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Review

Cell Penetrating Peptides in Drug Delivery

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Protein transduction domains (PTDs) are small cationic peptides that can facilitate the uptake of large, biologically active molecules into mammalian cells. Recent reports have suggested that PTDs may be able to mediate the delivery of cargo to tissues throughout a living organism. Such technology could eliminate the size restrictions on usable drugs, enabling previously unavailable large molecules to modulate *in vivo* biology and alleviate disease. In this article, we review the evidence that PTDs can be used both to deliver active molecules to pathological tissue *in vivo* and to treat models of disease such as ischemia, inflammation, and cancer.

KEY WORDS: cell penetrating peptides; protein transduction domain; drug delivery; drug carriers.

INTRODUCTION

The terms "protein transduction domain" (PTD) and "cell penetrating peptides" (CPPs) are typically used to refer to a class of small (<20 amino acid) cationic peptides that can traverse the plasma membrane of many, if not most, mammalian cells (for recent reviews see Refs. 1 and 2). The importance of these peptides for drug delivery lies in their ability to uniformly transport large, biologically active molecules (such as proteins or oligonucleotides) into ~100% of a population of mammalian cells growing under standard culture conditions. Further interest in these peptides was recently stimulated by the observation that PTDs can also facilitate systemic delivery of recombinant proteins to a large number of tissues in a living mouse (3). These results raised the possibility that large intracellular proteins linked to PTDs might be used therapeutically just as extracellular proteins (i.e., insulin, monoclonal antibodies) are currently used. In this review, we will discuss recent studies that have tested the ability of PTD-conjugates to modulate the biology of living organisms.

PROTEIN TRANSDUCTION DOMAINS

Among the most frequently used PTDs are the TAT, Antennapedia (Antp), and poly-arginine peptides. Protein transduction was first observed when the full-length HIV Tat protein was found to be capable of entering mammalian cells and activating transcription from an HIV long terminal repeat promoter construct (4,5). Subsequent studies defined the specific region of the protein necessary for cellular uptake (6). In a similar fashion, the Antp peptide was derived from a *Drosophila* homeodomain protein (7). Both of these peptides contain a large number of lysines and arginines, the basic nature of which appears to be essential for their cell-permeation properties. Indeed, independently derived PTDs often consist largely of positively charged amino acids (8), and even short peptides consisting entirely of arginines (9) or lysines (8) appear to cross plasma membranes.

Although both TAT and Antp have been extensively used as carriers for relatively small cargos such as peptides and oligonucleotides, TAT has been the predominant PTD used in the delivery of large molecules such as full-length proteins (2). Since their initial description (10), many in-frame TAT fusion proteins produced in bacteria have been shown to enter mammalian cells and carry out intracellular functions ranging from cytoskeletal reorganization to recombination of genomic DNA (for a recently compiled list of transducible TAT-fusion proteins, see Ref. 1). TAT peptides have even been used to effect the intracellular entry of much larger molecules such as 45-nm iron beads (11), lambda phage (12), and liposomes (13,14).

The mechanism by which PTDs enter mammalian cells remains an active area of investigation. The fact that neither sequence inversion nor synthesis with D-amino acids ablates the function of TAT and Antp indicates that they do not enter the cell by interaction with a chiral receptor (9,15). Instead, it seems likely that an electrostatic interaction takes place between cationic PTDs and the negatively charged polar heads of the phospholipids that constitute the plasma membrane. Ubiquitous, negatively charged proteoglycans such as heparan sulfate were recently shown to play a role in the cellular uptake of Tat protein (16). However, uptake of the shorter TAT peptide does not show the same dependence on such proteoglycans (17).

