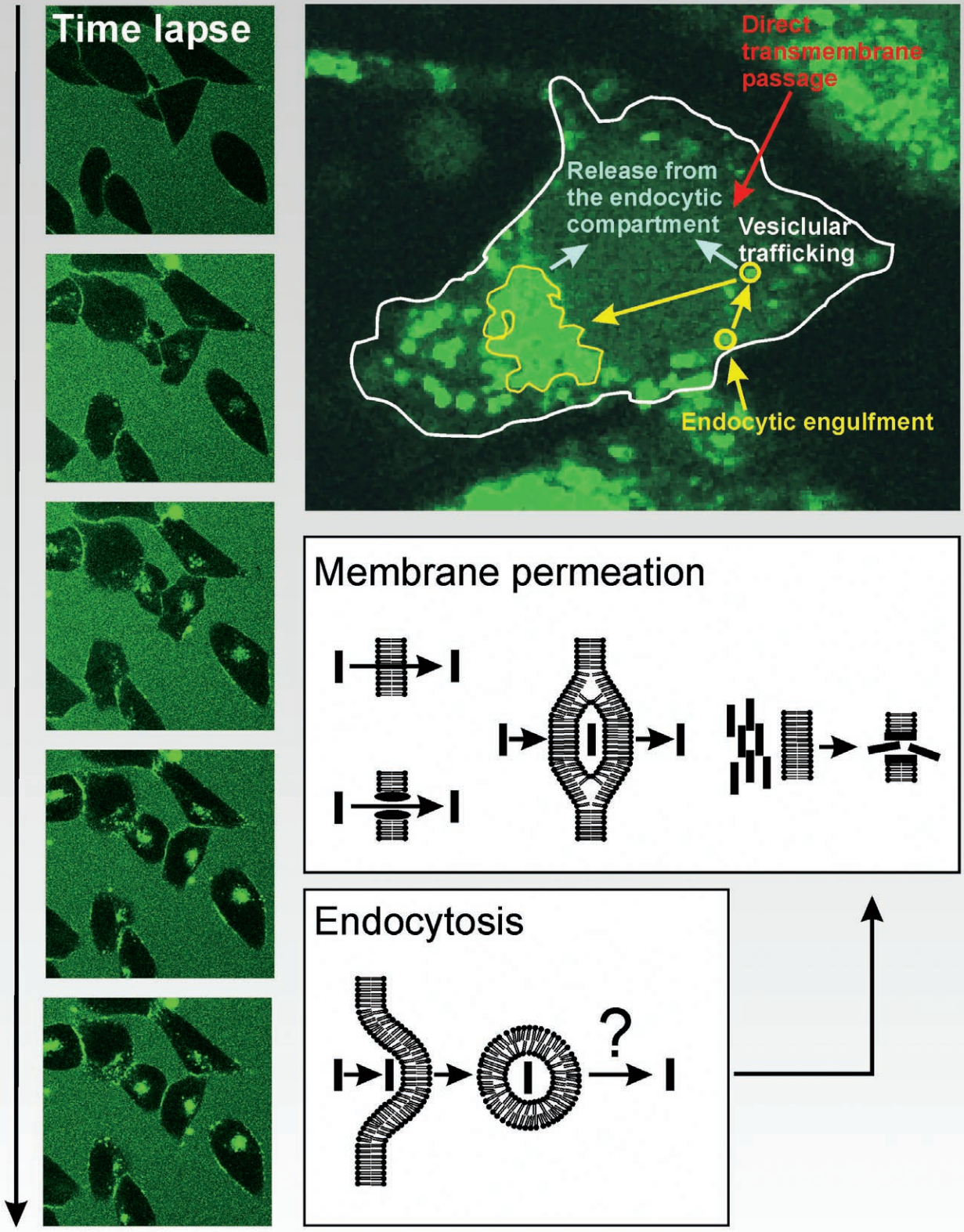
# Cell-Penetrating Peptides: Molecular Mechanism of Cellular Import




 DOI: 10.1002/cbic.200500044  

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# Break on through to the Other Side—Biophysics and Cell Biology Shed Light on Cell-Penetrating Peptides

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*Cell-penetrating peptides (CPPs) have become widely used vectors for the cellular import of molecules in basic and applied biomedical research. Despite the broad acceptance of these molecules as molecular carriers, the details of the mode of cellular internalization and membrane permeation remain elusive. Within the last two years endocytosis has been demonstrated to be a route of uptake shared by several CPPs. These findings had a significant impact on CPP research. State-of-the-art cell biology is now required to advance the understanding of the intracellular fate of the CPP and cargo molecules. Owing to their presumed ability to cross lipid bilayers, CPPs also represent highly interesting objects of biophysical research. Numerous studies have investigated structure–activity relationships of CPPs with respect to their ability to bind to a lipid bilayer or to cross this barrier. Endocytosis route only relocates the membrane permeation from the cell surface to endocytic compartments. Therefore, biophysical experiments are key to a mechanistic molecular understanding of the cellular uptake of CPPs. However, biophysical investigations have to consider the molecular environment encountered by a peptide inside and outside a cell. In this contribution we will review biophysical and cell-biology data obtained for several prominent CPPs. Furthermore, we will summarize recent findings on the cell-penetrating characteristics of antimicrobial peptides and the antimicrobial properties of CPPs. Peptides of both groups have overlapping characteristics. Therefore, both fields may greatly benefit from each other. The review will conclude with a perspective of how biophysics and cell biology may synergize even more efficiently in the future.*

## 1. From Permeation of the Plasma Membrane to Endocytosis and Back

With only ten years of age, the field of cell-penetrating peptides (CPPs) is currently witnessing a dramatic change of views. Up until 2003 it was commonly accepted that many CPPs enter mammalian cells by directly crossing the plasma membrane. This uptake was shown to be energy-, temperature- and receptor-independent; thereby, a role for endocytosis in cellular uptake was explicitly excluded. Furthermore, biophysical experiments also supported direct permeation of the plasma membrane as the mechanism of import. This import mechanism implicated a route of entry into mammalian cells that bypassed the hydrolytic activities present in the endocytic compartments and avoided the problem of escape from the endocytic compartment. However, the cell-biological experiments that formed the basis for this endocytosis-independent model were largely based on fluorescence microscopy performed with fixed, permeabilized cells. In 2003, data presented by Richard et al. challenged the model of a direct permeation of the plasma membrane by demonstrating that even mild fixation leads to an artefactual redistribution of internalized CPPs.[1] In living, nonfixed cells a large fraction of fluorescent CPPs was merely associated with the outer leaflet of the plasma membrane rather than being present within the cytoplasm. This membrane-associated population of CPPs accounted for the cellular fluorescence previously observed when cells were incubated with peptide at 4 °C. Richard et al. demonstrated that these peptides could be removed by trypsinization. Analysis of peptide uptake by live-cell microscopy demonstrated the involvement of endocytosis in the cellular internalization of the Tat peptide and the nonaarginine peptide.

Even though the paper by Richard et al. in 2003 may be considered as the breakthrough in raising general awareness about the relevance of endocytosis for the cellular uptake of CPPs, a number of earlier publications had conducted live-cell experiments and concluded that membrane permeation might not be the only mode of uptake. In fact, the first report on the ability of a truncated synthetic antennapedia protein to permeate into neurons and act as a transcription factor also included data from live-cell microscopy.[2] It was demonstrated that nuclear accumulation of the fluorescein-labeled protein was not a fixation-related artefact. The authors do not provide any concrete speculations about the transport mechanism across the plasma membrane. However, it is mentioned that the biological effects are strongly reduced after removal of polysialic acid residues present at the surface of the nerve cells.

In 2000, Scheller et al. presented a quantitative comparison of the uptake of different CPPs by using detection by confocal laser scanning microscopy in living cells.[3] These authors observed a partially vesicular distribution of fluorescence. Still, the use of state-of-the-art experimental procedures strongly

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supported a primarily nonendocytic uptake. Nevertheless, the authors stated "that nonendocytic as well as endocytic modes of uptake are involved" (in the uptake of the peptides). Hällbrink et al. employed conjugates in which penetratin, the Tat peptide, transportan, and model amphipathic peptide (MAP) were coupled to a fluorescent reporter group through a disulfide bridge to compare the uptake efficiencies of these different CPPs free from the bias of peptide export.[4] Only after reduction of the disulfide bridge was the fluorescence of the reporter group dequenched. This contribution is noteworthy for two reasons. First, the strategy for measuring import kinetics is highly elegant. Second, reducing conditions that release the reporter group from the CPP are only encountered in the cytoplasm, not in endolysosomal compartments. The authors proposed a kinetic model of CPP uptake according to which peptide in the medium is in equilibrium with peptide associated with the outer leaflet of the plasma membrane. Following translocation across the lipid bilayer, the membrane-associated peptide is in equilibrium with cytoplasmic peptide. The results were fully explained by a rapid translocation of conjugates across the plasma membrane. An intermitted entrapment in endocytic vesicles was not considered.

