CellPress

Feature Review

Mechanism Matters: A Taxonomy of Cell Penetrating Peptides

W. Berkeley Kauffman,[1,2] Taylor Fuselier,[1,2] Jing He,[1,2] and William C. Wimley[1,*]

The permeability barrier imposed by cellular membranes limits the access of exogenous compounds to the interior of cells. Researchers and patients alike would benefit from efficient methods for intracellular delivery of a wide range of membrane-impermeant molecules, including biochemically active small molecules, imaging agents, peptides, peptide nucleic acids, proteins, RNA, DNA, and nanoparticles. There has been a sustained effort to exploit cell penetrating peptides (CPPs) for the delivery of such useful cargoes *in vitro* and *in vivo* because of their biocompatibility, ease of synthesis, and controllable physical chemistry. Here, we discuss the many mechanisms by which CPPs can function, and describe a taxonomy of mechanisms that could be help organize future efforts in the field.

A Mechanistic Taxonomy of CPPs

In this review, we define 'cell penetrating peptides' (CPPs) (see Glossary) as the ∼1000 known peptides with the ability to enter cells or deliver cargo. They function by a diverse and mutable assortment of membrane activities. A single CPP is not, by virtue of its sequence, structure or amphipathicity, necessarily internalized via a singular mechanism. Instead, we argue that a peptide is capable of gaining access to the cytosolic compartment of a cell via multiple mechanisms and that varying the physical–chemical properties of a peptide can influence the relative contribution of these various mechanisms to the **uptake** of a peptide. Here we present the idea of a landscape of internalization mechanisms. In any particular situation, internalization will depend on fixed factors that include peptide sequence and physical chemistry but also on variable factors such as local peptide concentration, local lipid composition, the response of the cell to the peptide, and much more. Furthermore, the entry mechanism(s) of any CPP will depend very strongly on the characteristics of the cargo, such that each peptide–cargo combination should be considered a unique molecule.

We illustrate a possible landscape of CPP mechanisms in Figure 1 (Key Figure) based on the idea that the most crucial elements in describing the mechanism by which a CPP might translocate across membranes and deliver a cargo, under one specific set of conditions, are whether its internalization is mostly active (cell energy-dependent) or passive (cell energy-independent), and the degree to which it can disrupt membranes. The height of the landscape in Figure 1 is indicative of the toxic membrane activity presumed to be undesirable in a CPP: significant or long-lived disruption of the plasma membrane. By contrast, potent membrane lytic activity is less toxic when combined with a strong dependence on uptake because disruption of the endosomal membrane is less toxic than disruption of the plasma membrane. Ultimately, the low 'elevation' regions of the landscape are the areas that are potentially useful for cargo delivery by

Trends

The functional sequence space for cell penetrating peptides (CPPs) is vast. Recent data from computational, synthetic, and biological systems show that the mechanisms by which they bypass membranes are similarly diverse.

The CPP mechanism is mutable; it is not determined by the peptide sequence only. Many other experimental and biological factors are important, including local peptide concentration, local lipid composition, and the properties of the cargo.

The position of a CPP within the mechanistic taxonomy, under one set of conditions, can be described by the degree to which it is taken up by endocytosis, and the degree to which it can disrupt membranes.

Transformation from a peptide-centric approach to a mechanistic and cargo-centric approach may enable the CPP field to fulfill its long-held promise.

[1]Department of Biochemistry and Molecular Biology, Tulane University School of Medicine, New Orleans, LA 70112, USA
[2]These authors contributed equally.

*Correspondence: wwimley@tulane.edu (W.C. Wimley).