Early reports provided evidence that uptake of PTDs, such as TAT and Antp, occurs in an endocytosis-independent fashion (e.g., Refs. 6 and 7). However, recent work has indicated that some studies on the visualization of TAT uptake by standard methodologies, particularly methanol fixation, are particularly prone to artifacts. In fact, recent data from live

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cells suggest that a specialized form of endocytosis plays a major role in the uptake of TAT peptides (18) and TAT fusion proteins (19, Wadia and Dowdy, unpublished observations). Finally, although most tested mammalian cells have been found to be susceptible to transduction by PTDs, two epithelial cell lines appear to be resistant to TAT peptide entry (20). Further study of these cells should also provide mechanistic insights into PTD transit across the plasma membrane

IN VIVO APPLICATIONS OF PTDs

PTDs have proven their ability to manipulate the biology of cultured mammalian cells by delivering cargos such as peptides and recombinant proteins (1). However, the ability to deliver large, biologically active molecules to the interior of cells in a living organism would also be of tremendous benefit. To determine whether PTDs might facilitate such delivery, a TAT-β-galactosidase fusion protein was delivered to mice by intraperitoneal injection (3). Analysis of tissue sections revealed delivery of the fusion protein to many, if not most, tissues of the mouse, including the brain. Importantly, the X-gal assay used in this study demonstrated that the TAT-β-galactosidase, a 120-kDa protein enzyme, retained its activity *in vivo*.

In recent years, a number of studies have examined the ability of PTD-peptides and proteins to modulate the biology of cells and tissues *in vivo*. Many studies have focused on mouse models of ischemia or cancer. Although it might be expected that administration of PTDs would be more effective at protecting cells from isolated insults (such as ischemia) than at eliminating cancer cells with constitutively active programs to divide and avoid cell death, PTDs have been successfully used to treat mouse models of ischemia, cancer, and other diseases.

Ischemia

One of the most impressive examples of the therapeutic potential of TAT-fusion proteins has been provided by studies using a recombinant TAT-Bcl-xL protein. A member of the Bcl-2 family of proteins, Bcl-xL can act at the mitochondria to suppress apoptosis in multiple cell types. In one recent study (21), TAT-Bcl-xL (but not Bcl-xL) was shown to inhibit apoptosis in cultured neurons at concentrations (30–100 nM) much lower than those used with traditional small-molecule apoptosis inhibitors. The authors also demonstrated the *in vivo* efficacy of TAT-Bcl-xL by using a murine model of stroke in which cerebral artery occlusion leads to focal ischemia followed by neuronal apoptosis. The authors found that intraperitoneal administration of 9 mg/kg TAT-Bcl-xL protein could significantly decrease the size of the cerebral infarction (>60%) and reduce neuronal caspase-3 activity. The protein was effective even if it was administered at 45 min after the ischemic episode. In contrast, nontransducible Bcl-xL protein had no effect on neuronal apoptosis *in vivo*. These results were corroborated by another report in which intravenous TAT-Bcl-xL administration reduced neuronal apoptosis and infarct volume in a murine stroke model (22). In a separate paper, this group also showed that TAT-Bcl-xL was able to suppress axotomy-induced apoptosis in retinal ganglion cells after local delivery into the vitreous space of the eye (23). Furthermore, the TAT-Bcl-xL protein could still be detected in cultured cerebellar granule cells at 10 days after protein addition, indicating that not all proteins are so short-lived as to be poor candidates for therapeutics.

Other reports have provided more evidence for the therapeutic utility of TAT-Bcl-xL. In one example, a gain of function Bcl-xL mutant (termed FNK, which refers to the amino acid substitutions Y22F, Q26N, and R165K) was linked to the TAT PTD (24). The TAT-FNK protein was cytoprotective in cultured neurons and was detected in the brain after intraperitoneal administration to mice. Furthermore, intraperitoneal administration of TAT-FNK to gerbils (5 mg/kg) protected hippocampal CA1 neurons from cell death after transient global ischemia. Finally, another group has reported transduction of isolated pancreatic islet cells *ex vivo* and has shown that islet-derived insulinoma cells could be protected from apoptosis by TAT-Bcl-xL (25). These results have implications for current efforts to prevent islet-cell apoptosis during pancreatic islet transplantation as a treatment of diabetes.