With respect to the involvement of endocytosis in the uptake of CPPs, it was demonstrated that transport of doxorubicin conjugated to the arginine-rich SynB peptide into mouse brains was reduced by polylysine and protamine, which were employed as inhibitors of endocytosis.[5] For positively charged proteins like histone H1 or VP22, fixation artefacts had already been suggested in 2002.[6,7] Moreover, one of the first studies on the HIV-1 Tat protein clearly demonstrated the endocytic uptake of the protein.[8] At the beginning of 2003, Olsnes et al. stated that "experiments to exclude that the entry of endocytosed (Tat) peptide into the nucleus occurred after fixation and permeabilization are highly desirable".[9] Earlier, the same

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group had reported that neither the Tat basic domain nor the viral VP22 protein had been able to mediate membrane translocation of the Diphtheria toxin A fragment.[10] In this assay, import of the Diphtheria A toxin into the cytoplasm and its subsequent cytotoxicity provided a highly sensitive functional readout for the detection of the cytoplasmic delivery of a protein cargo. The authors therefore concluded that CPP-mediated protein import was inefficient compared to the natural Diphtheria toxin shuttle system.

Since 2003, endocytosis has become a focus in cellular CPP research. (For a review, see ref. [11].) Endocytosis comprises several different mechanisms. Therefore, it is not surprising that descriptive instead of mechanistic studies of the endocytic import of CPPs have played a major role over the past two years. In this context, a large number of cell-biology tools were acquired by CPP research. These tools, such as small-molecule inhibitors of endocytosis and intracellular trafficking, along with tracer molecules for labeling of endocytic pathways, originated largely from research on endocytosis. Even though most contributions currently favor endocytosis as the mode of uptake, direct membrane permeation has also received support for individual CPPs.[12, 13]

Endocytosis does not only add a further step to the order of events involved in CPP uptake. Endocytosis has a major impact on the biological applications of CPPs. Along the endolysosomal pathway, the CPP and cargo may encounter proteases that limit the biological activity of the cargo. Evidence has been presented that release of CPP–cargo conjugates into the cytoplasm is a bottleneck for the interaction of cargo peptides with cytoplasmic target proteins. Accordingly, disruption of endosomes enhances the nuclear delivery of Tat fusion proteins.[14] In addition, endosomal uptake of the calpain inhibitor calpastatin fused to the Tat peptide prevents the interaction of the inhibitor with calpain in the cytoplasm.[15] These observations are also interesting in the context of the physiological relevance of protein transduction, as one may ask along which pathway transcription factors reach their nuclear target.[16] However, for the antennapedia protein and other transcription factors even the very little protein taken up by direct membrane permeation may be sufficient to exert their function. Moreover, the CPP-mediated import of bioactive molecules is not only confronted with the entrapment of a significant part of the molecules inside endocytic vesicles. The endocytic import itself might interfere with cellular function. In our own work, we showed that the cationic CPPs penetratin, the Tat peptide, and nonaarginine induce the internalization of tumor necrosis factor (TNF) receptors 1 and 2 from the plasma membrane, thereby impairing the ability of the cell to respond to TNF-receptor-dependent stimuli.[17] Internalization of epidermal growth-factor receptors (EGFRs) was also observed.

Before the emergence of CPPs, analysis of the structure–activity relationship of peptides interacting with lipid bilayers had already been an area of intense research in the field of antimicrobial peptides. In analogy to research conducted on antimicrobial peptides, for CPPs most biophysical analyses have been based on the interaction of CPPs with phospholipid vesicles of different composition and size. The techniques employed for this purpose have encompassed fluorescence spectroscopy, fluorescence microscopy, circular dichroism, NMR spectroscopy, plasmon-waveguide resonance and impedance spectroscopy, polarized-light spectroscopy, polarization modulation infrared reflection spectroscopy (PM-IRRAS), ellipsometry, and neutron reflectivity.[18–25] In general, these studies aimed to clarify 1) the structure–activity relationships for the interaction of peptides with lipid bilayers, 2) the role of secondary structure in this interaction, 3) the correlation between the interaction with the lipid bilayer and the transit across the bilayer, and 4) the influence of lipid composition and additional factors such as membrane potential on peptide–lipid interactions and transit. However, in contrast to investigations on antimicrobial peptides, a large proportion of the biophysical analyses were performed without a direct correlation to biological activity. One possible reason may be that the integration of advanced cell biology represents a bigger hurdle than the implementation of antimicrobial and hemolytic assays.

The model assuming direct permeation of the plasma membrane as the route of cell entry established a link between cell biology and biophysics very early on. Biophysical experiments provided valuable information on the mechanistic details of the permeation of lipid bilayers by peptides. For penetratin, it carries a certain irony that the biophysical analyses confirmed the ability of this CPPs to cross a lipid bilayer and thereby probably distracted cell biologists from being more critical about their experimental design.[20] However, recent publications demonstrate that a link between both disciplines is highly productive in the context of endocytosis as well.

For nonendocytic internalization through direct permeation of the plasma membrane, three elementary steps had been distinguished: 1) association of the CPP with the plasma membrane, 2) permeation through the plasma membrane, and 3) release of the peptide into the cytoplasm. Alternative options have been discussed for each of these steps, depending on the CPP and the experimental study (see below). Cellular uptake of CPPs by endocytosis basically adds further events between steps 1 and 2, thereby resulting in a relocation of membrane permeation from the plasma membrane into the endocytic compartment.

Biophysics studies with well-defined model systems have contributed substantially to elucidating the structure–activity relationships of steps 1–3 in the context of direct membrane permeation. Future biophysical analyses will have to consider the chemical environment encountered by a CPP along the endolysosomal pathway. Acidification, for example, may change the protonation state of amino acid side chains, thereby affecting the interaction of these side chains with lipid bilayers. However, the chemical environment along the endolysosomal pathway is poorly defined. In order to understand the mechanistic details for the release of a CPP from an endosomal compartment, biophysics model systems need to mimic the conditions inside this compartment. For this reason, cell-biological experiments that address the role of the chemical environment along the endolysosomal pathway are a prerequisite for biophysical experiments. The objective will be to integrate both biophysics and cell-biology data, as exemplified by two recent contributions.[12, 26]

  

## 2. The Many Ways To Cross the Plasma Membrane

### 2.1. Cellular transport processes

The central role of cellular membranes is the creation of compartments for the organization of biological processes. Membranes generate a basis for regulating the composition of the intracellular medium by controlling the flow of nutrients, waste products, ions, etc. into and out of the cell and the individual compartments. Only molecules within a narrow range of molecular size, net charge, and polarity are able to directly cross the plasma membrane by passive diffusion.[27] For other small solutes, channels and transporters mediate transport across the plasma membrane. For large, hydrophilic macromolecules, it is generally assumed that endocytosis is the mode of internalization.[28]

In addition to maintaining a defined chemical environment for reactions inside the cell, the plasma membrane represents a line of defense against pathogens and molecules that interfere with cellular function and replication. Endocytic passage guides external molecules through compartments with high hydrolytic activity, thereby also exerting an important protective role.

Endocytosis comprises distinct pathways, which can be subdivided into two groups: phagocytotic and pinocytotic pathways (Figure 1 A).[29,30] Phagocytosis relates to the uptake of large particles and is restricted to cell types such as macrophages, monocytes, and neutrophils, which are specialized for the elimination of pathogens as well as infected and apoptotic cells. Pinocytosis, on the other hand, occurs in all cells and includes a variety of processes leading to the uptake of fluids, solutes, and membrane components. The regulation of these processes is highly complex and, despite the enormous progress in the analysis of the endocytic machinery, many details are still poorly understood. At least four different pinocytotic pathways can be distinguished: macropinocytosis, clathrin-mediated endocytosis (CME), caveolae-/lipid-raft-mediated endocytosis, and clathrin- and caveolin-independent endocytosis. These pathways differ with regard to the size of endocytic vesicles, the nature of the cargo, and the mechanism of vesicle formation.