CellPress

Key Figure

Mechanisms of Known Cell Penetrating Peptides (CPPs)
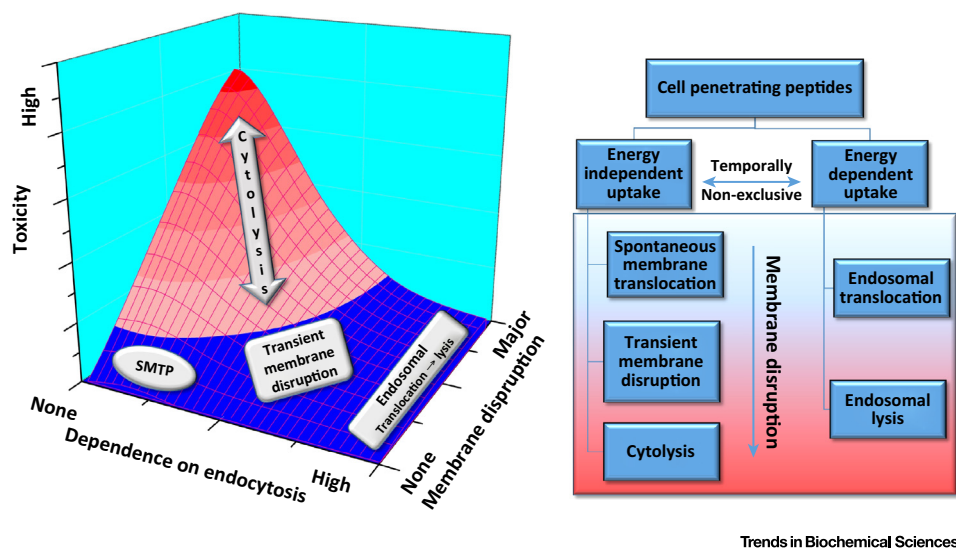

Trends in Biochemical Sciences

Figure 1. (A) A putative landscape for CPP mechanisms based on how they interact with cells and with cellular membranes. The *x*- and *y*-axes represent the mode of interaction with cell membranes, and the response of the cell to the peptide, respectively. Toxicity (*z*-axis) results from plasma membrane permeabilization, but not endosomal permeabilization. For this reason, low elevation regions of the landscape (blue) are useful for CPPs. (B) The taxonomy of CPP mechanism, which is a portion of the taxonomy for all membrane active peptides.

CPPs. In the context of Figure 1, we have defined five broad classes of activity, some of which might individually be best suited for the delivery of specific classes of cargo.

(i) *Potent plasma membrane lysis.* This is caused by peptides that permeabilize cell plasma membranes, and kill cells, at low concentration. CPPs are specifically selected not to have this property, although some acquire **cytolytic** activity at high concentration.

(ii) *Spontaneous membrane **translocation***. This occurs when a peptide passively translocates across membranes, at low concentration, without **endocytosis** or significant **membrane disruption**.

(iii) *Uptake (energy)-dependent membrane translocation.* This occurs when an **actively internalized** (endocytosed) **peptide** is triggered by changing conditions in the endosome to translocate across endosomal membranes after uptake without significant membrane disruption. The changing conditions can include decreasing luminal pH, transmembrane pH gradient, proteolysis, disulfide reduction, or a change in lipid composition.

(iv) ***Transient plasma membrane disruption***. This occurs when an interfacially active peptide has reached the conditions needed for localized or transient disruption of the plasma membrane structure or architecture. In this scenario, membrane disruption enables peptide and cargo delivery, but is not significant or long-lived enough to cause large-scale cytotoxicity.

(v) *Uptake (energy)-dependent membrane disruption.* This occurs when an actively internalized peptide is triggered to disrupt the endosomal membrane by local peptide concentration, endosomal pH, endosomal lipid composition, or other factors. Disruption can be small- or large-scale, and because the latter is not acutely toxic to cells, this mechanism can be useful for the delivery of actively uptaken cargo, including macromolecular cargo.

