN50 peptide)	inhibition of proinflammatory hu endothelial cell activation	n	h-region of FGF-4	(Kilgore et al., 1997)
NF-κB signal transduction	NLS of NF-κB (SN50 peptide)	inhibition of subcellular traffic of NF-κB in THP-1 and endothelial cells	n	h-region of FGF-4	(Lin et al., 1995)
NF-κB signal transduction	NLS of NF-κB (SN50 peptide)	inhibition of subcellular traffic of NF-κB, STAT1, NF-AT in Jurkat cells and IL-2 expression	n	h-region of FGF-4	(Torgerson et al., 1998)
NFκB signaling	NEMO binding domain	inhibition of NFκB activation in polymorphonuclear neutrophils	n	Tat	(Choi et al., 2003a)
Oxidative	Cu, Zn-SOD	protection of HeLa cells against	n	Tat	(Kwon et al.,

stress		paraquat (SO radical donor)			2000)
Oxidative stress	human catalase	protection against paraquat and H_2O_2 in PC12 and HeLa cells, penetration into skin	y	Tat, Arg_9	(Jin et al., 2001)
Pancreatic cancer	ras-p21 derived peptides	reversion to the untransformed phenotype in the ras-transformed pancreatic cancer cell line	n	Tat	(Kanovsky et al., 2003)
Pancreatic cell survival	heme oxygenase-1	increased survival of pancreatic islet in culture	y	Tat	(Ribeiro et al., 2003)
Pancreatic cell survival	Islet brain protein peptides	inhibition of JNK/cJun activation in pancreatic β cell line, protection against IL-1β-induced apoptosis	n	Tat	(Bonny et al., 2001)
Parkinson disease	TH encoding plasmid	tyrosine hydroxylase expression in the brain of Parkinsonian rats, relieve of symptoms after intravenous application	y	liposome with an antibody against the transferrin	(Zhang et al., 2003a)

				recept or	
peptide hormone signaling	NLS of NF-κB (SN50 peptide)	induction of stanniocalcin expression in neuroblastoma cells	n	h-region of FGF-4	(Yeung et al., 2003)
Pioneer work on protein transduction	β-galactosidase	enzymatic activity in all tissues, incl. the brain after IP injection	y	Tat	(Schwarze et al., 1999)
pioneering paper on transduction mechanism	β-Gal, HRP, RNAse A, Pseudom. exotoxin A	delivery *in vitro*, and into heart, liver spleen, lung, muscle *in vivo*	y	Tat	(Fawell et al., 1994)
Plant pathogen resistance	bacterial avirulence genes	hypersensitive cell death in *Arabidopsis* protoplasts	n	Pep-1	(Wu et al., 2003b)
Prolactin secretion	C-term of rab1, rab2, rab3A and rab3B	block of Ca triggered exocytosis in prolactin cells	n	Antp	(Perez et al., 1994)
Protein delivery into the brain	bFGF	delivery into the rat brain by intravenous injection	y	rat transferrin	(Wu et al., 2002)

				recept or antibo dy	
Protein phosphorylati on	MEK1 N-terminal peptide	Inhibition of ERK and ELK1 activation in NIH3T3 and PC12 cells	n	Tat and Antenn apedia	(Kelemen et al., 2002)
Protein uptake	albumin	increased uptake of albumin into sarcoma cells	n	polynu cleotid e	(Ryser et al., 1975)
Protein uptake	HRP	accumulation in mouse fibroblasts	n	poly-lysine	(Ryser et al., 1982)
Protein uptake	HRP, human serum albumin	accumulation in mouse fibroblasts	n	poly-lysine	(Shen and Ryser, 1978)
Ras signal transduction	dominant-negative Ras	inhibition of ERK and Ras activation in eosinophils	n	Tat	(Myou et al., 2002)
Reactive oxygen species	Cu/Zn SOD 21 nt antisense	promotion of apoptosis in PC12 cells	n	pAntp	(Troy et al., 1996a)
Reactive Oxygen	Superoxide Dismutase	increased viability of paraquat-treated fibroblasts	y	Tat, Lys_9	(Park et al., 2002a)

