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Review

Arginine-rich cell penetrating peptides: from endosomal uptake to nuclear delivery

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Abstract. Delivery of macromolecules into living cells by arginine-rich cell penetrating peptides (AR-CPPs) is an important new avenue for the development of novel therapeutic strategies. However, to date the mechanism of this delivery remains elusive. Recent data implicate endocytosis in the internalization of AR-CPPs and their macromolecular cargo and also indicate limited delivery of macromolecules into the cell cytoplasm and nucleus. Different types of endocytosis – clathrin-dependent endocytosis, raft/caveolin-dependent endocytosis and macropinocytosis – are all implicated in the uptake of AR-CPPs and their cargo into different cells. Cationic AR-CPPs dramatically increase uptake of conjugated molecules through efficient binding to surface proteoglycans. Whether this increase in binding can assure delivery of a sufficient amount of functionally active macromolecules into the cytoplasm and nucleus or whether there is a specific mechanism by which AR-CPPs facilitate the escape of conjugated cargo from endosomes remains to be understood.

Key words. Cell-penetrating peptide; protein transduction; arginine-rich peptide; heparan sulfate; endocytosis; TAT; penetratin.

Introduction

Recent advances in the understanding of cellular and molecular mechanisms underlying the development of various diseases offer new potential molecular therapy targets and, together with advances in biotechnology, increase the appeal and importance of various biomacromolecules such as DNA, RNA, peptides, proteins and their mimics as therapeutic instruments. In order to exert their biological action, macromolecular drugs have to be delivered into specific intracellular compartments – usually the cytoplasm or nucleus. The requirement for cytoplasmic or nuclear delivery poses a major challenge for the development of effective intracellular therapies based on the use of high molecular weight drugs because of the hydrophobic nature of the plasma membrane surrounding the cell, which effectively restricts the free exchange of hydrophilic molecules between the cellular interior and the extracellular medium. While many small hydrophobic molecules can passively diffuse through the membrane, small polar molecules essential for metabolism (ions, sugars, amino acids etc.) are delivered into the cell cytoplasm through the action of specific plasma membrane proteins – channels and transporters. Macromolecules, on the other hand, are generally directed into the endocytic pathway and end up in lysosomes, where they are degraded by lysosomal enzymes.

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Therefore, the development of macromolecular drugs is greatly hampered by intracellular delivery problems. It is not surprising that the discovery of several peptides that have been found to efficiently translocate covalently attached macromolecules through the plasma membrane by a receptor- and endocytosis-independent-pathway has attracted a lot of attention [1–3]. These peptides are often referred to as protein transduction domains or cell-penetrating peptides (CPPs). The latter name will be used throughout this review. The CPPs are a diverse group of different peptides including amphipathic helical peptides, such as transportan and its analogues, as well as highly cationic and hydrophilic arginine-rich peptides, such as TAT and penetratin. In this review, we will focus on the arginine-rich peptides, while transportan and other amphipathic helical peptides have been extensively reviewed elsewhere [4, 5].

The two most extensively used and studied AR-CPPs are TAT and penetratin. TAT is derived from the transcription activating factor of human immunodeficiency virus (HIV-1) [6–8], while penetratin is derived from the *Drosophila* homeodomain protein [9]. A number of other AR-CPPs, including short homoarginine sequences, have been described in the literature [10–12]. AR-CPPs have been successfully used to deliver various macromolecular cargos, including proteins, oligonucleotides, plasmid DNA, and even beads and liposomes (for an extensive list of different cargos delivered by various CPPs, refer to [13]) into mammalian cells. In many cases, cargo molecules have been found to induce biological responses, indicating delivery of cargo in a functionally active form [14–17]. Furthermore, TAT has been shown to facilitate delivery of conjugated protein into various tissues of live mice, when administered by intraperitoneal injection [17-20]. Recently, there have been numerous reports on the modulation of cells and tissues in vivo by TAT-conjugated proteins and peptides (reviewed in [21]). Despite the growing evidence that TAT and other AR-CPPs can be successfully used for cytoplasmic and nuclear delivery of various macromolecules, the mechanism of AR-CPP-induced translocation remains elusive.

In this review, we summarize and discuss recent data on the mechanisms of cellular uptake and of cytoplasmic and nuclear delivery of AR-CPPs. First, we outline the methodological challenges encountered in studies of the uptake mechanism for AR-CPPs and different cargo macromolecules covalently conjugated or non-covalently complexed with AR-CPP (AR-CPP-Cargo). Then, we discuss recent data on the involvement of various types of endocytosis and cell surface receptors in the cellular uptake of AR-CPPs and different AR-CPP-Cargos, and we analyze the mechanisms of endosomal escape and interactions of AR-CPPs with lipid bilayers. Finally, we highlight the most important unresolved questions and avenues for future research.