TAT peptides have also been used to reduce ischemia-mediated neuronal apoptosis *in vivo* (26). During ischemia, N-methyl-D-aspartate receptors (NMDAR) interact with the intracellular protein PSD-95, leading to nitric oxide production and cell death. Aarts *et al.* found that a NMDAR-derived peptide could be linked to TAT and delivered to cultured neurons. This TAT-NMR2 peptide then sequestered intracellular PSD-95, thereby blocking NMDAR-mediated apoptosis. Intravenous administration of TAT-NMR2 1 h after cerebral artery occlusion also reduced cerebral infarction volume and led to better neurological scores in rats. Importantly, this peptide had no effect on NMDAR-mediated currents, as *in vivo* NMDAR blockade is too deleterious to neurons to be used as a stroke therapy. The fact that both TAT-Bcl-xL protein (21,22,24) and TAT-NMR2 peptide (26) can systemically be delivered to neurons in the brain and inhibit apoptosis is a testament to the power and potential *in vivo* utility of large molecules linked to the TAT domain.

Damage to the heart due to ischemia is also a major cause of morbidity and mortality. Two groups have shown that TAT can be used to deliver large molecules into isolated rat hearts. One group was able to deliver peptides that selectively modulated different isoforms of protein kinase C (27) while another delivered a protein (TAT-ARC) that can bind and inhibit caspases 2 and 8 (28). In each report, perfusion with these transducible molecules reduced the amount of cell death in the heart following experimentally induced ischemia.

Cancer

A number of studies have examined the ability of transducible peptides and proteins to inhibit tumor growth *in vivo*. Given the lack of specificity of current cancer therapy and the limitations of gene therapy, PTDs appear to be an attractive means by which to introduce tumor suppressors or other pro-apoptotic proteins directly into growing tumors. However, tumors may be more challenging targets for therapy than ischemic tissue where therapeutic agents need only counteract a single ischemic event. In the case of tumors, however, a given therapy must find a way to slow down or kill cells that have extensively been selected to engage in proliferation and resist apoptotic stimuli. Despite this inherent challenge of

tumor treatment, progress has been made in the therapeutic delivery of intracellular proteins and peptides to tumors in living organisms.

Multiple groups have found that the *N*-terminus of the Smac protein can be linked to either TAT or Antp to facilitate cellular uptake (29–31). Smac is a mitochondrial protein that can inactivate the inhibitor of apoptosis (IAP) proteins, and all three studies found that Smac-TAT or Smac-Antp sensitized cells to pro-apoptotic stimuli. One group took these results a step further and tested the function of the Smac-TAT peptide in an intracranial glioblastoma xenograft model (30). The authors first showed that local administration of TRAIL, a death receptor ligand with specificity for tumor cells, reduced tumor volume and moderately extended the life of nude mice bearing established intracranial U87MG tumors. By contrast, local treatment with Smac-TAT alone had no effect on tumor growth. When TRAIL and Smac-TAT were coadministered, however, there was a synergistic effect on tumor volume and mouse survival. Whereas control mice all died of tumor burden by 35 days after tumor cell injection, mice treated with Smac-TAT and the highest doses of TRAIL survived beyond 70 days after the start of the experiment. Furthermore, histological analysis revealed no evidence of remaining tumor in the brain.

Two other reports have added to the evidence that PTDs may be useful in the local treatment of tumors. In one study, a TAT peptide derived from the *N*-terminus of p53 was shown to sequester mdm2 and stimulate p53 activity in tumor cells (32). Injection of the peptide into rabbit eyes harboring human retinoblastoma xenografts resulted in a high level of tumor cell apoptosis without significant toxicity to surrounding normal tissue. Another group linked a pro-apoptotic peptide $(KLAKLAK)_2$ to a cationic protein transduction domain (PTD-5) to facilitate cellular uptake (33). Although this peptide would not be expected to have any specificity for tumor cells, direct injection into subcutaneous murine fibrosarcomas inhibited tumor growth without evident toxicity to the mouse as a whole.