Species	(Cu,Zn-SOD)				
Renal Cell carcinoma	PKCdelta inhibitory peptide	block of renal cancer cell invasion and proliferation *in vitro*, partial renal tumor regression *in vivo*	y	Tat	(Datta et al., 2001)
Rheumatoid arthritis	KLAK anti-microbial peptide	apoptosis induction in synovial cells *in vitro* and in rabbit joints	y	HAP-1	(Mi et al., 2003)
RNA nuclear export	export shuttling domains of adapter proteins	inhibition of protein-protein interaction in HeLa cells	n	pAntp	(Gallouzi and Steitz, 2001)
ROS formation	gp91^{phox} docking sequence=9a a NAD(P)H oxidase inhibitor	decreased damage to the vascular wall in response to balloon injury	y	Tat	(Jacobson et al., 2003)
signal transduction of growth factor	aFGF	stimulation of DNA synthesis in cells expressing the diphtheria toxin receptor	n	Diphtheria toxin	(Wiedlocha et al., 1994)

Stat6 signal transduction	IL-4Rα SH-2 binding domain	Inhibition of IL-4-dependent Stat–4 phosphorylation	n	Antp	(Stolzenberger et al., 2001)
Stem Cell Proliferation	HOXB4 homeoprotein	*Ex vivo* expansion of hematopoietic stem cells	n	HOXB4	(Amsellem et al., 2003)
Steroidogenesis	CRAC domain of peripheral type benzodiazepine receptor	binding to cholesterol, inhibition of steroid production in Leydig cells	n	Tat	(Li et al., 2001)
T-cell activation	Cw-3 antigen peptide	sensitization of CTLs by treated dendritic cells	n	Antp/liposome	(Chikh et al., 2001)
TGF-β signaling	Smad-2 TGFβRI phosphorylation site	growth inhibition of Mv1Lu cells	n	Antp	(Yakymovych et al., 2002)
Thrombospondin regulation	gp91^{phox} docking sequence=9aa NAD(P)H oxidase	inhibition of Rac-induced thrombospondin expression in hu aortic endothelial cells	n	Tat	(Lopes et al., 2003)

	inhibitor				
T-lymphocyte activation	ovalbumin	presentation by antigen presenting cells, tumor regression	y	Antp	(Pietersz et al., 2001)
T-lymphocyte activation	ovalbumin	activation of ovalbumin specific cytotoxic T-lymphocytes	n	Tat	(Moy et al., 1996)
Topical delivery	Cyclosporin A	Inflammation inhibition in human an mouse skin	y	R_7	(Rothbard et al., 2000)
Tracking of cells *in vivo*	superparamagnetic nanoparticles	internalization into hematopoietic and neural progenitors	y	Tat	(Bhorade et al., 2000; Josephson et al., 1999; Lewin et al., 2000; Wunderbaldinger et al., 2002; Zhao et al., 2002)
transcription factor nuclear translocation	NLS of USF2	inhibition of IL-3 mediated survival in mast cells	n	h-region of FGF-4	(Frenkel et al., 1998)
Transfection	5.2 kb	luciferase reaction in COS-7	n	stearyl	(Futaki et al.,

	reporter plasmid	cells		-R_n and others	2001a)
Transfection	phage lambda DNA	luciferase and GFP expression in COS-1, VA13/2RA, 293, NIH/3T3, HeLa, A431 cells	n	Tat-coated lambda phage	(Eguchi et al., 2001; Nakanishi et al., 2003)
Transfection	plasmid DNA	Luciferase expression in HepG2 and COS-7 cells	n	oligolysines	(Wadhwa et al., 1997)
Transfection	reporter plasmid DNA	Luciferase expression in COS and HT1080 cells	n	side chain modified glycine 36 mer	(Murphy et al., 1998)
Transfection	reporter plasmid DNA	Luciferase expression in eukaryotic cells	n	Transferrin, linked to DNA-binding protein	(Wagner et al., 1990)