Methodological challenges in studies of arginine-rich CCPs

As already stated, despite significant interest many of the important results on the mechanism of AR-CPP uptake remain under debate. To a large extent, this is due to various methodological problems associated with the highly cationic nature of these CPPs. Fluorescence microscopy and fluorescence-activated cell sorter (FACS) analysis, the two experimental techniques most widely used in studies of the mechanism of CPP uptake, are prone to the artifacts associated with the highly cationic nature of AR-CPPs.

Because of the high positive charge, AR-CPPs efficiently bind to the negatively charged cell surface as well as to the glass or plastic surfaces of incubation chambers [22] and, despite their hydrophilic nature, cannot be easily removed by a few washes with buffered saline [23]. Recently, it was demonstrated that incomplete removal of cell-surface-bound peptide significantly affects the results of FACS analysis, since FACS analysis measures total cell-associated fluorescence and does not discriminate between bound and internalized molecules [23]. Surface-bound peptide contributes to detected cell-associated fluorescence signal, affects apparent cell association kinetics and masks the effects of various inhibitors on peptide uptake. The approaches proposed in the literature for efficient removal of surface-bound CPP and CPP-Cargos include protease digestion [23–25], competitive binding to polyaninonic glycosaminoglycans [26] and a combination of both [26]. In another approach, the fluorescence of non-internalized TAT labeled with fluorescein and 7-nitrobenzofurazan (NBD) was effectively quenched by dextran sulfate [27] and dithionite [28], respectively.

In some studies, analysis of AR-CPP uptake is preceded by cell fixation. Recently, it was shown that the formaldehyde or methanol fixation protocols commonly used to access cellular localization of internalized peptide and cargo could lead to artificial redistribution of peptide and conjugated cargo into the nucleus [23, 29–31]. While live-cell imaging indicates preferentially vesicular localization of internalized AR-CPPs and cargo, fixation leads to a broad cytoplasmic and nuclear localization [23, 29–31]. The effects of various fixation protocols vary depending on whether localization of free AR-CPP or AR-CPP-Cargo conjugate is studied. In the case of AR-CPPs conjugated or complexed with different proteins, no apparent effect of mild formaldehyde (2–4 %) fixation is observed [32–34] and only harsher methanol fixation leads to artificial redistribution of conjugate [30, 35]. In contrast, even mild formaldehyde fixation affects cellular localization of free AR-CPPs [23, 28, 36]. This artificial redistribution is due to the highly cationic nature of AR-CPPs. Methanol and, to a lesser extent, formaldehyde compromise the integrity of the cell membrane lipid

bilayer and allow redistribution of internalized AR-CPPs and AR-CPP-Cargos into the nucleus, where they are efficiently retained because of electrostatic interactions with negatively charged DNA.

Determining the intracellular localization of AR-CPPs and AR-CPP-Cargos is quite challenging. The majority of the studies follow the localization of fluorescently labeled peptides and conjugates using fluorescence microscopy. This approach relies on the stability of the link between AR-CPP and fluorophore, since localization is directly observed only of the latter. However, endocytosed AR-CPPs and AR-CPP-Cargos may be degraded upon delivery to late endosomes and lysosomes or inside the cytoplasm. In fact, it has been recently demonstrated that a large fraction of internalized TAT and penetratin peptides is degraded after a 2-h incubation [37]. In this situation, the fluorescent image no longer accurately characterizes the localization of intact AR-CPPs and AR-CPP-Cargos within the cell. While this may not be an issue with short incubation times, the contribution of degradation products is certainly a concern with longer incubations.

Recently, several approaches have been described that are based on the detection of biological activity of cargo molecules exerted upon their delivery into the cell cytoplasm or nucleus [15, 17, 25, 38–42]. In the most widely used assay Cre-recombinase, conjugated to the CPP of interest, is added to the cells that express reporter gene only after deletion of *loxP*-flanked STOP cassette that is specifically induced by Cre-recombinase [15, 17, 25, 40]. This method allows unambiguous and very sensitive detection of the delivery of the functionally active Cre-recombinase into the nucleus of living cells. Such approaches are clearly an indispensable asset for understanding the mechanisms of CPP-mediated cargo delivery. However, one has to be aware that these assays are based on powerful amplification cascades, and therefore quantitative information on the amount of macromolecules delivered can rarely be obtained.

Internalization of AR-CPPs and AR-CPP-Cargos involves endocytosis

Initially, the uptake of TAT, penetratin and other AR-CPPs was ascribed to a non-endocytic, energy- and receptor-independent pathway [8, 10, 43, 44]. This hypothesis was based on the very fast (within minutes), efficient and non-saturable delivery of peptides into the nucleus observed with fluorescence microscopy of fixed cells [8, 10, 43, 44]. The uptake was demonstrated to be insensitive to temperature, energy depletion and a number of inhibitors of endocytosis [8, 10, 43, 44]. On the basis of these data, it was proposed that AR-CPPs, despite their highly polar charged nature, translocate through the lipid bilayer of the plasma membrane by a novel mechanism involving the formation of an inverted micelle intermediate, bypassing the endocytic pathway, and delivering their cargo directly into the cytoplasm and nucleus of the cell [8, 44].

