Bioconjugate Chemistry

Review

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The Uptake of Arginine-Rich Cell-Penetrating Peptides: Putting the Puzzle Together

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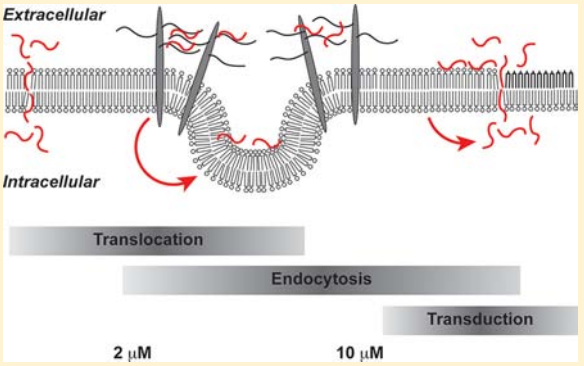
ABSTRACT: Over the past 20 years, cell-penetrating peptides (CPPs) have captured the attention of biomedical researchers, biophysicists, and (bio)organic chemists. These molecules efficiently enter cells and mediate entry of (macro)molecules that by themselves do not cross the plasma membrane. Since their discovery, models on the mechanism by which uptake occurs have seen major revisions. Starting from direct penetration across the plasma membrane, it later became apparent that for large molecular weight cargos in particular, endocytosis plays a role in uptake and furthermore that the route of uptake is a function of CPP, cell-type, cargo, and concentration. For the class of arginine-rich CPPs, this dependence on conditions has been elucidated in particular. As I will discuss here for this class of CPPs, a downside of this multitude of possibilities has been a lack of attention for commonalities in the observation of apparently distinct phenomena. At the same time, differences of apparently similar observations were not appreciated sufficiently. In addition, there has been insufficient acknowledgment of observations that are incompatible with the proposed models. Nevertheless, a considerable amount of data can be assembled into a quite coherent picture and the data that is left creates the basis for concrete future lines of research to resolve the questions that remain. Moreover, any uptake mechanism has its distinct structure−activity relationship for uptake giving room for the molecular design of molecules to preferentially direct uptake to either of them.



INTRODUCTION

Cell-penetrating peptides (CPPs) comprise a group of peptides of about 8 to 30 amino acids in length that are efficiently taken up by cells and mediate the uptake of conjugated cargo. A high density of positive charge is a characteristic of most CPPs.[1] For many CPPs, as for example in penetratin, these are present in an amphiphilic context. Another important class are the arginine-rich CPPs of which the TAT peptide and the oligoarginines are the most prominent members.

Penetratin and the TAT peptide were identified as so-called protein transduction domains of the homeodomain transcription factor Antennapedia[2] and of the transactivating protein TAT of the HIV, respectively.[3] It had been described before that both proteins have the capacity to reach the nucleus of cells after addition to the tissue culture medium.[4,5] In particular, work on penetratin supported the notion that these proteins as well as their transduction domains did so by directly translocating across the plasma membrane (see below). The original concepts were questioned when in 2003 Richard and colleagues followed the cellular uptake of fluorescein-labeled CPPs into living cells, demonstrating that uptake of these molecules occurred by endocytosis and that direct cytoplasmic entry was a misinterpretation as a consequence of a redistribution of peptides during fixation, which before had been used for the analysis of cellular peptide distribution.[6] This particular publication was of major significance in promoting the use of living cells in studies of peptide uptake. However, others had proposed a role of endocytosis before, and a considerable amount of data supporting direct permeation remained valid as it was not affected by fixation artifacts (reviewed in ref 7). Nevertheless, from then on research on import mechanisms focused on endocytosis. This was also due to the fact that applications of CPPs focused on import of macromolecular cargo, for which endocytosis is the preferred if not the only route of import.[8]

In spite of this change of focus, for biotinylated penetratin and arginine-rich CPPs strong evidence for direct translocation was provided with state-of-the-art mass spectroscopy techniques.[9,10] Furthermore, for penetratin it was shown that a fluorescein-labeled analogue entered by endocytosis, while a biotinylated one entered by direct translocation, proposing that fluorescent labeling may impede direct translocation across the plasma membrane and therefore escape detection by live-cell microscopy.[11] Interestingly though, from 2005 several papers demonstrated a rapid and nondisruptive direct cytoplasmic entry of fluorescently labeled arginine-rich peptides that occurred at high peptide concentrations, which are concentrations above 10 μM.[8,12−14] As detailed below, the mechanistic evidence obtained so far indicates that this uptake is fundamentally different from direct translocation at low concentrations. However, evidence so far also indicates that

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this route of uptake is limited to arginine-rich peptides conjugated to small molecular weight cargo.[8]

For arginine-rich CPPs, this Topical Review will first outline the differences between translocation at low concentrations and the so-called transduction at high peptide concentrations. Next to incubation of cells with CPPs at high concentrations, also other means to induce uptake with very similar characteristics have been described. I will point out commonalities of the different mechanisms to induce transduction that have not been discussed so far. Then, endocytic import will be reviewed in light of the data that cannot be accommodated by the current model for the induction of import. Finally, from this critical review of the available data directions of future research will be proposed.