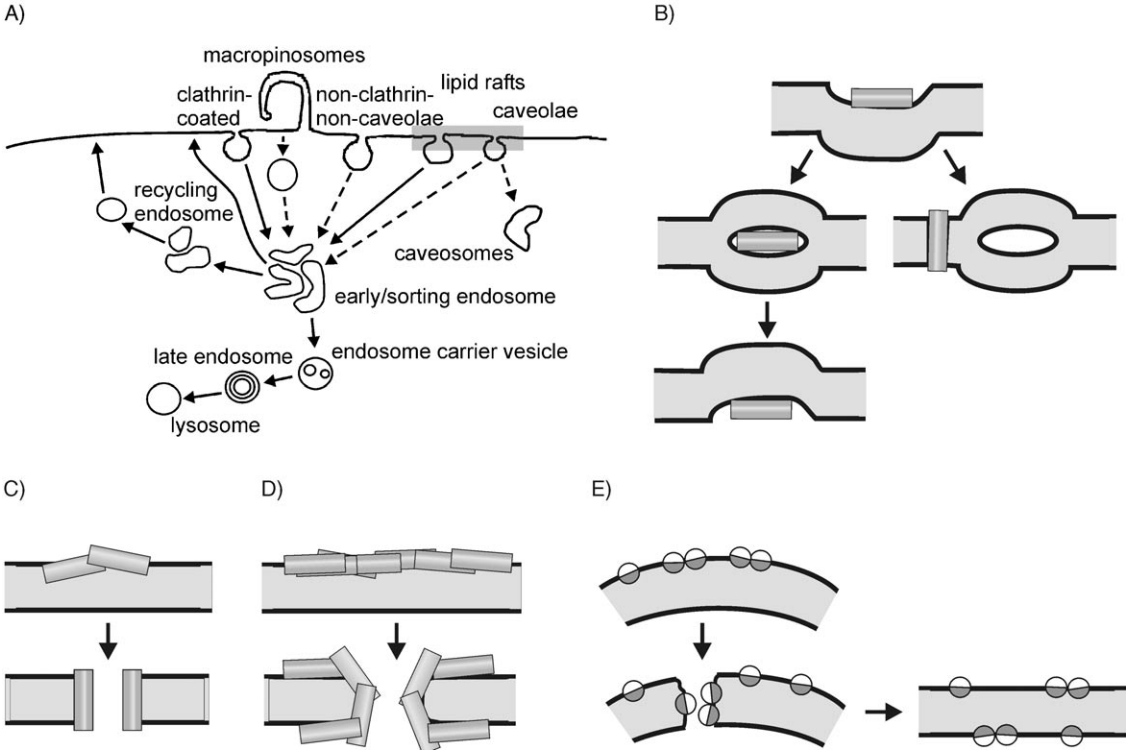


**Figure 1.** Proposed uptake mechanisms for membrane-active peptides. A) Cellular entry by endocytosis. For endocytosis through clathrin-coated vesicles, macropinosomes, and lipid rafts/caveolae, an involvement in the cellular uptake of CPPs has been shown already. Dotted lines refer to trafficking pathways that are still under dispute. The figure has been adapted from ref. [30,168]. B) Models for the membrane permeation of penetratin by inverted micelle formation. Association of the peptide with the plasma membrane disturbs the lipid bilayer so that the inverted micelle is formed. Translocation may either involve entrapment of the peptide within the micelle and release on the other side of the plasma membrane or formation of the micelle locally perturbs the bilayer and thereby induces the insertion of the peptide into the bilayer and membrane transfer. The figure was adapted from ref. [169]. C), D) Models proposed for the membrane permeation of membrane-active antimicrobial peptides. According to the barrel-stave model, a limited number of peptides first assembles on the plasma membrane and then inserts into the lipid bilayer; further peptides are then recruited and a pore is formed. In the carpet model, peptides accumulate on the plasma membrane to the point where the integrity of the plasma membrane is breached and pores are formed. The lipid head groups are always oriented towards the peptide.[131] E) Sinking-raft model for the uptake of membrane-active peptides. Amphipathic helical peptides form aggregates of limited size. The mass imbalance due to association of the peptides with only one face of the plasma membrane induces curvature that provides the driving force for translocation of peptides across the bilayer. In this panel, $\alpha$ helices are shown as cross sections, with the hydrophobic face in dark grey and the hydrophilic face in white. The figure was adapted from ref. [136].

  

Pathogens and their toxins have evolved sophisticated means for bypassing or stunning the hydrolytic guards in order to access the cytoplasm.[31–34] However, also a variety of endogeneous proteins, including growth factors and transcription factors, have been found in the nucleus after having been added externally to cells.[9] Therefore, entry of functionally intact macromolecules into the cytoplasm and nucleus also plays a physiologically significant role.[16]

### 2.2. Cellular import of membrane impermeable molecules in cell biology

In cell biology, the introduction of oligonucleotides, peptides, or entire proteins into cells enables interference with molecular processes inside the cell. The strategies developed for the import of cell-impermeable molecules can be roughly subdivided into three groups (reviewed in ref. [35]). The first group is based on a direct transfer of molecules into the cell by capillary microinjection, while the second one is based on a transient disruption of the plasma membrane, for example, by high-power electric pulses in electroporation or by incubation with pore-forming molecules, such as streptolysine O.

The third group relies on carriers as mediators of import. In this case, molecules that themselves lack the ability to enter cells are linked to shuttle molecules that possess this ability and thereby enter the cells piggyback. Especially for the introduction of recombinant DNA into mammalian cells, transfection reagents represent a well-established carrier-mediated approach. Transfection reagents improve the import efficiency of DNA molecules by 1) neutralization of negative charge and 2) conference of hydrophobicity, characteristics that facilitate the interaction with the plasma membrane. However, in spite of the hydrophobicity of these so-called lipoplexes, an uptake mechanism that involves internalization through endocytosis has received considerable support.[36] Similarly, encapsulation of molecules into liposomes incorporates the cargo into a high-molecular-weight complex with physicochemical characteristics that more closely match those of the plasma membrane. Evidently, these carriers exert their activity to a large extent by disguising the physicochemical characteristics of their cargo.

### 2.3. Cell-penetrating peptides as a carrier-mediated delivery strategy

CPPs are peptides consisting of roughly 10–30 amino acids (Figure 2, Table 1). Efficient cellular import has been achieved for cargos as diverse as peptides, proteins as large as 120 kDa,

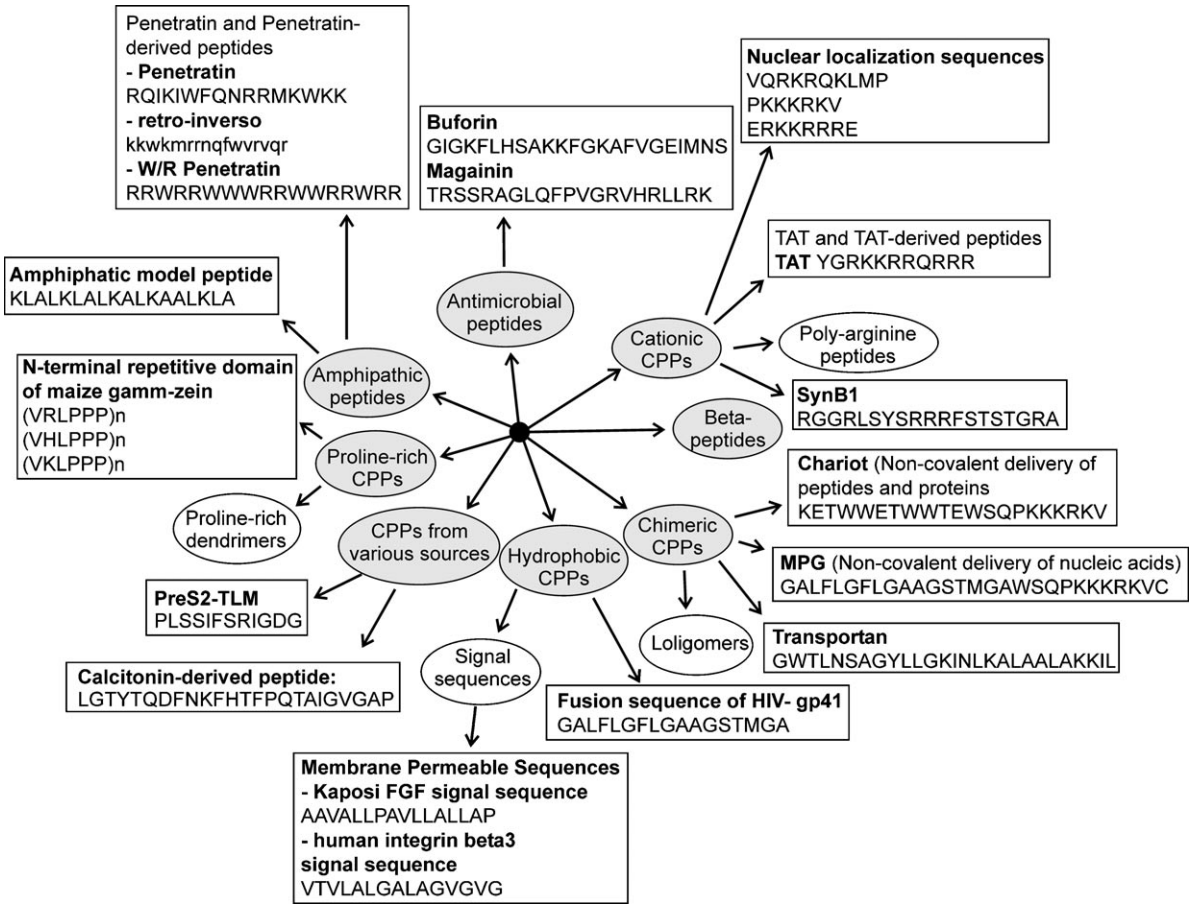


**Figure 2.** A family tree of CPPs. Some CPPs may be assigned to several categories, such as the chimeric CPPs that combine individual structural motifs. The darker shaded peptides were identified as the transduction domains of full-length proteins. Only a representative selection of peptides listed in Table 1 is shown.