As discussed above, the formaldehyde or methanol fixation used in these early studies could lead to artificial redistribution of peptide and conjugated cargo into the nucleus. In contrast to observations of fixed cells, live-cell imaging indicates punctate cytoplasmic localization of fluorescently labeled free TAT and oligo-arginine CPPs [23, 26, 28, 37, 45, 46]. Similarly, punctate localization was observed by means of live-cell imaging for various AR-CPP-Cargos: biotinylated TAT and penetratin bound to avidin (TAT-avidin and penetratin-avidin) [33, 34]; TAT and oligo-arginine conjugated to green fluorescent protein (GFP) [32, 35, 40, 47]; TAT conjugated to Cre-recombinase (TAT-Cre) [25, 40]; TAT or penetratin conjugated to peptide nucleic acid (PNA) [23, 48]; TAT, penetratin and oligo-arginine conjugated to oligonucleotide [41]; TAT conjugated to gold nanoparticles [49]; and TAT- and penetratin-modified liposomes [50]. Recent studies using live-cell microscopy and FACS analysis have demonstrated efficient inhibition of cellular uptake of different AR-CPPs and AR-CPP-Cargos at 4 °C [23, 25, 26, 28, 32, 34, 38, 45, 47–49, 51, 52] or using ATP depletion with various metabolic inhibitors [23, 28, 34, 38, 46, 51]. These recent studies using living cell microscopy and FACS analysis of internalized peptide demonstrate, in contrast to previous reports using fixation protocols, that AR-CPPs and AR-CPP-Cargos are internalized by endocytosis – a vesicular uptake process dependent on temperature and ATP. Note, however, that Thoren and coauthors, using live-cell microscopy, describe cytoplasmic and nuclear uptake of fluorescein-labeled R_7W (1–10 μM) into PC-12 and V79 cells that is not inhibited by ATP depletion and is even promoted at 4 °C. In contrast, penetratin was internalized in cells of both types in an ATP- and temperature-dependent manner [53].

Cell surface receptors for AR-CPPs

Interaction with cell surface receptors is the first step in endocytic uptake. Binding to plasma membrane through interaction with receptors greatly increases the efficiency of uptake compared with the uptake of the fluid phase markers. Several recent studies address the nature of the binding sites for AR-CPPs and AR-CPP-Cargos.

TAT peptide originates from full-length HIV-1 TAT protein (TAT-FP). Several studies have focused on the binding of TAT-FP to various cell surface glycosaminoglycans. Heparan sulfate proteoglycans have been implicated in internalization of TAT-FP [54, 55]. Externally added heparin, but not chondroitin sulfates, inhibits the cellular

uptake of TAT-FP, as does treatment with heparinase III, specific to heparan sulfate proteoglycans. In contrast, treatment with chondroitinase ABC and chondroitinase AC has no effect on the cellular uptake of TAT-FP [54, 55]. Moreover, the uptake of TAT-FP is greatly impaired in CHO mutant cell lines deficient in heparan sulfate biosynthesis [55]. Heparin – glycosaminoglycan, with a molecular weight ranging from 6 to 40 kDa – efficiently binds to TAT-FP and glutathione-S-transferase (GST)-TAT-FP fusion protein (GST-TAT-FP) with a dissociation constant (K_d) in the nanomolar range [56–58]. Heparin consists of alternating units of sulfated D-glucosamine and D-glucuronic acid and is similar to the heparan sulfate proteoglycans naturally present on the cell membrane. Only heparan sulfate competes with heparin for binding to TAT-FP and GST-TAT-FP, while other sulfated glycosaminoglycans, including chondroitin sulfate A/C or dermatan sulfate, do not affect binding similar to non-sulfated hylauronic acid and desulfated heparin [58]. These data implicate heparan sulfate proteoglycans as specific receptors for the cellular uptake of TAT-FP.

Peptides derived from the basic domain of TAT-FP compete with TAT-FP, and substitutions of arginins with alanins in this region greatly inhibit the uptake of TAT-FP [54]. Since TAT peptide originates from the basic domain of TAT-FP that is responsible for binding to heparin, one may suggest that heparan sulfate receptors are involved in the uptake of TAT and, possibly, other related AR-CPPs. Indeed, in Chinese hamster ovary (CHO) mutant cells that lack all proteoglycans (mutant cell line A-745) or are deficient in biosynthesis of heparan sulfate proteoglycans (mutant cell line D-677), cellular uptake of free TAT [24], of R_9 [36] and of TAT-modified liposomes [50] is greatly reduced, compared with the wild-type cells. On the other hand, the uptake of streptavidin- or avidin-bound biotinylated TAT and penetratin is reduced only in the A-745 mutant, with no appreciable reduction in D-677 cells [33]. Internalization of free TAT is also inhibited by treatment of wild-type CHO cells with heparinase III, while the same treatment has no effect on the uptake in the A-745 cell line [24, 27]. Addition of heparin inhibits the uptake of free TAT [27], TAT conjugated to both GST and GFP (GST-TAT-GFP) [32], TAT or penetratin-modified liposomes [33, 50, 51], TAT-avidin [33] and R_9 [36]. Heparin also inhibits DNA recombination induced by TAT-Cre [25].