DIRECT CYTOPLASMIC ENTRY OF CPPS—TRANSLOCATION VERSUS TRANSDUCTION

For the antennapedia homeobox domain, the initial experiments showed a homogeneous intracellular distribution of a fluorescently labeled protein as well as an increased neuronal differentiation at a concentration as little as 1 μM.[5] For the HIV TAT protein, transactivation at the viral promotor could be obtained at concentrations as low as 1 nM. Thus, for both proteins, transcriptional activation provided solid proof that the protein had reached the cytoplasm. However, for TAT activity was greatly enhanced by the lysosomotropic agent chloroquine which is generally considered evidence for endosomal uptake, and further evidence for endocytosis was provided a few years later.[15] In fact, the initial paper on antennapedia did not address the import mechanism. Experimental evidence of a direct membrane translocation of the antennapedia-derived penetratin peptide was only published some years later, when it was demonstrated that uptake occurred also at 4 °C and the intact peptide could be recovered from within the cells.[2] Receptor independence of uptake was further substantiated through uptake of a D-amino acid analogue.[16]

In spite of evidence for a role of endocytosis in uptake,[17,18] including the early work on the TAT protein,[4,15] from then on, the major part of work on the uptake mechanism and application of CPPs was conceptualized within the framework of direct membrane translocation (reviewed in ref 7). It was not until the publication of Richard and colleagues in 2003 that the pendulum swung to the other side and research was directed toward the role of endocytosis in uptake.[6,19] Endosomal sequestration also provided an explanation of many failed (various personal communications and own experience) attempts to exploit CPPs for cytoplasmic delivery of bioactive molecules. From this point, given the diversity of endosomal processes, cell types, cargos, and experimental techniques to study uptake, a consensus on common principles governing uptake was further away than ever.[19] To enhance delivery, activities were directed toward implementation of endosomal release strategies[20,21] with major success at least for the delivery of oligonucleotides.[22,23]

In 2005, Fabienne Burlina and co-workers presented a very carefully designed mass spectrometry protocol through which they could recover intact peptides from within cells and also quantitate the amount of peptide.[9] This protocol implemented the state of the art in eliminating a mere association of peptides to the plasma membrane as a confounding factor and also provided for a reliable discrimination of intact peptide and degradation products, which is difficult to achieve in fluorescence-based assays. In several follow-ups, this method was then employed to assess peptide import providing strong evidence for direct permeation[24,25]—on one hand, on the basis that intact peptide could be recovered, which was considered unlikely if import occurred by endolysosomal import, and on the other hand, through experiments that showed import following incubation at 4 °C.

Of particular interest with respect to the integration of concepts is a study of the same group in which the concentration dependence of import pathways was investigated for a series of arginine-rich CPPs and penetratin at concentrations lower than 12 μM. The authors concluded that at low micromolar concentrations uptake occurs by direct translocation across the plasma membrane, while at concentrations higher than 2 μM, a glycosaminoglycan-dependent endocytosis is induced.[10] In spite of questions that remain with respect to the induction of endocytosis (see below), this study provides a conceptional framework of direct, but rather inefficient translocation governed by peptide−lipid interactions which is then superimposed or replaced by endocytosis dependent on peptide−glycosaminoglycan interactions (also reviewed in ref 26) (Figure 1).