  

**Table 1.** Classification of CPPs according to their origin.

| Name | Comments | Sequence | Reference |
|---|---|---|---|
| Protein-derived CPPs: | | | |
| penetratin | derived from the third helix of the *Drosophila* antennapedia transcription factor (amino acids 43–58) | RQIKIWFQNRRMKWKK | [55] |
| Tat peptide | derived from the HIV-1 Tat protein | RKKRRQRRR | [60] |
| calcitonin-derived CPP | amino acids 9–32 of human calcitonin | LGTYTQDFNKFHTFPQTAIGVGAP | [110] |
| nuclear localization sequences | sequences from various proteins | VQRKRQKLMP, SKKKKTKV, GRKRKKRT, etc. | [141] |
| new polybasic CPPs | linear polybasic sequences like nucleic acid or heparin binding peptides, etc. | for example, RRRERRAEK, KCPSRRPKR | [51] |
| N-terminal repetitive domain of maize gamma-zein | | $(VRLPPP)_n$ $(VHLPPP)_n$ $(VKLPPP)_n$ | [142] |
| peptides from gp41 fusion sequence | | AVGAIGALFLGFLGAAG | [143] |
| preS2-TLM | PreS2 domain | PLSSIFSRIGDP | [64] |
| signal-sequence hydrophobic region (SSHR) | hydrophobic sequence derived from the fibroblast growth factor 4 signal sequence | AAVALLPAVLLALLAP | [40, 144, 145] |
| SSHR | hydrophobic sequence derived from the human integrin $\beta_3$ signal sequence | VTVLALGALAGVGVG | [146] |
| pVEC | CPP derived from the murine vascular endothelial cadherin | IAARIKLRSRQHIKLRHL | [147] |
| Vpr | CPP derived from the Vpr protein of HIV-1 | DTWPGVEALIRILQQLLFIH FRIGCQH | [148, 149] |
| CPP from pestivirus envelope glycoprotein | | RQGAARVTSWLGRQLRIAGKRLEGRSK | [150] |
| CPP derived from the prion protein | the N-terminal part (amino acids 1–28) of the mouse prion protein | MANLGYWLLALFVTMWTDVGLC KKRPKP | [151] |
| Antimicrobial peptides or CPPs derived from antimicrobial peptides: | | | |
| buforin | | TRSSRAGLQWPVGRVHRLLRK | [106] |
| magainin | | GIGKFLHSAKKWGKAFVGQIMNS | [106] |
| LL-37 | The human antimicrobial LL-37 peptide is able to transfer extracellular DNA into mammalian cells | LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES | [152] |
| SynB peptides | CPPs derived from protegrin 1, an antimicrobial peptide | RGGRLSYSRRRFSTSTGR, RRLSYSRRRF | [97, 153] |
| $S4_{13}$, $S4_{13}$-13 | CPPs derived from the antimicrobial peptide dermaseptin | ALWKTLLKKVLKA ALWKTLLKKVLKAPKKKRKV | [154] |
| proline-rich CPPs | proline-rich translocating peptides from the antimicrobial peptide bactenecin | for example, PRPLPFPRPG | [155] |
| Designed CPPs: | | | |
| transportan | chimera of mastoparan and the N-terminal fragment of galanin | GWTLNSAGYLLGKINLKALAALAKKIL | [121] |
| polyarginine CPPs | designed based on structure–activity relationships of the Tat peptide and comparison with other homopolymers | RRRRRRRR ($R_8$) | [65, 66] |
| KLA peptide/model amphipathic peptide (MAP) | $\alpha$-helical model amphipathic peptide, initially used for biophysics studies | KLALKLALKALKAALKLA | [63] |
| modeled Tat peptide | designed to increase the amphipathic character of the Tat peptide | YARAAARQARA | [72] |
| $\beta$-sheet-forming peptide | de novo designed amphipathic $\beta$-sheet peptide | DPKGDPPKGVTVTVTVTVTG KGDPKPD | [156] |
| retro–inverso forms of established CPPs | for example, penetratin | KKWKMRRNQFWVRVQR | [157] |
| W/R penetratin | functional analogue of penetratin | RRWRRWWRRWWRRWRR | [158] |
| MPG | peptide vector for the delivery of oligonucleotides into mammalian cells | GALFLGFLGAAGSTMGAWSQPKSKRKVC | [124] |
| Pep-1 | peptide carrier for the noncovalent delivery of proteins into cells | KETWWETWWTEWSQPKKKRKV | [61] |

  

oligonucleotides, plasmids, peptide nucleic acids (PNAs), small interfering RNA (siRNA), liposomes, and even nanoparticles.[37–47] In most of these applications, conjugation of only one carrier peptide to a large cargo renders the molecule import competent. However, a 10 amino acid peptide will have little impact on the physicochemical characteristics of a 120 kDa protein or a duplex, 21 nucleotide (nt) siRNA.[39,48] As a consequence, in contrast to the transfection reagents which disguise the physicochemical characteristics, most CPPs are well-defined pharmacokinetic modifiers that add a new moiety to a specific site of an otherwise unperturbed molecule. Therefore, even for peptide cargos, the import efficiency is strongly affected by the nature of the cargo.[49]

Rapid cellular uptake in combination with a highly defined molecular structure and ease of handling render CPPs highly attractive mediators of import.[50] Moreover, large collections of peptide-based transport vehicles are accessible by well-established automated procedures, thereby allowing detailed analysis of structure–activity relationships and a rational, straightforward approach for the generation of novel, optimized CPPs.[51,52]

The applications of CPPs exceed well beyond mere tissue-culture experiments. CPP–peptide conjugates and CPP fusion proteins that interfere with protein–protein interactions have been successfully applied as therapeutic agents in animal models.[53,54] In contrast to liposomes, CPP conjugates possess many beneficial drug-like characteristics. In the case of peptide cargos, covalent conjugates with CPPs can be synthesized by solid-phase synthesis and are analytically well defined.

*Where do CPPs come from?* Formally, the field of CPPs in its narrower sense started with the identification of the protein-transduction domain of the *Drosophila melanogaster* antennapedia homeodomain transcription factor.[55] However, Ryser and Hancock had already demonstrated in 1965 that addition of homopolymers of cationic amino acids ($\approx$100 kDa) to tissue-culture media containing radiolabeled albumin enhanced the uptake of the radiolabel into the cell.[56] In the 1970s, Shen and Ryser again demonstrated that covalent conjugation of poly-L-lysine to proteins and small molecules enhanced their cellular uptake and, in the case of methotrexate, the biological activity of the drug.[57,58]

About 10 years later, Frankel et al. observed that the HIV-1 Tat protein is taken up by tissue-culture cells. The internalized protein is then capable of transactivating the viral promoter.[59] A further 3 years later, the 60 amino acid polypeptide corresponding to the homeobox domain of the antennapedia transcription factor was shown to be internalized by neurons and to augment their morphological differentiation.[2]

For the HIV-1 Tat protein, the internalization was attributed to a basic domain comprising amino acids 48–60, while in the case of the antennapedia homeodomain, it was ascribed to a peptide of 16 amino acids corresponding to the third helix of the homeodomain.[55,60] As a CPP, the latter peptide is also referred to as penetratin. The findings obtained for the Tat and the penetratin peptide demonstrated that the efficient internalization of synthetic oligopeptides first observed by Ryser and Hancock in 1965 has a physiological correspondence in nature. During the following years, the compelling functional characteristics of the CPPs promoted the identification of further CPPs that were either based on small domains of naturally existing proteins or designed de novo.[61–63]

The field rapidly evolved into research areas that focused on 1) the cell biology of protein transduction, 2) the identification of novel transduction motifs, 3) applications of CPP conjugates in various cellular and animal systems, and 4) the investigation of the mode of internalization. Especially in the latter three areas, research has been primarily conducted on peptides small enough to be efficiently generated synthetically.

A total of about 30 different CPPs have been described so far. While some of these peptides are purely cationic, others are amphipathic with a large fraction of basic residues, and others again are fully hydrophobic.[38] Exceptions to these general characteristics exist, for example, the CPP derived from the PreS2 domain of the hepatitis B virus surface antigen.[64] Good solubility in aqueous buffers, short sequence length, accessibility through solid-phase peptide synthesis, high import efficiency, and low cytotoxicity are the criteria to be met by a CPP in order to be attractive as a molecular tool. Penetratin, the HIV-1 Tat-derived peptide, and the oligoarginine peptides have been used in the majority of cell-biology applications.[55,60,65,66]

## 3. Biophysics and Cell Biology Case by Case

Even though this review focuses on cationic and cationic amphiphilic CPPs, the biological and biophysical characteristics of these peptides are all but the same. In fact, the differences between these peptides may be more relevant for understanding the structural basis of uptake and trafficking than the properties they have in common. In addition, very different amounts of data, acquired with different experimental protocols, have been presented for individual peptides (Figure 3). In order to avoid confusion and to stress the differences between the individual CPPs, rather than trying to sketch a unifying picture, the information for each peptide will be summarized separately.

### 3.1. The Tat peptide and the Tat protein

The Tat protein is the most intensively studied protein from which a CPP has been derived. The same group that had first shown that the basic domain of the HIV-1 Tat protein (amino acids 48–60) directly translocated through the plasma membrane later revealed that these observations were due to fixation artefacts.[1,60] Interestingly, an endocytic uptake had already been demonstrated for the entire HIV-1 Tat protein in 1991.[8] Since the internalization mechanisms of both the Tat peptide and the Tat protein have been studied intensively and the results are strongly interconnected, both aspects will be discussed here.