Glossary

Actively internalized peptide: a peptide or peptide–cargo conjugate uptaken into cells by endocytosis.
Cell penetrating peptide (CPP): an exogenous peptide or peptide–cargo complex that can be detectably internalized into cells by any mechanism.
Cytolytic peptide: a peptide or peptide–cargo conjugate that causes significant or long-lived disruption of the cellular plasma membrane, leading to significant cytotoxicity.
Endocytosis: any active transport mechanism by which a cell engulfs material in a plasma membrane-derived, membrane-bound organelle. Major classes of endocytosis include clathrin- and caveolin-dependent endocytosis, as well as macropinocytosis and phagocytosis.
Membrane disruption: any process wherein the normally strict permeability barrier imposed by the lipid bilayer against polar molecules is diminished or circumvented. Disruption can be transient or long-lived, and the degree of permeabilization can range from minor (selective or short-lived) to catastrophic (non-selective or long-lived).
Transient plasma membrane disruption: the process by which a CPP transiently disrupts the cellular plasma membrane upon binding and accumulation on cell surfaces. Transient disruption may enable peptide or cargo internalization, but is not necessarily acutely cytotoxic.
Translocation: the process of crossing a membrane from one side to the other, independent of mechanism.
Spontaneous membrane translocation: the process by which a CPP translocates across membranes as monomers or small multimers. As we define it, spontaneous translocation occurs by a mechanism that neither causes nor requires membrane disruption.
Uptake: also referred to as 'entry' and 'internalization'. The process of entering a cell from the outside. From the perspective of cellular energy, uptake can be active (energy-dependent) or passive (energy-independent).

Much work has been done, mostly by trial and error, to modify sequences to improve internalization of CPPs, with some examples of success [1–6]. Here we discuss the many overlapping mechanisms by which actively and passively internalized peptides, and attached cargo, interact with lipid bilayer membranes and bypass or disrupt membranes to gain access to the cell cytosol.

Many CPPs are Actively Internalized

We define a 'cell penetrating peptide' as any peptide that can, to a measurable degree, enter the interior of living cells in cell culture, or deliver a membrane-impermeant cargo. Energy-independent passive internalization occurs when peptide–cargo conjugate directly bypasses the cellular plasma membrane to enter the cytosol. This can occur via transient membrane disruption [7] or spontaneous translocation [8], as discussed later. Energy-dependent, active internalization occurs when a CPP triggers uptake via endocytosis. Relevant uptake mechanisms are numerous and their cell biology has been thoroughly reviewed elsewhere [9,10]. Although specific biochemical mechanisms vary, in all cases, the cargo must be targeted to membrane-bound endosomes that are taken up into the cytosol.

Many pathogens take advantage of endocytic pathways to gain cellular access [11], so it is not surprising that the original CPP sequence, 'tat', derived from the arginine-rich motif of the DNA-binding *tat* transcription factor of human immunodeficiency virus (HIV) [12] enters cells by endocytosis. Entry of *tat* is temperature-dependent, dynamin-1-independent, and is reduced by cytochalasin D and amiloride, drugs that block macropinocytosis, suggesting that this is the primary mechanism of entry [12]. However, clathrin-mediated and caveolar endocytosis are likely to be also involved [13–15]. Other well-studied CPPs, including penetratin and the simple *tat* analog Arg_9, are also endocytosed [16].

Electrostatic interactions between cationic peptides and anionic cell surface molecules such as glycosaminoglycans (GAGs) and other glycoconjugates are crucial for the uptake of many CPPs, including *tat* [17–19]. Various CPPs are taken up with an efficiency that appears to be related to the binding stoichiometry between cell surface anions and peptide [17,19]. At the same time, however, the mechanism of cell entry is sensitive to the cargo molecule [15] and can change with small changes in peptide sequence [16]. Some CPPs can enter cells by active and passive mechanisms simultaneously [16].

The Diversity of Known CPPs

To access the cell interior, some CPPs may directly utilize active transporters [20] or cause endosomal lysis by the osmotic 'proton sponge effect' [21]. However, most CPPs likely function by physically disrupting or bypassing either the plasma membrane or the endosomal membrane, or both – actions that are ultimately determined by the physical chemistry of peptide–membrane interactions. Here, we discuss the range of physical–chemical properties of the many known CPPs to better understand the diversity among CPP mechanisms.

