Critical Review

Properties of Cell Penetrating Peptides (CPPs)

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Summary

Different approaches have been developed for the introduction of macromolecules, proteins and DNA into target cells. Viral (retroviruses, lentiviruses, etc.) and nonviral (liposomes, biolistics etc.) vectors as well as lipid particles have been tested as DNA delivery systems. However, all of them share several undesirable effects that are difficult to overcome, such as unwanted immunoresponse and limited cell targeting. The discovery of the cell penetrating peptides (CPPs) showing properties of macromolecules carriers and enhancers of viral vectors, opened new opportunities for the delivery of biologically active cargos, including therapeutically relevant genes into various cells and tissues. This review summarizes recent data about the best characterized CPPs as well as those sharing cell-penetrating and cargo delivery properties despite differing in the primary sequence. The putative mechanisms of CPPs penetration into cells and interaction with intracellular structures such as chromosomes, cytoskeleton and centrioles are addressed. We further discuss recent developments in overcoming the lack of cells specificity, one of the main obstacles for CPPs application in gene therapy. In particular, we review a newly discovered affinity of CPPs to actively proliferating cells.

Keywords Cell penetrating peptides; DNA delivery systems.

INTRODUCTION

Highly cationic peptides with low molecular weight, rich in basic amino acids, such as, arginine or lysine, or proline-rich, have been shown to cross the lipid layer barrier of the plasmatic membrane of several cells (1–8), usually impermeable for biological molecules. They were generically designated as cell penetrating peptides (CPPs) or protein transduction domains (PTDs). The number of known natural CPPs is restricted and they vary significantly in their origin, primary sequence and secondary structure. For instance, HIV-1 TAT peptide was isolated from the virus HIV (Human immunodeficiency virus) transcription activating factor (2, 9, 10). Antp43-58, also known as penetratin, was from Drosophila Antennapedia homeodomain transcriptional factor (4, 11, 12). pVEC was from murine vascular endothelial cadherin (13). Buforin II was a histone-derived antimicrobial peptide (8), and VP22 was from the Herpes simplex virus (HSV) type I structural protein (14–16), while oligoarginine peptides (17–20), model amphipatic peptide (MAP) (21) and transportan (22, 23) were designed. Their penetrating capacity is based on peptide sequences that are responsible for internalization capacity (9). CPPs internalization efficiency depends on the length of the peptide backbone, since stretches of six (R6) to eight (R8) arginine residues showed the highest internalization potential (19, 24). Presence of specific sequence (NLS - nuclear localization sequence) rich in basic residues (such as arginine and lysine) of the CPPs provides nucleus translocation (25).

When administered in non-toxic concentrations, the CPPs can be used to transport genes, therapeutic drugs and/or diagnostic probes into the intracellular compartment (26–29). The CPPs can be applied not only to genetically manipulate the mammalian genome, but also to provide an experimental model to study the mechanisms of translocation of macromolecules into the senescent cells (20, 30, 31).

A number of recent reviews discuss arginine- and lysine-rich peptides and their potential use in experimental biology (32–35). However, less is known about their penetrative specificity and also about the interaction with subcellular structures. Recently, we have described a novel CPP isolated from the venom of a rattlesnake that differs from those CPPs previously reported, due to both a selective penetration into previously reported, due to both a selective penetration into previously reported, due to both a selective penetration into previously reported, due to both a selective penetration into previously reported, due to both a selective penetration into previously reported, due to both a selective penetration into previously reported, due to both a selective penetration into previously reported, due to both a selective penetration into previously reported, due to both a selective penetration into previously reported, due to both a selective penetration into previously reported, due to both a selective penetration into previously reported, due to both a selective penetration into previously reported, due to both a selective penetration into previously reported, due to both a selective penetration into previously reported, due to both a selective penetration into previously reported, due to both a selective penetration into previously reported, due to both a selective penetration into previously reported, due to both a selective penetration into previously reported, due to both a sele...
and future applications of the CPPs as intracellular delivery system molecules and also as a tool for gene regulation.