In contrast to TAT-FP, uptake of AR-CPPs and their conjugates is inhibited not only by heparin but also by other sulfated glycosaminoglycans. Dextran sulfate inhibits the uptake of liposomes modified with TAT or penetratin [33, 50, 51]. Interestingly, while dextran sulfate also inhibits the entry of TAT-avidin, it does not affect uptake of penetratin-avidin [33]. Chondroitin sulfate A was found to have no effect on the internalization of TAT- or penetratin-modified liposomes [33] or on DNA recombination induced by TAT-Cre [25]. On the other hand, chondroitin sulfates B and C inhibit TAT-Cre-induced DNA recombination, although much less efficiently than does heparin [25]. These findings indicate that binding of AR-CPPs and AR-CPP-Cargos to heparan sulfate receptors is much less specific than binding of TAT-FP.

The values of the dissociation constant K_d of TAT peptide binding to heparin as measured with isothermal titration calorimetry vary from 0.37 [59] to 1.5 μM [60]. These values are considerably larger than K_d values in the nanomolar range measured for the interaction of heparin with TAT-FP at subsaturating conditions [57]. Also, the binding of both heparan sulfate and chondroitin sulfate B to TAT peptide is similar to the binding of heparin, with $K_d = 1.5$ μM [60]. R_9 binds to heparin slightly better, with a K_d between 120 [36] and 300 nM [61]. Interestingly, fluorescein-modified TAT has a K_d 10 times higher than unlabeled TAT [27], indicating that cargo may greatly influence the affinity and specificity of binding to cell surface proteoglycans. It has also been reported that binding to heparin of AR-CPPs labeled with fluorescent dye differently affects the fluorescence of the different attached dyes: while fluorescein is quenched upon binding to heparin [27], the fluorescence of tetramethylrhodamine is increased in the presence of heparin [36].

Taken together, these data indicate that heparan sulfate proteoglycans are important for the interaction of AR-CPPs and AR-CPP-Cargos with the cell surface and for their subsequent internalization. However, AR-CPPs and AR-CPP-Cargo conjugates interact with heparan sulfate proteoglycans with a lower affinity than does the TAT protein. Also, other proteoglycans and charged glycoproteins are involved in the uptake of AR-CPPs and AR-CPP-Cargos. It is not clear at this point whether interactions with cell surface proteoglycans are involved solely in the binding of AR-CPPs to the cell or whether they play an additional role targeting AR-CPPs and AR-CPP-Cargos into specific intracellular compartments, possibly facilitating their cytoplasmic and nuclear delivery.

Uptake of AR-CPPs and AR-CPP-Cargos involves several types of endocytosis

Endocytosis is a collective name for multiple pathways of vesicular uptake by cells. This uptake proceeds by phagocytosis, clathrin-dependent endocytosis and a number of clathrin-independent pathways that includes macropinocytosis and raft/caveolin-dependent endocytosis [62, 63]. Endocytosis is essential for the turnover of proteins and lipids of the plasma membrane, transmission of extracellular signals and uptake of many essential nutrients. In general, the content of the endocytic vesicles is delivered into lysosomes for subsequent degradation. However, many viruses and other pathogens have de-

veloped mechanisms to evade lysosomal degradation and utilize different types of endocytosis for cell infection [63]. Similarly, various protein toxins enter the cell through endocytic pathways [64].

The recent literature shows that cellular uptake of AR-CPPs and AR-CPP-Cargos is not restricted to a single type of endocytosis. Involvement of clathrin-dependent endocytosis [24, 28, 34, 46], caveolin-dependent endocytosis [32, 34, 47], and raft-dependent macropinocytosis [25, 26, 45] in the uptake of different AR-CPPs and AR-CPP-Cargos has been documented.

Clathrin-dependent endocytosis has been implicated in the uptake of TAT in HeLa, CHO and HepG2 cells [24, 46]. Live-cell imaging shows that TAT colocalizes with transferrin, a classical marker of clathrin-dependent endocytosis [23, 46]. Moreover, chlorpromazine and potassium depletion – specific inhibitors of clathrin-dependent endocytosis – efficiently inhibit the uptake of TAT in HeLa and CHO cell lines, while nystatin, an inhibitor of raft dependent endocytosis, has very little effect on the internalization of TAT in the same cell lines [24].