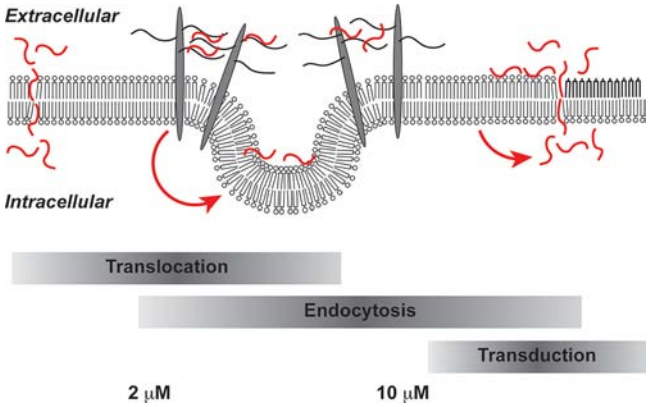

Figure 1. Concentration-dependent modes of import of arginine-rich cell-penetrating peptides. Intracellular red arrows indicate the requirement for the activation of molecular processes inside the cell, a characteristic that distinguishes transduction from translocation. For induction of endocytosis, cross-linking of glycosaminoglycan-bearing transmembrane proteins such as syndecans is shown. However, this model cannot account for the chirality dependence of peptide uptake and induction of endocytosis in cells that are poor in glycosaminoglycan expression. For transduction, the formation of a membrane microdomain, as is a consequence of ceramide formation, and import along the domain boundary is indicated. However, this model needs further experimental validation. Even though not addressed in detail, the mechanisms are not mutually exclusive. In the presence of endocytosis, one may assume that translocation still occurs.

Ziegler and Seelig revisited the concept that direct membrane permeation could not be detected for fluorescein-labeled peptides[11] by demonstrating that at a concentration of 100 μM the TAT peptide rapidly entered the cytoplasm. This uptake was associated with the formation of local aggregates of high peptide concentration at the plasma membrane.[14]

The same type of rapid cytoplasmic uptake from restricted membrane areas but already at a concentration of about 20 μM was reported for oligoarginines[12] and for the TAT peptide conjugated to small molecular weight cargo.[8,27] Cardoso and co-workers employed the term "transduction" for this mode of

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import. In 2007 our laboratory investigated in detail the concentration dependence of the uptake of arginine-rich CPPs and showed that in the concentration range of 10−20 μM a transition occurs from entry through endocytosis to a rapid cytoplasmic entry from areas of local enrichment at the plasma membrane that we coined nucleation zones (Figure 2).[13] Importantly, inhibition of endocytosis promoted nucleation zone-dependent uptake, demonstrating that the mechanism of uptake is a function of the availability of import routes.

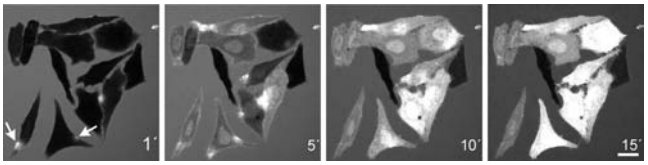

Figure 2. Rapid cytoplasmic uptake of R9. HeLa cells were exposed to Fluo-R9 at a concentration of 20 μM in the presence of serum and uptake was followed by time-lapse confocal microscopy at room temperature with frames recorded every 30 s. Four frames recorded at 1, 5, 10, and 15 min are shown. Arrows indicate the spatially confined areas of plasma membrane called nucleation zones from which the peptides spread throughout the cytoplasm. The scale bar corresponds to 10 μm. Reproduced with permission from ref 13.

It is somewhat surprising that in spite of the fact that this uptake only occurred at high peptide concentrations while at lower concentrations endocytosis was observed, there was no generalized notion that this uptake was fundamentally different from direct translocation at low micromolar peptide concentrations.[25] In particular, transduction could be inhibited by pharmacological inhibitors indicating that it involved the activation of specific cellular activities.[13] Later, we showed that this uptake involves the activation of acid sphingomyelinase and ceramide formation.[28] Ceramide formation is critical for this route of uptake, as inhibition of uptake by acid sphingomyelinase inhibitors and in acid sphingomyelinase knockout cells could be rescued by external addition of sphingomyelinase to form ceramide in the plasma membrane. This ceramide-dependent uptake showed the same restriction to low molecular weight molecules as described for transduction and the same bright zones at the plasma membrane, and given this commonality of characteristics, there is little reason to believe that transduction and nucleation zone-dependent uptake are different routes of import.

Ceramide is known to form membrane microdomains[29,30] with significant roles in the organization of membrane receptors in viral and bacterial infections.[31,32] Ceramide formation in the outer leaflet of the plasma membrane induces a negative (inward) curvature, while formation in the inner leaflet a positive (outward) membrane curvature.[33] The formation of membrane buds concomitant with transduction of CPPs was observed by fluorescence confocal microscopy as well as by electron microscopy.[13,34] Given the impact of ceramide on membrane curvature, formation of a bud is the opposite of what would be expected for formation of ceramide in the outer leaflet of the plasma membrane.[33] At present, the bud formation can therefore best be conceptionalized by a further impact of the arginine-rich peptide on lipid organization. In line with an outward bud formation contributing to uptake, direct uptake of octaarginine from localized membrane areas could also be induced by external addition of the peptide EpN18, corresponding to the N-terminal 18 amino acids of the protein epsin.[35] Inside the cell this protein induces positive membrane curvature (inward dips) in conjunction with the formation of clathrin-coated pits.