				s	
Transfection of neural cells	DNA	transfection of neuroglioma/neuroblastoma cell line	n	HC fragment of tetanus toxin	(Knight et al., 1999)
Translocation studies	β-Gal	transduction into salivary gland cell lines; retrograde labeling of rat submandibular gland	y	Tat	(Barka et al., 2000)
Translocation studies	^{99m}Tc	translocation into Jurkat leukemia cells	n	Tat and a total of 42 tested peptide variants	(Gammon et al., 2003)
Translocation studies	12-30 amino acid peptides	delivery of peptides *in vitro*	n	Diphtheria toxin N-termin	(Stenmark et al., 1991)

				al fragments	
Translocation studies	33 aa rab3A C-term	transduction into neurons and myoblasts	n	Antp HD	(Perez et al., 1992)
Translocation studies	51aa Cdc25C PPtase, 32 aa HIV-RT, GFP, β-Gal, antibodies	delivery into fibroblasts and other cells	n	Pep-1	(Morris et al., 2001)
Translocation studies	albumin	cellular uptake	n	poly-ornithine, poly-lysine, DEAE-dextran, histones	(Ryser, 1967; Ryser and Hancock, 1965)
Translocation studies	antisense oligonucleotides	correct splicing of an aberrant luciferase intron	n	Tat, Antp	(Astriab-Fisher et al., 2002)

Translocation studies	avidin-β-galactosidase	transduction into primary cells, cell lines, rabbit synovium and solid tumors	y	Tat and a number of synthetic cationic peptides	(Mi et al., 2000)
Translocation studies	barnase, a prok. ribonuclease	toxicity in a fibroblast cell line	n	Pseudomonas exotoxin A	(Prior et al., 1992)
Translocation studies	biotin, fluorescein, oligonucleotides	delivery into the cell nuclei; labeling of lymphocytes *in vivo*	y	anti-DNA mAb	(Ternynck et al., 1998)
Translocation studies	biotin, peroxidase; luciferase reporter	Penetration in to a variety of cell lines *in vitro*; transfection of 3T3 cells	n	fragments of polyreactive	(Avrameas et al., 1998)

	plasmid			anti-DNA antibodies	
Translocation studies	biotin/avidin	delivery into a melanoma cell line	n	Islet-1 HD derived peptide	(Kilk et al., 2001)
Translocation studies	biotin-streptavidin-coupled β-galactosidase	delivery of β-galactosidase into CHO, HeLa and a number of other cells	n	Tat, lysine and arginine homopolymers	(Mai et al., 2002)
Translocation studies	biotinyl group	transduction into Bowes' melanoma cells	n	transportan	(Pooga et al., 1998a)
Translocation studies	Dehydrofolate reductase	transactivation of LTR-CAT reporter in HeLa cells	n	full length Tat	(Bonifaci et al., 1995)

Translocation studies	DNA, heparan sulfate, just incubated with the Tat-PTD	accumulation of DNA and glycosaminoglycans into endosomes and the nucleus	n	Tat	(Sandgren et al., 2002)
Translocation studies	eGFP	fluorescence in myoblasts and in muscle periphery and in blood vessels after in vivo application	y	Tat	(Caron et al., 2001)
Translocation studies	eGFP	fluorescence in HeLa, COS-1 and Jurkat cell *in vitro*	n	Tat	(Fittipaldi et al., 2003)
Translocation studies	FITC	Uptake into Jurkat cells and blood cells in vivo after IP application	y	Tat derivatives	(Ho et al., 2001)
Translocation studies	fluorescein	inflammatory reaction after injection into striatum or lateral ventricles	y	Antp	(Bolton et al., 2000)
Translocation studies	fluorescein	translocation into aortic endothelial cells	n	KLALKLALKALKAALKLA	(Scheller et al., 1999)
Translocation studies	fluorescein	fluorescence in pure, cell-free unilamellar vesicles	n	pAntp	(Thorén et al., 2000)

Translocation studies	fluorescein	Fluorescence observed by fluorescence correlation microscopy	n	pAntp, bFGF MTS	(Waizenegger et al., 2002)
Translocation studies	fluorescein	fluorescent labeling of HeLa cells	n	Tat in α and β conformation	(Umezawa et al., 2002)
Translocation studies	fluorescein or ^{125}I	Uptake into melanoma cell line	n	Antp, transportan, and derivatives	(Lindgren et al., 2000)
Translocation studies	fluorescein, carbonic anhydrase, 17 kDa myoglobin	fluorescent labeling of macrophage cell line	n	Tat, D-Tat, R_n, HIV-1Rev, FHV coat, BMVag, c-	(Futaki et al., 2001b)