A different type of endocytosis, macropinocytosis, is implicated in the uptake of TAT in Namalwa cells. Extraction of cholesterol by methyl-β-cyclodextrin, known to affect both raft/caveolin-dependent endocytosis and macropinocytosis, inhibits the uptake of TAT in Namalwa cells [26]. Since uptake was also inhibited by cytochalasin D, an inhibitor of F-actin polymerization, these findings argue for the involvement of macropinocytosis in the uptake of TAT in this cell line [26]. Similarly, macropinocytosis has been implicated in the uptake of R_8 in HeLa cells [45] and of TAT-Cre in T cells [25]. In both cases, uptake was inhibited by methyl-β-cyclodextrin and cytochalasin D [25, 45]. In addition, no colocalization was observed with transferrin [45] or caveolin-1 [25], indicating that a clathrin- and caveolin-dependent pathway might play only a limited role in the uptake of R_8 and TAT-Cre in cells of these types.

Another endocytic pathway, raft/caveolin-dependent endocytosis, is implicated in the uptake of GST-TAT-GFP and of TAT-avidin and penetratin-avidin in HeLa and HL3T1 cells [32, 34, 47], as evidenced by extensive colocalization of GST-TAT-GFP with caveolin-1 and cholera toxin subunit B – markers of caveolin-dependent endocytosis [32, 47]. On the other hand, transferrin shows very little colocalization with GST-TAT-GFP, indicating that a clathrin-dependent pathway is not involved in the uptake of this conjugate in HeLa and HL3T1 cells [32, 47]. In addition, vesicular uptake of GST-TAT-GFP follows its slow aggregation on the cell surface, and the velocity of endosomes, containing GST-TAT-GFP, is much slower (3 μm/h) than that of transferrin-containing vesicles (0.5 μm/s) [47]. Involvement of the caveolin-dependent pathway is also supported by the inhibition of GST-TAT-GFP uptake by methyl-β-cyclodextrin treatment of HeLa cells [32]. Methyl-β-cyclodextrin treatment also inhibits the uptake of TAT-avidin and penetratin-avidin [34].

In addition, AR-CPPs in some cases upregulate specific endocytic pathways. In particular, R_8 induces F-actin polymerization in HeLa cells [45], and TAT promotes the uptake of unconjugated 70-kDa neutral dextran, a fluid phase marker internalized mostly by macropinocytosis [26], indicating that both peptides activate macropinocytosis.

To conclude, the existing literature implicates various pathways of endocytosis for the uptake of free and/or cargo-conjugated AR-CPPs. Specific pathways of endocytosis contribute to the uptake to a different extent, depending on the presence and type of attached cargo and cell type used. In part contribution of different pathways of endocytosis may depend on the size of the attached cargo, since the uptake of polystyrene beads gradually shifts from the fast clathrin-dependent pathway to slower raft-dependent pathways upon increase in the size of the beads [65]. Thus, while the variability in the experimental conditions used complicates direct comparison of the data obtained by different groups, existing data suggest that different cell types may utilize various types of endocytosis for the uptake of different AR-CPPs and AR-CPP-Cargos.

Cytoplasmic and nuclear delivery of AR-CPPs and AR-CPP-Cargos

After endocytic uptake, AR-CPPs and AR-CPP-Cargos remain separated from their ultimate destination, the cytoplasm and nucleus, where delivered macromolecules would be able to induce relevant biological responses. It is thus important to understand the mechanisms of intracellular trafficking and endosomal escape of AR-CPPs and their conjugates. Recently, several groups have reported cytoplasmic and nuclear delivery of fluorescently labeled AR-CPPs [37, 46]. Potocky et al. report that while no cytoplasmic and nuclear fluorescence was observed in the presence of a 1.5-μM concentration of fluorescein-labeled TAT, a 15-min incubation of HeLa cells with 7-μM fluorescein-labeled TAT and β-$(VRR)_4$ peptides resulted in diffuse cytoplasmic and nuclear staining in about 50 (for TAT) to 75% (for β-$(VRR)_4$) of all cells [46]. An increase of endosomal pH upon incubation with 50 mM NH_4Cl inhibited cytoplasmic and nuclear delivery of peptides [46], indicating that peptide escape depends on acidification of endosomes. Similarly, cytoplasmic delivery of fluorescein-labeled TAT, R_9 and penetratin is observed after a 2-h incubation with the MC57 cell line [37]. Bafilomycin A and chloroquine, which are inhibitors of endosome acidification, inhibit cytoplasmic delivery [37], confirming the importance of acidification for peptide release. Lack of cytoplasmic

delivery of 10-kDa dextran in the presence of these AR-CPPs indicates that their escape is not accompanied by a major destabilization of endosomes. Surprisingly, no cytosolic fluorescence is observed for any of the peptides in HeLa cells [37], in contrast to the findings of [46]. Incubation of MC57 cells with the Golgi-disrupting drugs nordihydroguaiaretic acid and brefeldin A prior to R_9 and penetratin addition inhibits the uptake of peptides, while incubation of HeLa cells with the same drugs after incubation with TAT and penetratin results in the appearance of cytoplasmic fluorescence, implicating retrograde transport in the cytoplasmic delivery of AR-CPPs [37]. Importantly, however, a large fraction of TAT and penetratin is degraded within the cell, and while fluorescent products of penetratin degradation are retained inside cells, products of TAT degradation effectively escape from them [37]. It is possible that peptides are degraded within endosomes (in a low pH-dependent manner) and that fluorescently labeled degradation products then escape the endosome or even the cell. Moreover, escape of fluorescein from endosomes can be facilitated by low pH, since at low pH fluorescein is uncharged and can readily diffuse through the membrane [66]. These reservations aside, these papers present interesting data on the intracellular trafficking of AR-CPPs.