Although a number of lethal tumors are treated by local administration of chemotherapeutics, many more lethal tumors are disseminated throughout the body, thus necessitating the systemic delivery of anticancer agents. Early *in vivo* experiments demonstrated that TAT proteins are delivered to a large number of organs after intraperitoneal (IP) injection (3), suggesting that systemic delivery to a primary tumor or to multiple metastases should be feasible with transducible agents. If this is true, it should be possible to design transducible agents that can be delivered to virtually any type of cancer.

A handful of papers is beginning to provide evidence that systemic delivery of transducible agents can modulate tumor biology *in vivo*. In a particularly clever series of experiments, Harada *et al.* fused the oxygen-dependent degradation domain (ODD) of HIF-1α to the TAT-β-gal and the TAT-Caspase-3 fusion proteins (34). The ODD domain stimulates degradation of HIF-1α under normoxic but not under hypoxic conditions. Thus, the authors hypothesized that a TAT-ODD fusion protein would be stable in the hypoxic core of tumors but would be degraded and nonfunctional in normal tissue. Indeed, after IP injection of TAT-ODD-β-gal into tumor-bearing nude mice, only the hypoxic regions of the tumors showed evidence of TAT-ODD-β-gal protein. By contrast, TAT-β-galactosidase protein could be detected throughout tumors after IP delivery. Furthermore, TAT-ODD-β-gal was undetectable in mouse liver after IP injection, again unlike the parental TAT-β-gal protein. Next, tumor-bearing mice were given IP injections of a TAT-ODD-Caspase-3 protein. TAT-ODD-Caspase-3 was able to reduce tumor growth without causing the toxic side effects that would be expected from delivering active caspase-3 to an entire mouse. Thus, it is possible to use functional domains to modulate the type of tissue in which TAT-fusion proteins are active and in this way increase their tumor specificity.

Two other reports have shown that systemic delivery of cell-penetrating peptides can be used to inhibit specific tumors *in vivo*. In the first case, TAT was fused to a peptide derived from the VHL tumor suppressor that inhibits IGF-I receptor signaling in renal cell carcinomas (35). IP administration of TAT-VHL peptide slowed the growth of subcutaneous renal cell carcinoma tumors in nude mice, primarily through inhibition of cell proliferation rather than by induction of apoptosis. Peptide treatment appeared to reduce tumor invasion into the underlying tissue as well. This study also provides strong immunohistochemical evidence that the TAT-VHL peptide is homogeneously delivered to the tumors after IP injection. A second report found that IP delivery of an Antp-p16 fusion peptide moderately inhibited the growth of pancreatic cancer cells growing as peritoneal and as subcutaneous tumors in nude mice (36). Although p16 functions primarily as an inhibitor of cell cycle progression, the authors found that Antp-p16 slowed tumor growth by inducing apoptosis of cancer cells *in vivo*. In neither study was any toxicity to normal tissue observed (35,36), indicating that cancer cells may be much more sensitive to the effects of transducible tumor suppressor peptides than nontransformed cells. If this proves to be a general rule, cell penetrating peptides may prove to be a powerful tool in the design of anticancer agents that target tumors while sparing normal tissue.

Other Disease Models

PTDs have also been used to modulate blood pressure (37) and inflammation (38,39) *in vivo*. In the first example, the TAT peptide was linked to a peptide that blocks the assembly of the NAD(P)H oxidase complex. This peptide (gp91dsTAT) was then administered to mice with angiotensin II (Ang II), which normally stimulates assembly of the NAD(P)H oxidase complex in vascular cells. Coadministration of gp91dsTAT impaired the generation of O_2- in the vasculature and partially blocked the increase in systolic blood pressure caused by Ang II injection (37). Using a similar strategy, May *et al.* designed a peptide (termed NBD for NEMO binding domain) that could block the interaction of NEMO (NF-κB essential modifier) with the IKK (inhibitor of κB-kinase) complex (38). As this interaction is necessary for the activation of NF-κB in response to proinflammatory stimuli, they were able to use an Antp-NBD peptide to block the response of cultured cells to inflammatory stimuli such as TNF-α. Furthermore, they demonstrated that administration of Antp-NBD reduced the inflammatory response *in vivo* in ear edema and in peritonitis models of inflammation. At least in these models, the Antp-NBD peptide was as efficacious as the established anti-inflammatory drug dexamethasone. Finally, Bucci *et al.* fused a peptide from the eNOS binding