**Milestones on the Investigation of CPPs Intracellular Localization**

To visualize intracellular localization of peptides, they are usually conjugated with fluorescent dyes such as, Cy3, Cy5, FITC, etc. Using confocal microscopy study it was shown that peptides could rapidly penetrate, within 2–5 minutes, into the cell and localize in the cytoplasm and/or nuclei. Recently, the nuclear localization of some peptides was questioned due to the artifact produced by fixation process (38–44). To avoid fixation procedure artifacts analysis of the peptide nuclear localization preferentially using living cells has been proposed (40, 44, 45). Alternatively, ‘peptide competition assay’ (PCA) based on subsequent treatment of the cells with native and fluorescent-conjugated peptides has been used for cationic peptides (46). Briefly, cells, pre-treated (for 5–15 min) with native (non-labeled) peptide and subsequently treated for more than 5–15 min with fluorescent-conjugated peptide, demonstrated a fluorescent signal restricted to the cytoplasm, indicating a saturation of the binding sites within the nuclei by the native peptide, even after fixation (Fig. 1A). Conversely, cells treated only with fluorescent-conjugated peptide and then fixed, showed strong nuclear and cytoplasm fluorescent labeling (Fig. 1B). This method can also be use in vivo when both native and fluorescent-conjugated peptides are subsequently injected in mice, as was applied for crotamine in order to demonstrate its nuclear localization (36). Interestingly, a lack of crotamine nuclear localization is also observed in fixed mouse primary fibroblasts, mitotically inhibited by irradiation (Fig. 1C).

**CPPs Subcellular Localization**

CPPs cytoplasm and nuclear localization has been discussed in several reports (2, 4, 11, 18, 19, 27, 40, 47–50). However, to date there are only two proteins VP22 and crotamine, from HSV and rattle snake, respectively, exhibiting proven differential subcellular localization pattern, such as binding with cytoplasmatic or nuclear structures, and a mitosis dependent cytoplasm-to-nucleus transfer (14, 36, 51, 52). Cytoplasm to nucleus transfer of HSV VP22 301-amino-acid protein during cell division has been shown (51). Cells transfected by green fluorescent protein (GFP)-tagged VP22 express this protein at high level in cytoplasm where it binds, reorganizes and stabilizes microtubules. This feature is similar to the overexpression of microtubule associated proteins (53). Such microtubules interaction was shown to be related to the cell cycle: cells with microtubules bundling by GFP-VP22 did not progress through mitosis (51). Moreover, the authors showed that VP22 has the ability to translocate from the cytoplasm to nucleus via binding to the condensing chromatin at early stages of mitosis. It remains bound to chromosomes during all mitotic stages and can be found in daughter cells after cytokinesis. Further investigation of specific regions of the VP22 open reading frame demonstrated the presence of different domains in VP22 molecules that are responsible for the microtubules and chromatin binding (52).

Crotamine shared subcellular localization similar to VP22. It remains specifically bound to chromosomes and centrioles during mitosis, both in vitro and in vivo. According to our data, crotamine reaches the nucleus within 5 min during S/G2 transition. Crotamine binds to the chromosomes stained by DAPI (DNA binding dye) with no overlapping for the crotamine and DAPI staining (Fig. 1E). At the end of telophase, crotamine was observed in the cytoplasm of dividing cells (Fig. 1D). The pattern of crotamine labeling of subcellular structures suggests its employment, for example, to distinguish normal and tumor cells based on the number of centrioles or chromosomes.

The majority of cationic peptides conjugated with fluorescent markers demonstrated localization within the nuclei (2, 4, 23, 45, 50, 54). However, their specific subcellular localization such as interaction with cytoplasmic or nuclear structures has not been reported.

**Internalization of Macromolecules Mediated by CPPs**

CPPs conjugated with other proteins or peptides have the ability to penetrate into the cells. The cells rapidly internalize such complex in a concentration dependent manner with maximum intracellular concentration within approximately 15 min. Biological activity of introduced proteins was detected even at low ambient concentrations of approximately 25–200 nM (5). Internalization of peptide-protein complex was shown for many cell types (54–64). Successful transduction was observed even in osteoclasts and mononuclear cells from peripheral blood that are usually impermeable for retroviral transfection (65, 66). Intracellular concentration of the CPP-protein complex can be controlled with high accuracy by its concentration in culture medium regardless of the cell type.