*The Tat peptide*: Concerning the initial step of uptake, that is, the association with the plasma membrane, a remarkable agreement between biophysics and cell biology exists. It was shown that the Tat peptide possesses a much lower affinity for lipid membranes than for glycosaminoglycans, a result that makes the latter interaction a more probable candidate for the

  

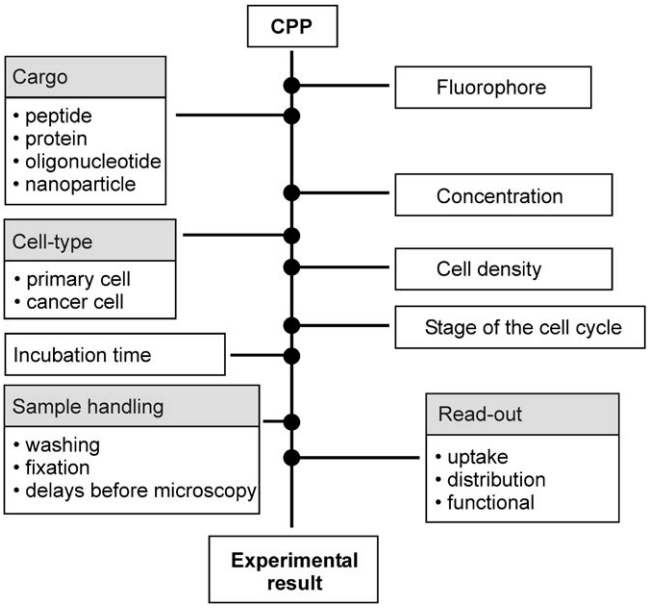


**Figure 3.** Factors potentially contributing to contradictory results in cell-biology CPP experiments. For most cell-biological experiments, fluorescently labeled analogues of CPPs are employed. For penetratin, different fluorophores had little impact on the relative uptake effciencies of analogues labeled at different positions within the peptide. However, differences in the cellular distribution of fluorescence were observed.[49] It is not yet clear whether and to what degree the import mechanism of a CPP alone differs from that of a CPP–cargo conjugate. For the intracellular peptide distribution, a cell-type dependence has been observed. Moreover, cancer cells were killed more efficiently than normal cells by an amphiphilic peptide.[170] At a given peptide concentration, cell density strongly affects the loading efficiency.[171] In G1 phase, uptake of a conjugate of the Cre-recombinase with the SSHR was only half as efficient as uptake during other phases of the cell cycle.[172] The interpretation of results on endocytic trafficking is strongly dependent on incubation time. With incubation times that are too long, the peptide and proteolytic fragments may equilibrate in different endocytic compartments. In this way, information on the route of endocytic trafficking may be lost. If cells are washed insufficiently before analysis by flow cytometry, peptides associated with the plasma membrane may be mistaken for peptides taken up into the cells. Fixation may strongly affect the distribution of molecules inside the cell. However, it is obvious that a CPP conjugated to a high-molecular-weight protein will behave differently towards fixation than a CPP alone. Moreover, incubation for too long after removal of peptide from the incubation medium may allow exit of peptides or peptide fragments from the cells. If this leakage occurs preferentially for peptides in the cytoplasm, than erroneous results on peptide distribution will also be obtained. Finally, the results will strongly depend on the read-out. Functional read-outs, such as the determination of the activity of the Cre-recombinase, will only detect intact protein reaching the nucleus. Even though the understanding of the cellular trafficking of CPPs has benefited enormously from the use of fluorescently tagged CPPs in live-cell microscopy, one should be aware that the subcellular distribution of the fluorescent dye may not represent that of the CPP but rather that of a proteolytic fragment.

binding to the plasma membrane than the interaction with zwitterionic and anionic lipids.[67–69] The cell-biological relevance of the interaction with carbohydrates on the cell surface was confirmed by the demonstration that mutant cells defective in glycosaminoglycan synthesis failed to internalize conjugates of the Tat peptide with a high-molecular-weight cargo.[70] Essentially the same observation had been made earlier for the Tat peptide fused to green fluorescent protein (GFP).[71]

The relevance of the secondary structure of the Tat peptide for its internalization has been a matter of intense debate. The formation of an amphipathic $\alpha$-helix is considered essential for the interaction of many membrane-active peptides with a lipid bilayer. The Tat peptide was suggested to possess similarities with such amphipathic $\alpha$ helices.[72] However, circular dichroism measurements for the Tat peptide and for other arginine-rich peptides in methanol revealed that most of these peptides had little or no secondary structure.[66] Consistent with this finding, arginine-rich oligomers are highly permissive towards structural modifications. Peptides containing aminohexanoic acid spacers and polyguanidinium peptoids are taken up by mammalian cells.[73–75] Evidence was also provided showing that the Tat peptide possesses characteristics of a poly-(proline) II helix in aqueous and membrane-mimicking micellar sodium dodecylsulfate (SDS) solutions.[76]

With regard to the second step, that is, cellular entry, the agreement between biophysics and cell biology has not been established yet. Fluorescently labeled Tat peptide and Tat–PNA constructs accumulate in endocytic vesicles that originated from clathrin-dependent endocytosis.[1,77] This finding raised the question of how the peptide exits the endocytic compartment and enters the cytoplasm. It was shown that for the Tat peptide endosomal acidification is required for this process.[78,79] In contrast to the rather slow endocytosis-mediated uptake of the Tat peptide demonstrated in these studies, time-lapse microscopy of a fluorescein-labeled Tat peptide in mouse fibroblasts revealed the formation of dense aggregates on the cell surface and a rapid increase of fluorescence in the cytoplasm and the nucleus within seconds.[50] Interestingly, even though microscopy was performed in living cells, in contrast to the work of Richard et al.,[1] only a few endocytic vesicles could be observed. The images in this contribution resemble those of earlier reports demonstrating "capping" or aggregation of cell-surface proteoglycans upon ligand binding.[80] The cellular basis for these apparently conflicting results still needs to be resolved.

Biophysical analyses have shown that, like other cationic CPPs, the Tat peptide rapidly traverses the membranes of giant unilamellar vesicles, whereas it is unable to translocate across lipid membranes of large unilamellar vesicles.[67] The molecular basis for this difference is unknown. At this point, it is fully unclear whether and, if so, in what respect these observations are relevant for the pH-dependent endosomal release of the peptide inside cells.[78,79,81]

*The Tat protein*: The Tat protein, once internalized and present in the cytosol of eukaryotic cells, is able to transactivate the transcription of several genes that are under the control of the viral promoter.[59] This attribute provides a robust functional assay for comparing the entry of bioactive protein into the cytoplasm of mammalian cells under different experimental conditions.

In agreement with the findings for the Tat peptide mentioned above, endosomal acidification is also necessary for the Tat protein to enter the cytoplasm.[81] Interestingly, the in vivo anti-HIV effect of the antimalaria drug chloroquine is based on its ability to neutralize endosomes and, thereby, prevent Tat

  

from entering and affecting T cells.[82] Vendeville et al. used an elegant combination of cell biology and biochemistry to investigate the release of the Tat protein.[81] Endosomes loaded with the Tat protein and other marker proteins were purified from Jurkat cells and experiments were performed in a cell-free system. It was shown that the chaperone Hsp90 is required for endosomal exit of the protein. In addition to pharmacological inhibitors, neutralizing antibodies could also be employed in these cell lysates.

However, the contribution of the individual endocytic pathways for uptake is still under dispute. Fittipaldi et al. identified caveolar endocytosis as a route of uptake.[83,84] Consistent results were obtained for a functional assay based on the transactivating activity of the Tat protein and for Tat–GFP fusion proteins. Vendeville et al. provided evidence for clathrin-mediated endocytosis.[81] Tat-peptide-mediated protein import was reported to be clathrin- and lipid-raft-mediated,[85] lipid-raft-mediated macropinocytosis,[83] and purely lipid-raft dependent.[86,87] It remains to be established whether the full-length Tat protein, the Tat peptide, and Tat peptide–protein conjugates share common import pathways.

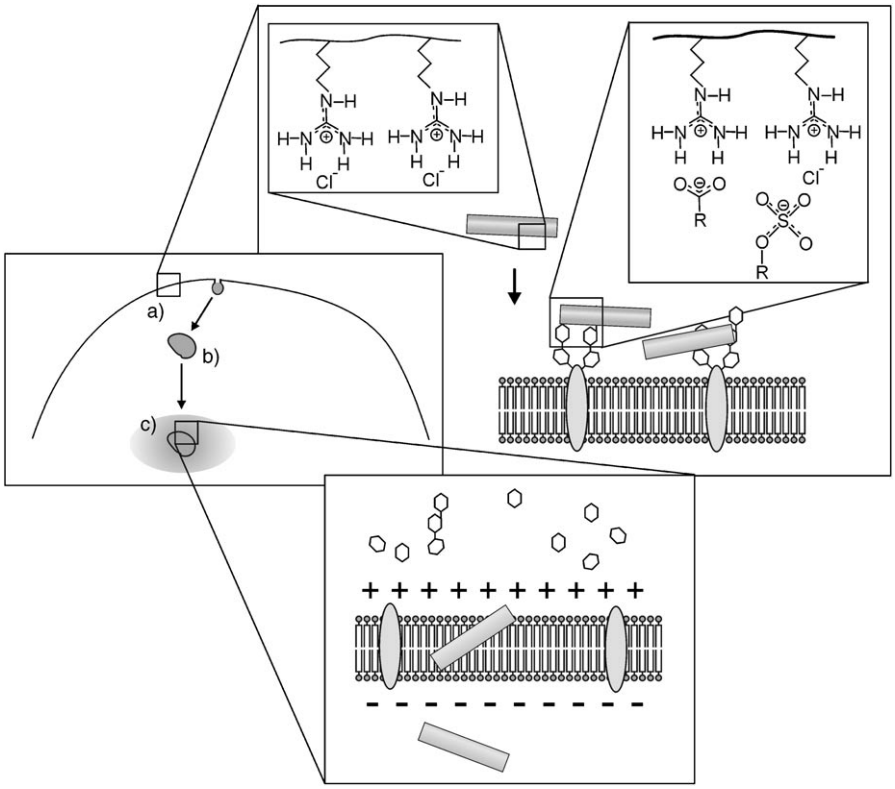


**Figure 4.** A literature-based model for the cellular uptake of arginine-rich CPPs. The initial membrane association is mediated by interaction of the guanidinium groups with negatively charged groups on the cell surface, rich, for example, in glycosaminoglycans (a). The CPP is endocytosed and acidification is required for the heparanase-dependent degradation of heparin in the endosomes (b).[88] Next, the peptide translocates across the lipid bilayer by a transmembrane-potential-driven process (c).[12,26] A potential-driven translocation should require that not all charges of the peptide are compensated through complexation.