In contrast to incubation with fluorescently labeled peptide, incubation of cells with different AR-CPP-Cargos such as conjugates with GFP, Cre-recombinase and oligonucleotides does not lead to any detectable nuclear or cytoplasmic staining even after 24 h [25, 35, 40, 41, 47, 67]. Only dendritic cells, which are professional antigen-presenting cells, show cytoplasmic staining (no nuclear staining) after a 12-h incubation with TAT-Ub-PEP fusion proteins labeled with Alexa Fluor 594 dye [67]. The apparent inconsistency with numerous reports of the efficient delivery of functionally active cargo molecules into the cytoplasm and nucleus most likely reflects the very high sensitivity of functional assays based on the powerful amplification cascades. Recently, several groups have measured the biological activity of cargo molecules in the cell cytoplasm and nucleus to study the mechanism of cytoplasmic and nuclear delivery of different AR-CPP-Cargos. TAT-mediated delivery of plasmid DNA is inhibited by ATP depletion and at 4 °C and involves endocytosis [38, 39]. TAT-Cre-induced DNA recombination is also inhibited at 4 °C [25]. Interestingly, both TAT-Cre-induced DNA recombination [25, 40] and TAT-mediated transfection with plasmid DNA [39] are greatly promoted by the endosome-disrupting agent chloroquine, indicating that a major fraction of internalized AR-CPP-Cargos remain entrapped in the endosomes. Functional delivery of TAT-Cre [25] as well as of p53 conjugated to R_9 [68] is effectively promoted by the fusion peptide derived from influenza hemagglutinin HA2. These data indicate that cytoplasmic and nuclear delivery of cargo molecules by arginine-rich proteins is a relatively inefficient process, with the majority of the macromolecular cargo remaining trapped within vesicular compartments. In fact, the functional delivery of oligonucleotides conjugated either to TAT, penetratin, or R_9 [41] or diphtheria toxin A subunit conjugated to TAT [42] is too inefficient to be detected. This inefficiency might reflect the low sensitivity of the methods used in these studies or, alternatively, the efficiency of nuclear or cytoplasmic delivery may depend on the properties of conjugated cargo.

The apparent difference between the efficiency levels of cytoplasmic and nuclear delivery for AR-CPPs and AR-CPP-Cargos may be due to several factors. First, AR-CPP-Cargos are usually used at lower concentrations that might be insufficient for efficient endosomal escape. Second, AR-CPP-Cargos might escape from endosomes less efficiently simply because of size restrictions. Third, cargo molecules may adversely affect the ability of AR-CPPs to mediate endosomal escape by hindering their interactions with endosomal membranes. Finally, the escape of AR-CPP might be greatly overestimated because of the release of fluorescent degradation products from endosomes.

Interactions of AR-CPPs with protein-free artificial lipid bilayer membranes

As mentioned above, endocytosed AR-CPPs and AR-CPP-Cargos remain separated from the cytoplasm by the endosome membrane. Eventually, AR-CPPs and cargo macromolecule have to cross the lipid bilayer of the cell membrane to gain access to the cell cytoplasm. Several recent works have focused on direct interactions of AR-CPPs with the lipid bilayer, since such interactions may play an important role in the mechanism by which AR-CPPs cross the lipid bilayer and deliver their cargo into the cytoplasm.

TAT, penetratin and oligo-arginines bind exclusively to negatively charged liposomes. The binding of oligo-arginins and TAT is mostly electrostatic in its nature and does not induce conformational changes in the peptides (these peptides are mostly random-coiled both in solution and on the liposome membrane) or measurable perturbations of the lipid bilayer [61, 69]. On the other hand, penetratin changes its conformation upon binding to lipid bilayers containing anionic lipid phosphatidylglycerol (PG), from a mostly random-coiled structure in the buffer to more α-helixal and β-sheet conformations on the lipid bilayers [70–73]. However, penetratin does not insert into the lipid bilayer, remaining on the surface [72, 74]. Addition of cholesterol inhibits the binding of penetratin to liposomes composed of zwitterionic phosphatidylcholine (PC) and anionic phosphatidylserine [75]. TAT, R_7W and penetratin induce aggregation of large unilamellar vesicles (LUVs) composed of different proportions of

PC and PG (3:1 or 3:2) at a high peptide-to-lipid ratio (>1/15) [69, 76, 77].