				Fos, cJun, etc., chemically crosslinked to the cargo	
Translocation studies	fluorescently labeled oligonucleotide	uptake into cells *in vitro*	n	FGF-4	(Dokka et al., 1997)
Translocation studies	fluorophore	delivery into T-lymphocytes	n	Antp	(Fenton et al., 1998)
Translocation studies	fluorophore	accumulation in the nucleus of HeLa cells	n	dermaseptin + NLS	(Hariton-Gazal et al., 2002)
Translocation studies	fluorophore	transduction into melanoma and different epithelial cells	n	pVEC, Antp	(Elmquist et al., 2001)
Translocation studies	fluorophore	transduction observed in non-fixed cells	n	Tat, Antp, R_7	(Thoren et al., 2003)

Translocation studies	fluorophore	fluorescence in HeLa, macrophage and COS-7 cell lines	n	Tat, HIV-1 Rev, Arg_8, Arg_{16}, FHV coat	(Suzuki et al., 2002)
Translocation studies	fluorophores and an immunosuppressive peptide	delivery of fluorophores into Cos7, HeLa, PC12, NIH3T3 cells, primary hippocampal neurons, inhibition of NFAT activation	n	GST from Schistosoma japonicum	(Namiki et al., 2003)
Translocation studies	GAL4 DNA binding domain linked to VP16 transactivating domain	luciferase expression	n	Tat, VP22, BioPORTER, TransIT	(Ye et al., 2002)
Translocation studies	GFP	fluorescence in cultured cells	n	Fushitarazu and engrail	(Han et al., 2000)

				ed homeo domai ns	
Translocation studies	GFP	translocation into COS-7 cells and muscle bystander cells	n	HSV-VP22	(Derer et al., 1999)
Translocation studies	GFP	HeLa cervical carcinoma and fibroblastic TM 12 cell fluorescence	n	manga inin, buforin and Tat	(Takeshima et al., 2003)
Translocation studies	GFP	delivery into Drosophila S2 cells.	n	oligoar ginine, oligoly sine	(Han et al., 2001)
Translocation studies	GFP	fluorescence in HeLa cells	n	Tat and derivati ves, Lys_9, Arg_9	(Park et al., 2002b)
Translocation studies	GFP and peptides	delivery into the nuclei COS-1 cells	n	HSV-1 VP22	(Elliott and O'Hare,

					1997; Elliott and O'Hare, 1999)
Translocation studies	GFP, avidin-TRITC, Biotin-streptavidin-Texas red, antibody	Transduction into BRL or COS-7 cells	n	Transportan	(Pooga et al., 2001)
Translocation studies	Hoxa5 homeoprotein	transduction to the nucleus of neurons and fibroblasts	n	Antp	(Chatelin et al., 1996)
Translocation studies	HSV-TK, Bcl-rambo, Smac/DIABOLO, GFP	apoptosis promotion in A549 cells by 400 nM Tat-Smac	n	Tat	(Yang et al., 2002b)
Translocation studies	NBD- and TAMRA-fluorophores	translocation into K562 cells	n	D- and L-SynB1 and SynB3, $pAntp_{43-58}$	(Drin et al., 2003)
Translocation	NF-κB p50	activation of Igκ and TNFα	n	HSP70	(Fujihara and

studies		production in pre-B cells			Nadler, 1999)
Translocation studies	NLS of NF-κB, integrinβ_3 cytoplasmic domain	inhibition of subcellular traffic of NF-κB in Jurkat cells; inhibition of adhesion of erythroleukemia cells	n	h-region of FGF-4 or integrin β_3	(Zhang et al., 1998)
Translocation studies	ovalbumin epitope	triggering of CTL response	n	Anthrax toxin N-term fragment	(Ballard et al., 1998)
Translocation studies	peptide nucleic acids	delivery of fluorescein-labeled peptides into various cell types	n	Tat, ANTP	(Koppelhus et al., 2002)
Translocation studies	Pseudomonas exotoxin	Toxicity in carcinoma cell lines and smooth muscle cells	n	bFGF, aFGF	(Gawlak et al., 1993)
Translocation studies	rhodamine	Uptake into CHO cells	n	Loligomers	(Sheldon et al., 1995)
Translocation studies	rhodamine, FITC entrapped in liposomes	delivery into lung carcinoma and breast tumor cells and cardiac myocytes	n	Tat attached to liposo	(Torchilin et al., 2001)