### 3.2. Oligoarginine peptides

Oligoarginine peptides are a group of CPPs that are structurally closely related to the Tat peptide. In 2000, it was demonstrated that "polyarginine enters cells more efficiently than other homopolymers".[65] Still, despite the great structural similarity between these compounds, not all findings regarding the Tat peptide and the oligoarginine peptides have been congruent. In contrast to the Tat motif, for which uptake by endocytosis is strongly supported by several independent studies, the uptake mechanism for the oligoarginine peptides is still under debate.

*Endocytosis-dependent internalization*: Colocalization experiments and pharmacological intervention studies have supported an endocytosis-dependent uptake.[78,88,89] Futaki and coworkers recently reported an important role for macropinocytosis.[89] However, the impact of the inhibition of macropinocytosis on the reduction of uptake depended on the chain length of the oligoarginine peptides, a result indicating that additional pathways differentially contribute to the uptake of these molecules.[89] The inability of nonaarginine to enter cells deficient in heparan sulfate suggests that binding to heparan sulfate is necessary for internalization.[88] These researchers proposed a model according to which the oligoarginine-mediated delivery of molecules into mammalian cells involves 1) binding to cell-surface heparan sulfate, 2) uptake by endocytosis, 3) release upon heparan sulfate degradation, and finally 4) leakage from endocytic vesicles (Figure 4). With regard to the mechanism of endosomal release, two other contributions showed that, for fluorescein-labeled nonaarginine and a fluorescein-labeled $(VRR)_4$ peptide consisting of β-amino acids, endosomal acidification is required for accessing the cytoplasm.[78,79]

*Membrane permeation and the role of membrane potential*: The importance of arginine residues for the efficient uptake of a large number of CPPs stimulated further research on how the physicochemical and structural characteristics of the guanidinium group may mediate membrane translocation of nonlipophilic molecules.[65,90,91] It was shown that the guanidinium group has a high propensity to form complexes with negatively charged molecules, thereby increasing the lipophilicity of the molecule. Formation of such complexes with negatively charged lipid head groups at the plasma membrane promotes the partitioning of guanidinium-group-rich oligomers into the lipid bilayer.[92,93] Release at the other site of the bilayer occurs by the reverse reaction. This model of membrane permeation

  

is not far away from the original "inverted micelle" concept (Figure 1B).[55] According to this model, basic residues within the CPP interact with the negatively charged phospholipids in the plasma membrane, thereby causing a local invagination of the plasma membrane that leads to local reorganization of the lipid bilayer and the formation of an inverted micelle. Transfer across the membrane would either occur through transient entrapment within this micelle or through perturbation of the lipid order close to the micelle.

In contrast to this solubility-driven mechanism, Terrone et al. demonstrated the importance of a transbilayer electric potential as a driving force for membrane permeation.[26] Diverse lipid compositions enabled a substantial potential-dependent (inside negative) uptake of different cationic CPPs (penetratin, $Arg_6$-Gly-Cys, and $Lys_6$-Gly-Cys) into large unilamellar vesicles.[26] Remarkably, the lysine-containing peptide was taken up as efficiently as the arginine-containing peptide. When the importance of arginine residues for cellular uptake of cationic CPPs as stated by many different groups is considered, one therefore has to ask to what degree these experimental conditions reflected the physiological conditions encountered by the peptides.[12,92] In a related study, Rothbard et al. stressed the ability of the guanidinium head groups to form bidentate hydrogen bonds with hydrogen-bond-acceptor functionalities on the cell surface.[12] With the assumption that not all charges are neutralized, the resulting still positively charged complexes then partition into the lipid bilayer and migrate across at a rate proportional to the electric transmembrane potential. At the inner leaflet of the plasma membrane the complexes dissociate and the peptides enter the cytosol.[12] This hypothesis was substantiated by demonstrating a reduction of peptide uptake into Jurkat cells in the presence of depolarizing drugs. However, at this point it cannot be excluded that the potential just promotes the association of the CPP with the plasma membrane.

When import was investigated in the absence of a transbilayer potential, import of oligoarginine peptides was only observed for giant unilamellar vesicles but not large unilamellar vesicles.[67] The fluid and dynamic structure of giant unilamellar vesicles in comparison to large unilamellar vesicles might account for these differences.[67,94]

The findings on the significance of a transmembrane potential correspond to cellular results reported for fibroblast growth factors 1 and 2 (FGF-1 and -2). These proteins are capable of crossing cellular membranes and reaching the cytosol, similarly to CPPs and CPP–protein conjugates. However, it should be emphasized that FGF translocation occurs from within intracellular vesicles.[95] Dissipation of the vesicular membrane potential blocks translocation.[96] Arginine residues are highly abundant and clustered in the amino terminal portion of the FGF-2 protein. However, the lack of such a stretch of cationic amino acids in FGF-1 raises the question of whether this domain is in fact the relevant structural characteristic.

### 3.3. Penetratin

*Endocytosis*: Endocytosis of penetratin was demonstrated[78,97] shortly after the contribution of Richard et al. in 2003.[1] Inhibitors of metabolism or endocytosis impaired the uptake of penetratin.[97] Moreover, similarly to the Tat peptide, penetratin promotes the endocytosis of high-molecular-weight cargo upon binding to cell-surface glycosaminoglycans, and endosomal acidification is involved in the release of the peptide into the cytosol.[78] A strong propensity to bind to the plasma membrane had been shown to be crucial for cellular uptake.[91]

*Membrane permeation*: Thoren et al. demonstrated by fluorescence microscopy that penetratin can traverse a pure lipid bilayer of giant unilamellar vesicles and that translocation does not involve pore formation.[20] The inability of penetratin to form pores under these experimental conditions clearly distinguished this CPP from other antimicrobial membrane-active peptides such as melittin.[20] With results rather contrary to the study of Thoren et al., Drin et al. reported that penetratin is not sufficiently helical and amphipathic to cross the bilayer of the phospholipid membrane of large unilamellar vesicles.[18] These researchers concluded that penetratin does not belong to the family of amphipathic $\alpha$-helical peptides whose members are able to translocate through lipid bilayers through pore formation. Possibly, these discrepancies may be explained by the different model systems, that is, giant versus large unilamellar vesicles, consistent with the differences observed with giant versus large unilamellar vesicles for the analogues of the Tat peptide.[67] With regard to the molecular mechanism of membrane translocation for penetratin, a two-step process was postulated[98] that resembles the one proposed for the oligoarginine peptides by Matile and co-workers.[92,93] By employment of a phase-transfer assay, it was demonstrated that negatively charged lipids promote the transfer of penetratin from a hydrophilic into a hydrophobic environment, probably through charge neutralization. Phase transfer by charge neutralization was also observed with a variant penetratin (Trp6Phe). However, penetratin, but not the mutant version, was internalized by living cells. This finding underscores the fact that charge neutralization and phase transfer represent only a first step in the internalization process and that the tryptophan residue at position 6 plays a critical role in the translocation step.[55,99]

Analogous to the findings described for the oligoarginine peptides, the presence of an electric transmembrane potential also seems to be a major prerequisite for the membrane transit of penetratin.[26] Peptide uptake is accompanied by only minor perturbations of the overall barrier function of the lipid bilayer, a fact consistent with the inability of penetratin to form pores. Binder and Lindblom proposed an "electroporation-like" mechanism for the uptake of penetratin, according to which the asymmetric distribution of penetratin between the outer and inner surfaces of a charged lipid bilayer causes a transmembrane electrical field.[100] This field alters the lateral and curvature stress acting within the membrane.[100] Above a threshold these effects induce internalization of penetratin through transient inversely curved structures. However, given the importance that other research attributed to the presence of a transmembrane electric field, one may rather assume that the peptide perturbs the already existing electric field.

*Secondary structure*: Circular dichroism spectroscopy showed that penetratin is randomly structured in aqueous buffers.[101]

  

Binding of the peptide to model membranes induces distinct secondary structure, the nature of which depends strongly on the experimental conditions, such as the membrane charge and the peptide/lipid ratio.[21,99,102] Both β-sheet and α-helical structures have been reported, although lipid binding increased the α-helix content of the peptide in most studies.[19,22,24,102–104] Notably, the degree of membrane perturbation caused by penetratin was related to its secondary structure.[21] In the helical state the peptides have little effect on the membrane. Under conditions in which penetratin is converted into β structures, the peptide causes membrane perturbation. In comparison to transportan (see below), penetratin appears to penetrate deeper into the membrane. Polarized-light spectroscopy measurements indicated that penetratin adopts an orientation parallel to the membrane surface.[23] However, evidence has been presented that the penetratin/lipid interaction is governed by electrostatic interactions, a result indicating that amphiphilicity and α-helix content may be misleading parameters in analyses of structure–activity relationships.[24]

The overall structural characteristics of penetratin and antimicrobial peptides such as magainin are very similar. In line with this structural similarity, Langel and co-workers showed that some eukaryotic CPPs, among them penetratin, possess antimicrobial properties.[105] On the other hand, the antimicrobial peptides magainin and buforin were shown to possess CPP-like properties (see below).[106]

### 3.4. Model amphipathic peptide (MAP)

Unlike the previously mentioned CPPs, the sequence of the MAP is not derived from a sequence naturally occurring in a protein but was designed to yield an amphipathic designer peptide. Conjugation to MAP significantly increased the bioavailability and bioactivity of PNAs, thereby underlining the potential of this peptide as a drug carrier.[107]

In an α-helical conformation, this designer peptide is amphiphilic along the length of the helix.[108] In the original contribution it was stated that cellular uptake of this CPP occurs primarly through nonendocytic uptake and depends primarily on helical amphipathicity.[109] However, a later study from the same group showed that both amphipathic and nonamphipathic peptides of similar amino acid composition are internalized by mammalian cells to about the same extent.[3] Rather than the amphipathicity being decisive for import, it was suggested that more amphipathic peptides interacted more strongly with molecular structures inside the cell, thereby decreasing the rate of exit from the cells.[3]

### 3.5. Calcitonin-derived carrier peptides

Cell-penetrating properties have also been described for the human 32 amino acid hormone calcitonin (hCT) and for peptides derived from its sequence.[110] The C-terminal peptide (amino acids 9–32; hCT (9–32)), which lacks the receptor-activating N terminus, was shown to mediate the efficient transport of cargos such as proteins, plasmid DNA, and the antineoplastic agent daunorubicin into different cell lines.[111–113] In particular, the peptide has been attracting attention for the delivery of bioactive substances through the nasal mucosa.