The impact of different proteins on the specific cellular events was evaluated using CPP-protein complex. It was demonstrated, for instance, that T cells were capable of internalizing the epitope of antigenic peptide that localizes on their surface linked to MHC class I complex (65). Later, another group showed transduction of E1A (60kDa) protein fused with TAT into the primary human lymphocytes, where the protein binds to its target cell endogen proteins p130 and pRB retaining the activity even when fused with TAT (5). Furthermore, TAT-eGFP (enhanced green fluorescent protein) was internalized by primary culture of myoblasts. These myoblasts were isolated from patients affected by Duchene muscular dystrophy and showed strong fluorescence, 4 h after treatment (6). Chimeric VP22-SV40 large T antigen fusion protein causes terminal differentiation of C2C12 cells into myotubes when it accumulates in the nucleus. It was capable of inducing S-phase and mitosis in these differentiated cells, thus opening novel approaches for tissue regenerating techniques (67). In another example a complex...
of TAT and GDH (human glutamate dehydrogenase gene) protein underwent correct refolding and retained normal activity unaffected by the presence of TAT following introduction into PC12 cells (68).

Effective CPPs internalization into cell in vivo has also been shown. TAT fused with heterologous proteins has been shown to penetrate into various mouse tissues (5). Activation of endogenous T cells was detected after intraperitoneal injection of Antp – antigenic complex in mice (65). Hemato-encephalic barrier that exists to protect cerebral tissues from most drugs delivered through the body tissues was crossed by the Antp-antisense-peptide-nucleic-acid oligomers complex in rats (27) and, furthermore, biologically active enzyme was delivered into mouse cells and tissues via intraperitoneal injection of TAT-β-galactosidase (5). It is important to note that different studies demonstrated delivery of molecules of approximately 129 kDa in size into a wide range of cells and tissues in mammals as well (27). More recent studies have extended CPPs applications to deliver a variety of non-protein cargos (29, 55, 57, 68).

Using CPPs to deliver a recombinant-deficient virus allowed the lowering of the effective virus titer, enhancement

Figure 1. Intracellular localization and cell specificity of crotamine, a CPP from the venom of South American rattlesnake. (A, B) DAPI, Crotamine and 5-BrdU nuclear localization in the proliferating cells peptide competition assay. (A – B) Peptide competition assay in mouse bone marrow cells: a fluorescent signal is restricted to the cytoplasm (A). In (B) nuclear and cytoplasm fluorescent labeling is observed. (C) Lack of nuclear localization of crotamine visualized in non-dividing mouse embryonic fibroblasts. (D) At the end of cell division (telophase) crotamine localization is restricted to the cytoplasm. Asterisk indicates nucleus. (E) Cy3-conjugated (red) crotamine (arrow) labeling observed on DAPI stained chromosomes (blue). (F – R) DAPI (blue), Crotamine (red) and 5-BrdU (green) labeling and overlapping (yellow) of Cy3-crotamine labeling and 5-BrdU incorporation observed on the chromosomes of actively proliferating (AP) cells. (F – I) Metaphase. (J – M) Anaphase. (N – R) Telophase. (D – R) Epifluorescence (EF), Magnification ×800. (S) Crotamine labeling of AP cells (arrowhead) observed in differentiating culture of ES cells. (T) Same as in (S) showing alkaline phosphatase activity in AP, undifferentiated cells. Arrows indicates non-dividing, differentiated cells. A – D, S, T = overlay of digital interference contrast (DIC) and fluorescent confocal microscopy (DIC + Fcm); A – D bars = 100 μm. S, T bars = 25 μm.
of the uptake, improvement of the gene expression, a decrease in cytotoxicity and immune response in vitro and in vivo (69).

To date, viral vectors were considered the most effective. However, they showed undesirable immune response, and were capable of transfecting only a limited number of cell types, not even mentioning the risk of oncogenesis. On the other hand, the CPPs-proteins complex not only showed high delivery efficiency irrespective of the cell type, but also a high specificity was observed and it is also unlikely to cause malignant transformation.

**Cell and/or Tissue Specificity for Molecule Delivery**

A number of cargo molecules including proteins, nucleic acids, small molecules, and particles have been shuttled into living cells by the CPPs (33, 34, 50, 55–60). Also, the effects of the cargo molecules to the cellular uptake have been evaluated (54, 61–63).