				me surfac e	
Translocation studies	rhodamine, plasmids	uptake into different cell lines	n	loligom er 4	(Singh et al., 1998)
Translocation studies	Superoxide Dismutase (Cu,Zn-SOD)	increased viability of paraquat-treated HeLa cells	n	Tat	(Eum et al., 2002)
Translocation studies	TNF-R1 peptide, EGFP	inhibition of c-Raf-1 kinase in HeLa cells, fluorescence in primary bone marrow and plant cells, PreS into liver *in vivo*	y	PreS2 PTD	(Oess and Hildt, 2000)
Transmitter release	H3 domain of HPC-1/syntaxin 1A	Suppression of transmitter release in PC12 cells	n	Tat	(Fujiwara et al., 2001)
Ubiquitin-proteasome pathway	NLS of NF-κB (SN50 peptide)	inhibition of chymotrypsin-like activity induction in mytubes	n	h-region of FGF-4	(Whitehouse et al., 2003; Whitehouse and Tisdale, 2003)
Vaccine design	CTL antigen	presentation on the surface of APC, autoimmunity/diabetes in a mouse model	y	loligom er	(Kawamura et al., 2002)

Vaccine research	specific antigens	presentation on CD8+ antigen specific cells, antigen-spec. CTLs in vivo	y	Tat	(Kim et al., 1997)
Vascular smooth muscle relaxation	HSP20 phosphopeptide analogues	relaxation of coronary artery smooth muscle	n	Tat	(Flynn et al., 2003)
Vasoconstriction	angiotensin receptor domain	increased firing rate in hypothalamus and brainstem neurons	n	Tat	(Vazquez et al., 2003)
Vasoconstriction	*C. botulinum* C3 exoenzyme	GTPase RhoA inhibition, disassembly of actin stress fibers in aortic smooth muscle cells and contraction of aortic rings	n	Tat	(Sauzeau et al., 2001)
Vasoconstriction	$gp91^{phox}$ docking sequence=9aa NAD(P)H oxidase inhibitor	decreased angiotensin II induced blood pressure elevation and ROS formation after IP injection	y	Tat	(Rey et al., 2001)
Vasodilation	cGPK-1α inhibitory	Vasodilation inhibition	n	Tat and	(Dostmann et al., 2000)

	peptide			Antennapedia	
VEGF-induced vascular permeability	Rac antagonist	Inhibition of VEGF-induced epithelial fenestration	n	Tat	(Eriksson et al., 2003)
Viral gene delivery	Ad-GFP, Ad-βgal, Ret-GFP, Ad-eNOS, Ad-VEGF	Enhancement of Adeno- and retroviral infection by about 10 X in COS-7 cells, artherial endothelium and muscles *in vivo*.	y	pAntp, Tat	(Gratton et al., 2003)
Viral gene delivery	GFP	enhanced spread of adenovirally delivered GFP in retina and striatum	y	HSV-1 VP22	(Kretz et al., 2003)

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FIGURES

Fig. 1. The procession of the Trojan Horse

Sketch for the painting

Giovanni Domenico Tiepolo, 1760. Image copied from http://www.calliope.free-online.co.uk/troy/falltroy.htm

Fig. 2. Systemic application of Tat-Bcl-x_L reduces tissue damage after focal cerebral ischemia. The recombinant protein was intravenously applied before a 2 h thread occlusion. Here, tissue damage and swelling was assessed by TTC staining. The white area is ischemic. Compared to control, Tat-Bcl-x_L treatment reduced infarct size to about 1/3 (E. Kilic, G.P.H. Dietz and M. Bähr, unpublished, cf. (Kilic et al., 2002)).