It should be stressed that addition of EpN18 induced direct and rapid cytoplasmic uptake at a peptide concentration at which for the peptide alone, endocytosis was observed. In the same way, also with external addition of sphingomyelinase, rapid uptake could be induced at a peptide concentration of 5 μM of R9, well below the threshold for induction of transduction of around 20 μM. These observations indicate that various means exist to induce and/or contribute to a membrane disturbance which at higher concentrations is autonomously caused by the arginine-rich peptide alone. This also indicates that for transduction there is an induction and a transfer step.

As pointed out above, transduction is a characteristic of arginine-rich CPPs. For penetratin this uptake could only be induced at a considerably higher concentration (100 μM) with concomitant cholesterol depletion of the plasma membrane using methyl-β-cyclodextrin.[13] The amphipathic CPP TP10[36] induces massive membrane disturbance and toxicity at concentration of 25 μM (Table 1).[37]

The original contribution by Ziegler and Seelig[11] introduced a notion that transduction was associated with acute cellular toxicity. However, one should note that in this publication much higher concentrations of peptide (50−500 μM) were used than in the other work described here (typically below 20 μM). At these lower concentrations, signs of neither a

Table 1. Cell-Penetrating Peptides with Different Uptake Characteristics[a]

CPP/class	sequence	membrane staining	uptake mechanism
	Cationic Amphipathic		
TP10	Fluo-AYLLGKINLKALAALAKKIL-NH_2	++	membrane insertion/endocytosis/massive membrane disruption (above 10−20 μM)[37,56−58]
Penetratin	Fluo-RQIKIWFQNRRMKWKK-NH_2	-	translocation[c]/endocytosis[10,11,13]
	Arginine-Rich		
R9	Fluo-RRRRRRRRR-NH_2	-	translocation (below 2 μM)[c]
TAT	Fluo-YGRKKRRQRRR-NH2	-	endocytosis (below 10−20 μM/transduction[10,13,53] (above 10−20 μM)[b]
hLF	Fluo-KCFQWQRNMRKVRGPPVSCIKR-NH_2	-	

[a]The lactoferrin-derived CPP shows transduction in spite of a rather small arginine content. The two cysteine residues need to form a disulfide bridge for activity as a CPP. All peptides are indicated as peptide amides (−NH_2) with an N-terminal carboxyfluorescein moiety (Fluo-). [b]Indicated concentrations depend on the type of peptide, cell type, and incubation conditions.[12] [c]Data obtained for biotinylated analogues.

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compromise of membrane integrity nor acute toxicity could be observed.[13]

Local membrane areas of increased fluorescence were also discernible in confocal fluorescence micrographs of cells in which cytosolic uptake of arginine-rich CPPs was induced by preincubation of cells with pyrenebutyrate.[38] This import route shares with transduction the restriction to arginine-rich peptides.[39] However, with this method also considerably larger molecules have been delivered to the cytosol.[38,40] Time lapse analyses should be instructive to reveal to which degree this route shares further characteristics with transduction.

TRIGGERING OF ENDOCYTOSIS—GAPS IN A CONCEPT

Following the recognition that endocytosis is a major route of import, research into the endocytic pathways that contribute to uptake intensified. Kaplan and Dowdy made the highly noteworthy observation that TAT and other CPPs increased the cellular uptake of the fluid phase endocytosis marker dextran in a dose-dependent manner indicating that the peptides induced macropinocytosis.[41] A similar observation but in favor of an induction of clathrin-dependent endocytosis was presented by our laboratory in the same year, showing that penetratin, TAT, and R9 induced the endocytosis of TNF receptors and the EGF receptor.[42] Quite evidently, CPPs activate cellular processes that lead to endocytosis.

The observation that cationic CPPs interact with negatively charged glycosaminoglycans in solution[43] and on the cell surface[44−46] and that removal of glycosaminoglycans (GAGs) reduced uptake initiated experiments into the possible molecular mechanism connecting GAG binding and uptake. Nakase and co-workers as well as Gerbal-Chaloin and co-workers showed for TAT and R8, and for MPG, respectively, that uptake of these peptides induced a Rac-dependent actin reorganization in a heparan sulfate proteoglycan dependent manner.[45,47] These observations created a strong mechanistic framework involving molecules that are well-established players in the induction of macropinocytosis and therefore, since then, have received little critical evaluation.