domain of caveolin-1 to Antp (39). The Antp-Cav peptide blocked the production of nitric oxide in cultured endothelial cells by inhibiting eNOS. Intraperitoneal administration of Antp-Cav reduced vascular leakage and interstitial edema in two mouse models of inflammation.

LOW-MOLECULAR-WEIGHT DRUG–PTD CONJUGATES

Although cell penetrating peptides have primarily been used to facilitate cellular uptake of large molecules, these peptides have also been conjugated to small molecules in order to enhance *in vivo* delivery to specific tissues. For example, cyclosporin A (CsA) fails to penetrate the skin after topical delivery, but treatment of inflammatory skin disease by systemic CsA is partially limited by its numerous side effects. Linkage of an arginine heptamer to cyclosporin A (R7-CsA) enabled delivery of CsA to the cells of the epidermis and dermis by topical administration (including dermal T cells) (40). Conjugation to R7 did not disrupt the functionality of CsA, as topical R7-CsA reduced inflammation in a mouse model of contact dermatitis. Untreated inflammation in the same mouse remained unchanged, indicating that topically administered R7-CsA was not delivered systemically to a significant extent.

Doxorubicin is an anticancer drug that can readily be exported from tumor cells by the efflux pump P-glycoprotein (P-gp). P-gp is expressed in multidrug resistant (MDR) tumor cells as well as in the endothelium that forms the blood–brain barrier. Consequently, doxorubicin's effectiveness in treating brain and MDR-amplified tumors is markedly reduced by P-gp activity. In an effort to bypass P-gp-mediated efflux in these settings, doxorubicin has been linked to the Antp peptide. This Antp–dox conjugate displayed enhanced brain uptake in an *in situ* brain perfusion model (41). Further studies showed that this enhancement was specifically due to P-gp bypass and not general disruption of other aspects of the blood–brain barrier, because this enhancement did not occur in P-gp deficient (mdr1a [–/–]) mice (42). Moreover, Antp–dox conjugates displayed higher cytotoxicity than free dox against cultured MDR tumor cells (42). These data indicate that cellular uptake of cell-penetrating peptides may not be regulated by the P-gp pump, and therefore peptide–drug conjugates may be useful in the delivery of drugs to tissues in which P-gp activity is rate-limiting.

CONCLUSIONS

Numerous recent studies have now revealed the potential ability of PTDs to modulate the biology of living organisms and alleviate disease. However, many questions remain unanswered. The immunogenecity and long-term side effects of PTD administration still require much investigation. Whatever the immunogenecity of these short basic peptides, it seems likely that it will be far less than that of the viral vectors that have been so extensively studied for the purposes of gene therapy in preclinical models and in clinical trials. Clearly, an immune response has not prevented some degree of efficacy in the relatively short-term mouse studies described above. Nevertheless, if immunity to one particular PTD were to develop in long-term trials, the abundance of different PTD peptides would allow for replacement of the original peptide thereby sidestepping the problem.

Pharmacokinetic studies of PTDs have been reported (43), but many more will be needed. In particular, it will be necessary to address the effects of specific cargo on the biodistribution of PTD conjugates. Such studies may ultimately lead to the elucidation of general rules regarding cargo properties that either favor or hinder *in vivo* delivery. In addition, PTDs have been optimized for delivery to cells growing on plastic dishes, but further optimization for *in vivo* applications may also be valuable. Although there is clearly much more preclinical work to be done, the results described here indicate that intracellular proteins and peptides linked to PTDs may one day be as therapeutically efficacious as the many small-molecule drugs in use today.

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