In Madin–Darby canine kidney (MDCK) monolayers truncated linear sequences of hCT penetrated the plasma membrane and exhibited a "punctuated" cytoplasmic distribution.[114] The internalization is temperature-, time-, and concentration-dependent, which is indicative of endocytic uptake.[114,115] Colocalization and pharmacological intervention studies in HeLa cells provided evidence for lipid-raft-mediated endocytosis.[115]

In aqueous buffers, full-length hCT is described to be largely unstructured.[116] A potentially amphipathic region between residues 8–22 is important for bioactivity. Remarkably, replacement of these amino acids by a different sequence able to form an amphipathic α helix also yielded a biologically active molecule.[117] In micelles, hCT assumes an amphipathic α helix from residues 9–16, followed by a β turn.[118] A different study showed that upon interaction with neutral and negatively charged liposomes hCT adopts a β-sheet conformation.[110]

hCT (9–32) was described to preferentially interact with negatively charged phospholipids.[119] Solid-state NMR-based analysis indicated that hCT (9–32) is not capable of penetrating lipid membranes.[120] Consistent with these findings, a second study showed that hCT (9–32) does not insert spontaneously into lipid bilayers.[119] Based on this purely biophysical study, the authors concluded that endocytosis should be the probable mode of uptake for this peptide.

### 3.6. Transportan

Transportan is a chimeric 27 amino acid CPP composed of 12 residues derived from the neuropeptide galanin connected through a lysine residue to 14 residues corresponding to the wasp-venom peptide mastoparan.[121] The transportan peptide was originally supposed to enter cells independently of endocytosis.[121] A recent study demonstrated that transportan-mediated protein transduction involves both clathrin-dependent and -independent endocytosis.[85] Similarly to the Tat peptide and penetratin, transportan has been applied to the delivery of various bioactive cargos into the cytoplasm of mammalian cells in culture and even in animal models.[43,44,122] Solution NMR studies showed that transportan forms a well-defined α helix in the C-terminal mastoparan part. The N-terminal domain has a weaker tendency to form an α helix.[123] In contrast to penetratin, transportan is always helical, independent of the vesicle surface charge.[21] Oriented circular dichroism spectroscopy suggests that transportan in its helical state lies parallel to the vesicle surface.[21]

### 3.7. MPG and Pep-1

As carrier molecules, MPG and Pep-1 share the remarkable characteristic that efficient import of cargo molecules does not require covalent linkage. Instead, both CPPs form noncovalent complexes with their respective cargos. Both vectors are chimeric molecules that combine structural domains from different sources.

  

*MPG*: The 28 amino acid bipartite amphipathic peptide MPG is composed of 17 amino acids derived from the fusion sequence of HIV-1 gp41, a 3 amino acid spacer, a 7 amino acid nuclear localization sequence (NLS) of SV40 large T antigen, and a C-terminal cysteine amide. The peptide efficiently promotes the intracellular delivery of single- and double-stranded nucleic acids including siRNA, independent of the endosomal pathway.[45,124]

Upon interaction with phospholipids, the otherwise unstructured peptide assumes a β-sheet conformation.[125] It was proposed that translocation proceeds through the transient formation of a transmembrane pore-like structure.[125] The noncovalent formation of MPG/cargo complexes is also associated with a partial conformational change of MPG into a sheet structure. Analyses by atomic force microscopy showed that analogues of MPG ($P_{(\beta)}$ and $P_{(\alpha)}$) disrupt the lipid organization of monolayers and that the conformational state of the CPP itself can influence the uptake by lipid vesicles.[126]

*Pep-1*: Pep-1, also known as Chariot, is a 21 amino acid peptide, consisting of an 11 amino acid hydrophobic motif containing five tryptophan residues and a second domain corresponding to the NLS of the SV-40 large T antigen, linked through a three amino acid spacer.[61] The first domain interacts with macromolecular cargos and is required for efficient targeting of the complexes to the plasma membrane. The NLS improves the intracellular delivery and solubility of the peptide. Pep-1 and the analogue Pep-2 have been applied successfully to the delivery of proteins, peptides, and PNAs into mammalian cells.[61,127] It was shown that membrane crossing of Pep-1 involves formation of a transient transmembrane pore-like structure. Pep-1 interacts strongly with lipids and this interaction is associated with a conformational transition, whereas complexation with its cargo does not induce conformational changes.[125] With results rather contrary to this work, another study suggested that the main driving force for Pep-1 translocation was the charge asymmetry between the outer and inner leaflet of biological membranes. Translocation occurred only in the presence of a negative membrane potential and was enhanced by the presence of anionic lipids.[128]

## 4. Cell-Penetrating Peptides versus Antimicrobial and Membrane-Active Peptides

Hundreds of membrane-active peptides with antimicrobial activity (from 9 up to ≈100 amino acids in length) have been isolated from natural sources or designed de novo. These peptides can be subdivided into several groups based on their structure, sequence length, and the absence or presence of disulfide bridges.[129,130] With respect to their specificity, some are toxic to microorganisms but not to mammalian cells and some are toxic to both microorganisms and mammalian cells, such as the bee venom melittin.[131]

When it is considered that the same repertoire of biophysical techniques has been applied to antimicrobial peptides and CPPs, it is surprising that a comparison of these classes of peptides in cell biology has only started recently. Detailed analyses of structure–activity relationships in biophysical and biological test systems exist for several antimicrobial peptides. For this reason, CPP research should greatly benefit from an integration into the wider context of membrane-active peptides. One of the first contributions to take this step demonstrated that some CPPs are capable of entering bacteria and exerting antimicrobial effects.[105] TP10, a 21 amino acid deletion analogue of transportan, inhibited growth of *Candida albicans* and *Staphylococcus aureus*. pVEC, another cationic CPP, inhibited *Mycobacterium smegmatis* growth at low micromolar concentrations, below the levels that harmed human HeLa cells. On the other hand, the antimicrobial peptides magainin and buforin were shown to be able to translocate across human plasma membranes.[106]

Conversely, research on CPPs in eukaryotic cells has stimulated testing of peptides for CPP-like activities in prokaryotes. Uptake of β-decaarginine peptides was reported for gram-positive *Bacillus megaterium* as well as for gram-negative *Escherichia coli*.[132] The absence of endocytosis in bacteria renders these cells a highly attractive system for addressing the direct membrane permeation of CPPs. An earlier study demonstrated that small cell-wall/membrane-active peptides covalently conjugated to PNAs improve the in vivo antisense potency of the PNAs targeted to bacterial RNA. These conjugates cured HeLa cell cultures of an *E. coli* infection without any apparent toxicity to the human cells.[133] In this context, one should note that antimicrobial peptides, instead of simply breaching the bacterial integrity, might exert more specific inhibitory activities on cell-wall synthesis, on protein, nucleic acid synthesis, or on enzymatic activities inside the cell.[130] According to these speculations, antimicrobial peptides may be considered as multifunctional bacterial CPPs.

### 4.1. Interaction of antimicrobial peptides with lipid membranes

Essentially two mechanisms have been proposed for the insertion of antimicrobial peptides into lipid bilayers, the "barrel-stave" and the "carpet" mechanisms (Figure 1C and D). According to the barrel-stave model, membrane-active peptides first associate on the surface of the bilayer, followed by insertion into the bilayer and formation of a bundle that spans the bilayer (Figure 1C). Recruitment of further peptides yields a barrel-like pore made of helical peptides as staves. In this model the overall structure of the plasma membrane remains unperturbed. For the bee venom melittin, this mechanism has been questioned recently and a "toroidal" model has been proposed, in which the lipid monolayer bends continuously through the pore so that the water core within the pore is lined by both the peptides and the lipid head groups.[134]

The carpet or detergent-like mechanism describes the mode of action of, for example, dermaseptin S. According to this mechanism, positively charged peptides that are either monomeric or oligomeric cover the negatively charged membrane in a carpet-like manner. The peptides orient themselves so that their hydrophobic face is oriented towards the lipids and the hydrophilic face is oriented towards the phospholipid head groups. When a concentration threshold is reached, the pep-

  

tides permeate the membrane by locally disrupting the bilayer structure (Figure 1D; reviewed in ref. [131]).