However, the mechanism of CPPs uptake has yet to be elucidated, and little is known about their cell specificity. The majority of known CPPs are not cell or tissue specific. This is a foremost obstacle for the clinical application of the CPPs. In fact, only recently, we discovered the first CPP that has high level of affinity to actively proliferating (AP) cells (36). Selective penetration of crotamine into AP cells was confirmed by 5-BrdU incorporation studies, which are routinely used to identify actively proliferating cells. Crotamine and 5-BrdU labeling co-localized within the nucleus on chromosomes (Fig. 1F – R). In AP cells, crotamine was also observed in cytoplasm, indicating 5-BrdU and crotamine have different mechanisms involved in cell selection. However, both marked a pool of AP cells, suggesting that crotamine might be used as a marker of AP cells in vitro and in vivo. Consistent with its affinity to AP cells, we showed that crotamine can penetrate into undifferentiated mouse embryonic stem (ES) cells in vitro (36). In differentiating mouse ES cells, crotamine labeling overlapped with the pool of undifferentiated actively proliferating cells exhibiting a high level of alkaline phosphatase activity (Fig. 1S,T). This finding suggests that CPPs may potentially be used for selective transfection of ES cells, thereby augmenting or replacing electroporation currently employed to deliver a molecule of interest. Moreover, our data show that in developing pre-implantation embryo, crotamine is preferentially accumulating in the inner cell mass and, therefore, may be used to target those cells for payload delivery. Low cytotoxicity of crotamine at its effective concentrations makes it a particularly valuable representative of CPPs (36). Therefore, one of the important areas of our research is elucidating the mechanisms governing the transmembrane transfer of crotamine.

**MECHANISMS OF MEMBRANE TRANSLOCATION OF CPPS**

The mechanism of uptake of cationic CPPs is still unclear and some existing concepts, particularly the role of endocytosis in this process has been revised (40, 42, 70, 71). Until recently, it was believed that internalization of CPPs involves the mechanism of endocytosis similar to the one described for bacterial and plant toxins that include their own transmembrane transfer apparatus (72). The inability of natural CPPs, to penetrate into the cells at low temperature seems to support the notion that its internalization requires endocytosis (54). On the other hand, several studies have shown CPPs internalization under conditions when endocytosis is inhibited (2, 4, 20). Moreover, the large variety of CPPs and the absence of shared epitopes indicate that their primary sequence may not play a significant role in their properties and that the transfer is not mediated by specific receptors (73). Recent studies have demonstrated the involvement of the electrostatic interaction with the extracellular matrix of the cell followed by endocytosis (40–42). This has been shown even for internalization process of oligocationic CPPs such as TAT peptides and penetratin, previously shown to translocate via a direct and energy-independent mechanism (4, 20). However, weakly cationic CPPs seem to enter into the cells by a lipid raft-mediated uptake without interacting with the extracellular matrix (74, 75). Curiously, Henriques et al. have suggested a physical mechanism governed by electrostatic interactions between the CPP and the membrane, which is dependent on negative transmembrane potential across the bilayer for a CPP-mediated protein uptake (76).

Many membrane-associated toxic peptides exhibiting amphipathic character appear to translocate across membranes by pore formation (77). On the other hand, CPPs with a more limited amphiphilic character, such as the Antennapedia homeodomain-derived peptide penetratin, HIV TAT or even polyarginine-based sequences do not appear to form classical pores in lipid bilayers (2, 4, 20, 32). Recently, an in vivo experiment with the cationic CPP HIV-1 TAT has also demonstrated the involvement of membrane-associated heparan sulfate in the uptake mechanism (45). The interaction of one of the most efficient CPP, the oligoarginine, with both heparan sulfate and lipid vesicles was also demonstrated (78). A systematic structure-activity relationship study of the antimicrobial peptide buforin II revealed that its cell-penetrating efficiency depends on z-helical content with a proline hinge as a key structural factor for the cell-penetrating property. A single amino acid substitution for proline changes buforin to a membrane-active peptide, and conversely the insertion of a proline-hinge region can switch a membrane-permeabilizing peptide to a cell-penetrating one (79). Furthermore, more recently, Sadler et al. (7) have shown that proline-rich peptides, which process hydrophobic character, represent a potentially new class of cell-permeate peptides probably because of their ability to interact with the lipid tails that constitute the cell membrane.

**CONCLUSIONS**

During recent years CPPs-based technologies have become a useful tool in biological research due to their noninvasive
and efficient delivery of biologically active molecules into cells in vitro and in vivo. The latest discovery of CPPs with an affinity to actively proliferating cells opens new vistas in cancer and developmental biology research. Despite major advances in CPPs studies, the mechanisms of CPPs action remain poorly understood. It seems to be specific for each individual CPP and it is likely to remain a focus of research in this area for the years to come.

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REFERENCES