While no translocation of TAT through the lipid bilayer has been observed [78], several groups have demonstrated translocation of penetratin through the protein-free lipid bilayer, and several mechanisms of such translocation have been proposed. An electroporation-like mechanism of penetratin translocation through charged lipid bilayers has been proposed by Binder et al. [74, 79]. These authors suggest that binding of penetratin to the outer monolayer neutralizes the surface charge of anionic lipids and leads to the development of a transmembrane electrical field that is known to result in the formation of transient pores in protein-free lipid bilayers [80–82]. Such a translocation was observed by means of isothermal titration calorimetry only for liposomes containing more then 50 % PG and at peptide-to-lipid ratios higher then 1/20 [74, 79]. On the other hand, the induction of transmembrane potential by valinomycin in the presence of transmembrane gradients of sodium and potassium resulted in efficient translocation of NBD-labeled penetratin for LUVs of different anionic lipid compositions at the much lower peptide-to-lipid ratio of 1/1000 [83]. Interestingly, though, penetratin translocation shows little correlation with lipid mixing or leakage of water-soluble dye [83].

A liposome size-dependent mechanism of translocation was recently proposed [84, 85]. Translocation of fluorescein-labeled penetratin into giant unilamellar vesicles (tens of microns in diameter) made of soy bean lipid extract has been observed by fluorescence microscopy [84–86]. On the other hand, no translocation or content dye leakage is observed on LUVs (100 nm in diameter) composed of PC:PG in a 3:2 mixture [77, 84–86]. However, it should be noted that the giant vesicles used in this study are permeable to a 20-mer oligonucleotide, albeit to a smaller extent than to penetratin [84, 85]. It is therefore unclear whether the observed translocation of penetratin is a specific property of the system used.

Another mechanism, based on dynamic counter-ion charge neutralization, was recently proposed for the translocation of poly- and oligo-arginins. It was demonstrated that in the presence of amphiphilic anions poly- and oligo-arginins redistribute into the bulk organic phase and facilitate transfer of carboxyfluorescein from water into the organic phase [87, 88]. In the presence of amphiphilic anions together with phosphate anions polyarginine induces transfer of carboxyfluorescein through the bulk organic membrane [87]. Similarly, poly- and oligo-arginines induce the escape of carboxyfluorescein from liposomes containing anionic lipids [87]. It was later demonstrated that in the presence of amphiphilic anions, poly- and oligo-arginines induce the escape of carboxyfluorescein from uncharged LUVs [89, 90].

These studies demonstrate that under certain conditions AR-CPPs can translocate through the anionic lipid bilayer. The mechanism of this translocation is still unclear. In addition, the extent to which these data are relevant for the mechanism of cytoplasmic delivery of AR-CPPs and, more important, AR-CPP-Cargos remains to be understood.

Conclusion

Cytoplasmic and nuclear delivery of macromolecules remains a major challenge for the development of effective intracellular therapies based on the use of high molecular weight drugs. Recent data from the use of live-cell imaging and FACS analysis together with assays based on the functional activity of cargo macromolecules have provided important new insights into the mechanisms of AR-CPP and AR-CPP-Cargo uptake. Our current, and clearly insufficient, understanding of the key steps in the delivery of AR-CPPs and their macromolecular cargo into the cytoplasm is summarized in Figure 1. Recent data strongly indicate that endocytosis of AR-CPPs and AR-CPP-Cargo conjugates is the first step in their delivery into the cell cytoplasm. However, it appears that AR-CPPs and AR-CPP-Cargos are rather promiscuous in terms of both surface receptor usage and the specific type of endocytosis utilized. While heparan sulfate proteoglycans appear to be the main cell surface receptors for AR-CPPs, other sulfated glycosaminoglycans are likely to be involved in cell surface binding. Similarly, three different types of endocytosis – clathrin-dependent endocytosis, raft/caveolin-dependent endocytosis and macropinocytosis – have been implicated in the uptake of different AR-CPPs and AR-CPP-Cargos in various cell types. It remains to be understood whether all these endocytic pathways contribute equally to the delivery of functionally active cargo to the cytoplasm. Alternatively, there might be a single specific endocytic pathway responsible for delivery into the cytoplasm, while AR-CPPs internalized by other pathways are destined only for degradation inside the endocytic compartment. The related question as to whether a specific cell surface receptor is responsible for targeting AR-CPPs and AR-CPP-Cargos into specific endosomes is also open. Another related question, which has been reviewed in detail by Brooks et al. [91], is to what extent different properties of the macromolecular cargo influence the mechanism of delivery to the cytoplasm and nucleus.