Nevertheless, several observations question this concept. First, removal of GAGs only partially inhibits peptide uptake by 20−80%.[45,48] Second, efficient uptake of CPPs that strongly binds GAGs is also observed for cells that are poor in GAGs such as Jurkat T cell leukemia cells.[49] Jurkat cells have only about 10% of the expression level of GAGs present on HeLa cells.[37] Third, D-peptides show only about half the uptake efficiency of their L-amino acid counterparts in spite of the same binding affinity for heparan sulfate proteoglycans.[50] Also with respect to the linking of GAG clustering and induction of uptake, the picture is ambiguous. Contrary to a direct connection of clustering and endocytosis,[51,52] a PEGylated variant of an arginine-rich CPP failed to cluster GAG but nevertheless showed uptake by endocytosis.[44] For analogues of the amphipathic CPP TP10 strong aggregation of GAGs on the cell surface compromised uptake.[37] However, for a collection of variants of a CPP derived from human lactoferrin[53] there was a strong negative correlation of stoichiometry of binding between peptides and heparan sulfates and uptake efficiency as measured by isothermal titration calorimetry. These latter observations could best be explained by a stronger cross-linking of the glycocalyx through those peptides with only a few binding sites per GAG chain. Stoichiometry did not correlate with the efficiency to cluster heparan sulfates, though, as measured by dynamic light scattering spectroscopy.[49]

FUTURE STEPS

Over the past years, a wealth of data on import mechanisms of CPPs has been acquired. These achievements would receive even more recognition if it was appreciated that the diversity of import pathways does not represent a lack of coherence, but instead a finely nuanced picture of import mechanisms, each with its own structure−activity relationship and molecular mechanisms of uptake. While the cationic amphipathic CPP TP10 strongly inserts into the plasma membrane and leads to acute membrane disturbance at concentrations above 10 μM, penetratin shows little membrane insertion and instead induction of endocytosis. For arginine-rich CPPs no enrichment in the plasma membrane is observed. For these CPPs a transition from direct translocation at low concentrations to induction of endocytosis and then to transduction occurs. Endocytosis as well as transduction are reactions of the cell to the interaction with the peptide. Even though both translocation and transduction lead to a direct entry of peptides into the cytosol, according to current evidence, dependence on the activation of cellular processes is an exclusive characteristic of the latter.

For both endocytosis and transduction, identification of the molecular events that trigger uptake still remains a major challenge. Cross-linking of GAGs is a highly attractive concept to explain the induction of endocytosis. Nevertheless it is insufficient to explain the stereoselectivity of uptake. Furthermore, a concept is still required to explain the presence of endocytosis in GAG-deficient cells. A simple explanation for the latter could be that in GAG-deficient cells, uptake of CPPs only occurs by constitutive endocytosis following interaction with the plasma membrane, while in cells carrying GAGs, endocytosis is induced. However, this hypothesis still needs experimental validation. The recent publication by Amand and colleagues is a first step toward dissecting the interplay of CPP interactions with the glycocalyx and transfer to the lipid bilayer.[52] With respect to transduction, so far, there is no indication with respect to the molecular processes activating this route. In general, the understanding of the molecular events underlying the activation of acid sphingomyelinase is still very sketchy.[54] The transition from endocytosis to transduction can best be explained by assuming that the capacity to clear peptide from the cell surface by endocytosis is limited. Above the 10−20 μM concentration threshold, peptide accumulates at the plasma membrane and triggers transduction. The lowering of the threshold by endocytosis inhibitors supports this model.

Further elucidation of the molecular details of the individual uptake routes will benefit from a consequent application of the present knowledge on concentration dependencies and specific structure−activity relationships for each individual process. Present research mostly aims to elucidate the uptake pathway of a given CPP for a limited number of cell types. Inevitably, this approach faces the potential simultaneous presence of several uptake routes for the given CPP which will compromise an understanding of the molecular details of a particular uptake route. Instead, it would be helpful to select a CPP in such a way that uptake is restricted as much as possible to a particular route. For example, D-nonaarginine shows a reduced capacity to induce endocytosis, but the full capacity to induce nucleation zone-dependent uptake.[50] The growing realization that each uptake route has its own structure−activity relationship also

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gives room to optimize CPPs and other molecular transporters with respect to a particular route of uptake, which will increase delivery efficiencies and provide the possibility to incorporate selectivity for cell types possessing a particular route. The incorporation of tryptophans to enhance the interaction with GAGs may serve as one example in this direction.[55] Finally, while research into the uptake mechanisms induced by CPPs provides highly interesting fundamental insights into membrane organization and dynamics, the relevance of the individual uptake routes for delivery of various types of cargos also needs to be explored.

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Notes

The authors declare no competing financial interest.

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