The "sinking-raft" model provides a framework for the translocation of peptides across a membrane (Figure 1E).[135,136] According to this model, amphipathic helical peptides form transient aggregates of limited size (a few peptides), in which the hydrophilic faces of the helices are oriented towards each other and the hydrophobic faces are oriented towards the lipophilic core of the lipid bilayer. This raft then sinks into the lipid bilayer and disassembles on the opposite face of the bilayer. The driving force for translocation is provided by the induction of local curvature due to the association of the peptide with only one face of the membrane. In contrast to the previous models, the peptides retain an orientation parallel to the bilayer surface. Moreover, the integrity of the plasma membrane is only transiently breached.

#### 4.2. Cell-penetrating versus antimicrobial peptides

The initial finding that penetratin traverses a pure lipid bilayer without forming pores clearly distinguished this CPP from antimicrobial peptides.[20,137] Moreover, CPPs such as Tat and penetratin are considerably less toxic and haemolytic than most antimicrobial peptides.[60,114] Nevertheless, recent findings underline common functional characteristics of CPPs and antimicrobial peptides. Both groups of peptides interact with and perturb lipid bilayers. However, despite compelling similarities between both groups (some CPPs are actually derived from antimicrobial peptides; see Table 1), not all CPPs are antimicrobial and vice versa.

For membrane-active antimicrobial peptides, the carpet and barrel-stave mechanisms represent clear concepts about membrane interaction and perturbation that have been derived from experimental data. Antimicrobial peptides for which the uptake mechanism has been investigated may therefore provide references for narrowing in on the uptake mechanism of CPPs. Magainin was demonstrated to be internalized rapidly into mammalian cells exhibiting a cooperative concentration dependence of uptake.[106] This finding suggested pore formation through a barrel-stave mechanism similar to the one described for model membranes as an intermediate step in cellular entry. Furthermore, translocation was accompanied by cytotoxicity. In contrast, buforin, another cationic antimicrobial peptide, translocated by a less concentration-dependent mechanism without showing any significant toxicity. Even though the uptake of the Tat peptide showed some similarity to the buforin uptake, Tat uptake was more temperature and energy dependent, a fact indicating that each peptide enters the cells by a different mechanism.[106]

## 5. Summary and Outlook

The inhibition of cellular import of cationic CPPs by gramicidin A, which reduces the electric transmembrane potential, is a recent example of the stimulation of cell-biological experiments by biophysical results.[12,26] The contributions by Rothbard et al.[12] and Terrone et al.[26] demonstrate that the link of biophysics and cell biology in elucidating the import mechanism of CPPs needs to reach beyond a mere mimicking of lipid composition. Now that evidence is presented that CPPs discriminate between endocytic pathways, biophysical experiments will have to implement all available knowledge on the biochemical nature of the microdomains from which these pathways originate and on the endocytic vesicles inside the cell.[84] For example, the unconventional phospholipid lysobisphosphatidic acid (LBPA) is abundant in late endosomes and has not been detected elsewhere in the cell. The lipid is involved in protein and lipid trafficking through late endosomes.[138] The distribution of LBPA may also account for the trafficking and translocation of CPPs through endosomal compartments. In vitro experiments with vesicles should therefore directly investigate the effect of incorporation of this lipid on peptide transport. Moreover, given the relevance of the interaction with heparan sulfate for peptide import, the question arises to what degree model systems lacking these glycoproteins reflect the physiological situation.

In addition, the implementation of biochemical assays with purified intracellular compartments, exemplified by Vendeville et al.,[81] will enable 1) the definition of the chemical environment in these compartments in more detail, 2) the analysis of the integrity of the CPP, and—through reconstitution experiments—3) the definition of factors in the cytoplasm that are required for exit (Figure 5). Nevertheless, one should be aware that model systems may miss essential aspects of the interaction of a CPP with a cell. The induction of macropinocytosis by oligoarginines demonstrates that CPPs elicit cellular responses and that the uptake is at least modulated by the response of the cell to these molecules.[139]

Currently, biophysics and cell biology aim to understand the import mechanism of CPPs. However, instead of being the object of study, CPPs may become highly valuable probes for endocytosis research as well. Biophysical analyses that aim to mimic the intracellular environment imply that the chemical nature of this environment is known. A failure to induce membrane passage in a model system therefore also implies a lack of understanding of the chemical conditions inside the cell. Conversely, once cell-biological experiments identify the entry point of a CPP into the cell, the successful reconstitution of membrane transfer in a model system may yield new insights into the chemistry of the respective cellular compartment. Moreover, once the trafficking pathways for individual CPPs have been defined, these molecules may serve as tracer molecules.

During the last few years the development of so-called "biologicals", non-small-molecule protein-, peptide-, and oligonucleotide-based drugs has seen a remarkable expansion of activity. Anticancer therapy based on the application of antibodies, such as Herceptin, the antibody directed against the Her2 receptor, or peptide-based pharmaceuticals, such as Fuzeon for the treatment of HIV infections, clearly illustrate that the control of biological function by proteins and peptides is not restricted to an academic setting but offers highly relevant options for the development of modern therapies. However, given, for example, the problems associated with the efficient

  

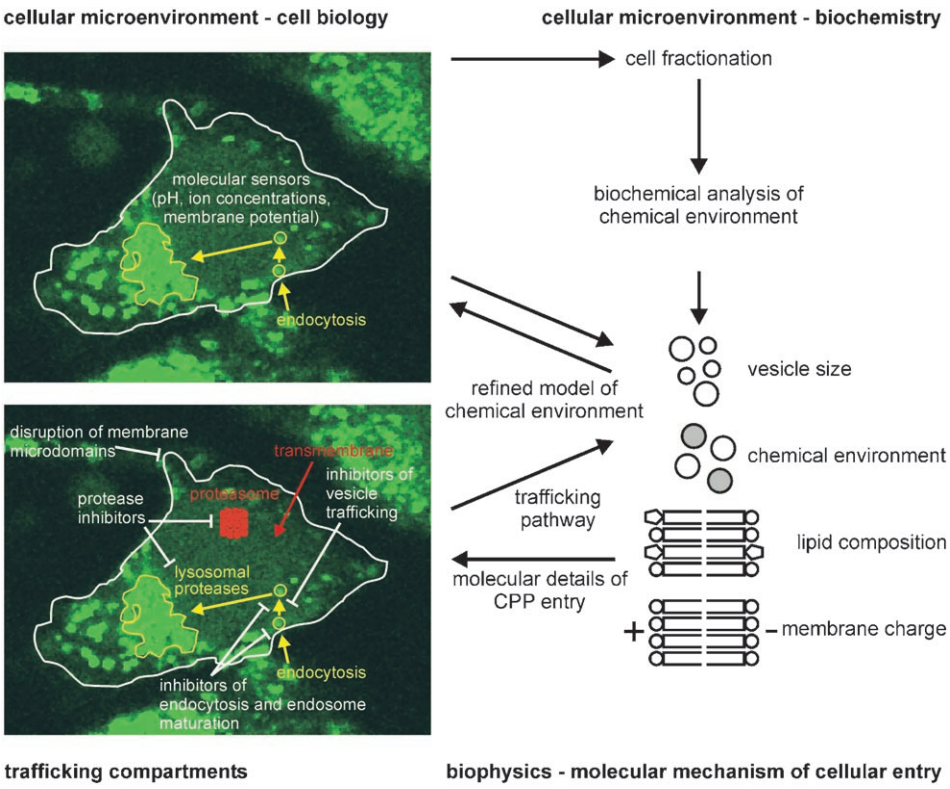


**Figure 5.** The combination of cell biology, biochemistry, and biophysics in the analysis of the uptake and trafficking of CPPs. The intracellular trafficking pathway may be resolved through a combination of fluorescently labeled CPPs and CPP conjugates for detection of import in functional assays with inhibitors of endocytosis, intracellular trafficking, and proteolytic breakdown. In order to elucidate the molecular mechanism of import, model systems for biophysical experiments will have to reproduce the intracellular chemical environment encountered by the peptide along the trafficking pathway. This information will be provided through cell-biological experiments that utilize fluorescent probes for pH value, membrane potential, and ion concentration, as well as through biochemical analyses of organelles isolated by cell fractionation. Discrepancies in the results obtained by biophysics and cell biology indicate a mismatch in the experimental conditions. In this way, cell biology/biochemistry and biophysics synergize in defining the conditions inside the cell that are relevant for the import of CPPs.

targeting of siRNAs in vivo, it is obvious that cellular targeting is still one of the major challenges in fully exploiting the potential of biologicals.

Cell-type-specific targeting strategies addressing cell-surface receptors will definitely play an important role in the targeting of biologicals. However, the use of CPPs as pharmacokinetic modifiers and a combination of strategies will certainly gain significance. The intramolecular masking of a CPP by a negatively charged stretch of amino acids and proteolytic liberation of the active CPP at cancer cells that release a metalloproteinase is one elegant example of a combination of strategies.[140]

The picture of endocytic uptake of CPPs is still sketchy. It is not yet clear to what degree CPPs discriminate between the different endocytic pathways and, if they do, what the consequences are for intracellular degradation and cytoplasmic release of cargos. However, once the structure–activity relationships of CPPs are understood, it is very likely that different CPPs will serve as pharmacological modifiers with highly discriminatory abilities for different compartments. Given the significance of the endolysosomal compartment for therapeutic intervention, cytoplasmic delivery will not in all cases be the ultimate goal.

## Acknowledgements

*This work was supported by the Volkswagen Foundation (Nachwuchsgruppen an Universitäten).*

**Keywords:** cell-penetrating peptides · cellular uptake · endocytosis · peptides · protein transduction

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Received: February 1, 2005
Published online on October 27, 2005

  