Interactions of cationic AR-CPPs with negatively charged glycosaminoglycans on the cell surface greatly enhance the endocytic uptake of conjugated macromolecules. However, recent data indicate that the delivery of cargo to the cytoplasm and nucleus is very limited and clearly much less efficient than suggested by early studies. It appears that the escape of internalized AR-CPP-Cargos is a limiting step in the delivery of functionally active cargo,

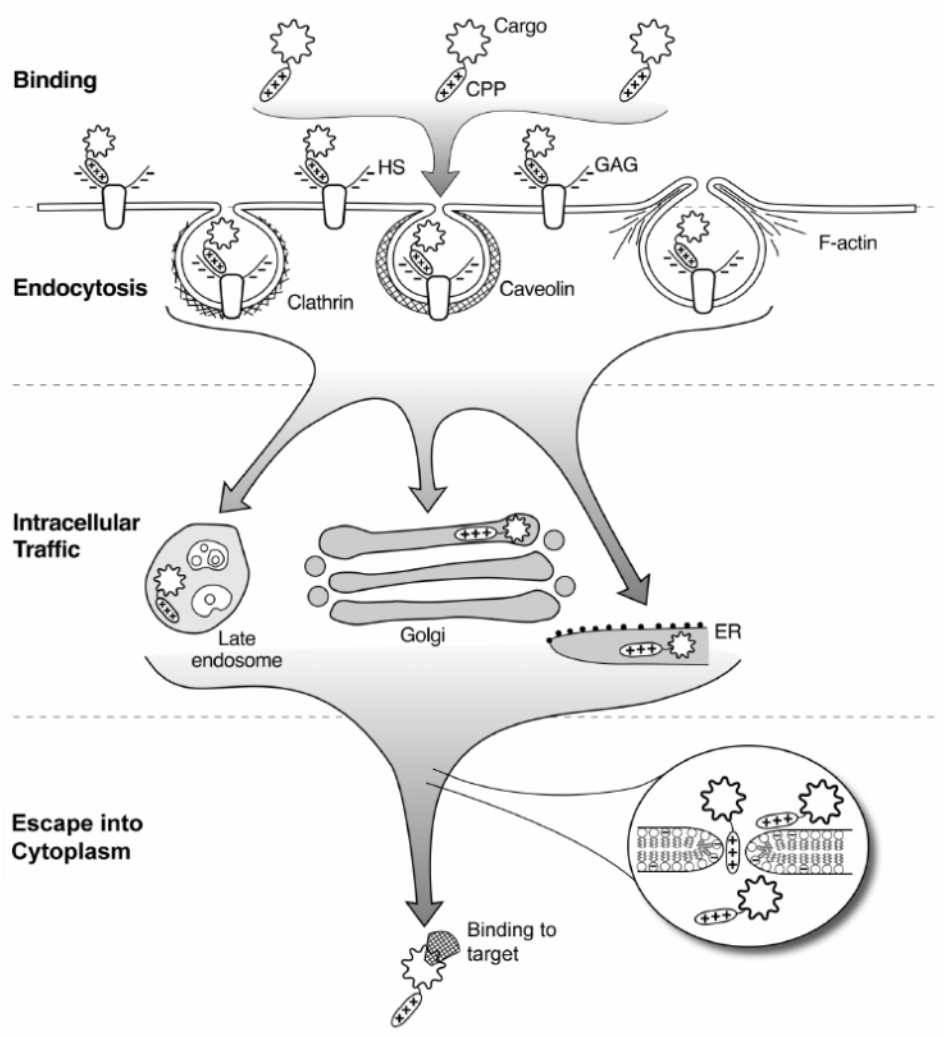

Figure 1. Delivery of cargo into cell cytoplasm by cationic arginine-rich cell penetrating peptides (CPPs). The cartoon summarizes multiple pathways, by which CPP-conjugated cargo first binds to the negatively charged cell surface glycosaminoglycans (GAG), including heparan sulfates (HS), and then enters the cell by clathrin-dependent endocytosis and/or by caveolin-dependent endocytosis and/or by F actin-dependent macropinocytosis. Trafficking through late endosomes and/or Golgi and/or endoplasmic reticulum (ER) delivers the CPP-conjugated cargo into the cytoplasm. To reach its intracellular target, CPP cargo must cross the bilayer. This stage might involve CPP-induced opening of transient pores in the lipid bilayer.

and understanding the mechanisms of this escape is one of the major open questions in the field. There are indications that the escape of AR-CPPs into the cytoplasm depends on endosome acidification and retrograde transport to the Golgi [37, 46]. However, it is unclear whether this is true for AR-CPP-Cargos and whether macromolecules have to be delivered into specific organelles for their subsequent release into the cytoplasm. It remains to be understood whether AR-CPPs play any role in endosomal escape or whether their function is restricted to efficient delivery into the endosomes. Experiments on protein-free lipid bilayers may provide important insights into the role of the AR-CPPs in this process.

In conclusion, recent data have provided important insights into the role of endocytosis and cell surface proteoglycans in the AR-CPP-mediated delivery of functionally active macromolecules into the cell cytoplasm and nucleus. However, the main question remains puzzling: How do arginine-rich peptides promote the transport of macromolecules across the membrane? We believe that future studies combining assays based on the functional activity of delivered cargo, live-cell imaging and experiments on the protein-free lipid bilayer will provide important new insights into this intriguing and important phenomenon.

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