We compiled a set of ∼950 sequences that have been reported to enter cells and deliver polar cargo. Approximately 90% of the sequences are from a CPP database [22] [i] established in 2012. We supplemented the list with ∼100 additional sequences found in the recent literature. After removing redundant sequences, the database contains 747 unique CPPs. Their efficiencies and mechanisms vary widely [22]. Unfortunately, most of these peptides deliver only dyes (63%) or biotin and other small molecules (33%) as proof of principle. Delivery of larger or useful cargos is very rare in the CPP literature, with proteins comprising only 4% of cargoes [22]. The exception is the delivery of oligonucleotides through complexation with CPPs, which is now routine in many cell types [23]. We will not review oligonucleotide delivery here. Approximately 45% of the CPP sequences are homologous or partially overlapping with other database entries, as many

CellPress

researchers have studied variants of a few well-known CPPs; ~35% of the sequences are described in only one or a few papers, or studied by only one laboratory. The size and diversity of the database suggests that CPP behaviors are not highly dependent on specific primary sequences and that the functional sequence space for CPPs is probably enormous, at least with respect to entry of dye-labeled peptides into cells.

The distributions of the important physical properties of the known CPPs are shown in Figure 2, along with the values of some well-studied CPPs. Most CPPs are small, cationic peptides, with a median length of 14 residues and a median charge of +5 (Figure 2A,B). A median of 35% of CPP residues are cationic (Figure 2C), although this number ranges from 0% to 100%. Given the relatively tiny sequence diversity that is possible for the highly cationic peptides, they are extremely overrepresented. The overall interfacial hydrophobicity [24] of most of the known CPPs is not favorable for spontaneous partitioning into zwitterionic membranes (Figure 2D), distinguishing CPPs from other types of membrane active peptides, which are more hydrophobic [25]. For most CPPs, membrane binding requires electrostatic interactions or peptide amphipathicity, which promotes membrane binding by coupling it to structure formation [26]. Indeed, although most CPPs are not highly amphipathic, a subset of CPPs are, either when folded into an α-helix (Figure 2E, see penetratin), or when folded into a β-sheet structure (Figure 2F).

The sequence space for CPPs that can deliver a conjugated dye molecule 'cargo' to cells is very large, therefore the discovery of novel CPPs, or new variants of known CPPs, may be a relatively trivial task, even by trial and error. In the end, as we discuss later, it will probably be more useful to focus future efforts on the discovery and engineering of peptides that exploit particular mechanisms, with the goal of enabling the efficient delivery of particular cargoes of genuine utility.

Archetypal Cell Penetrating Peptides

A small number of CPPs have been especially well studied or have mechanisms that represent the diversity of CPP mechanism discussed here. We introduce them next and review some of their characteristics.

Tat

The *tat* sequence is a naturally occurring CPP derived from the *tat* transcription factor of HIV. Mutational analysis of the full-length HIV-tat protein revealed a short, highly basic, and unstructured N-terminal sequence (GRKKRRQRRR) that was necessary and sufficient for cell entry [27] and cargo delivery [28]. The mechanism of cellular entry for *tat* initially was thought to be energy-independent [28]; however, a general consensus has emerged that endocytosis is the primary mechanism of entry [17,29].

Oligoarginine

In an effort to understand and simplify the CPP motif of *tat*, researchers assessed the ability of synthetic oligoarginines to translocate into cells. Oligomers with between 6 and 12 arginines, the most common amino acid in CPPs [22], entered cells, and nona-arginine (Arg_9) was found to be the optimal length [30]. Entry of Arg_9 into live cells is not dependent on chirality [31] but it is dependent on the backbone spacing between arginine residues [32]. Other homo-polycationic peptides of similar length (e.g., Lys_9) do not show the same ability to enter cells [30]. Arg_9 enters cells by endocytosis at low concentration ($\leq 5\,\mu M$) [33], but can cross the plasma membrane directly at high concentration ($\geq 10\,\mu M$) [30,34].

Penetratin

Like *tat*, penetratin was discovered in a mutational analysis of a naturally occurring *trans*-activating protein [35]. The antennapedia homeoprotein (pAntp) of *Drosophila* contains a helical
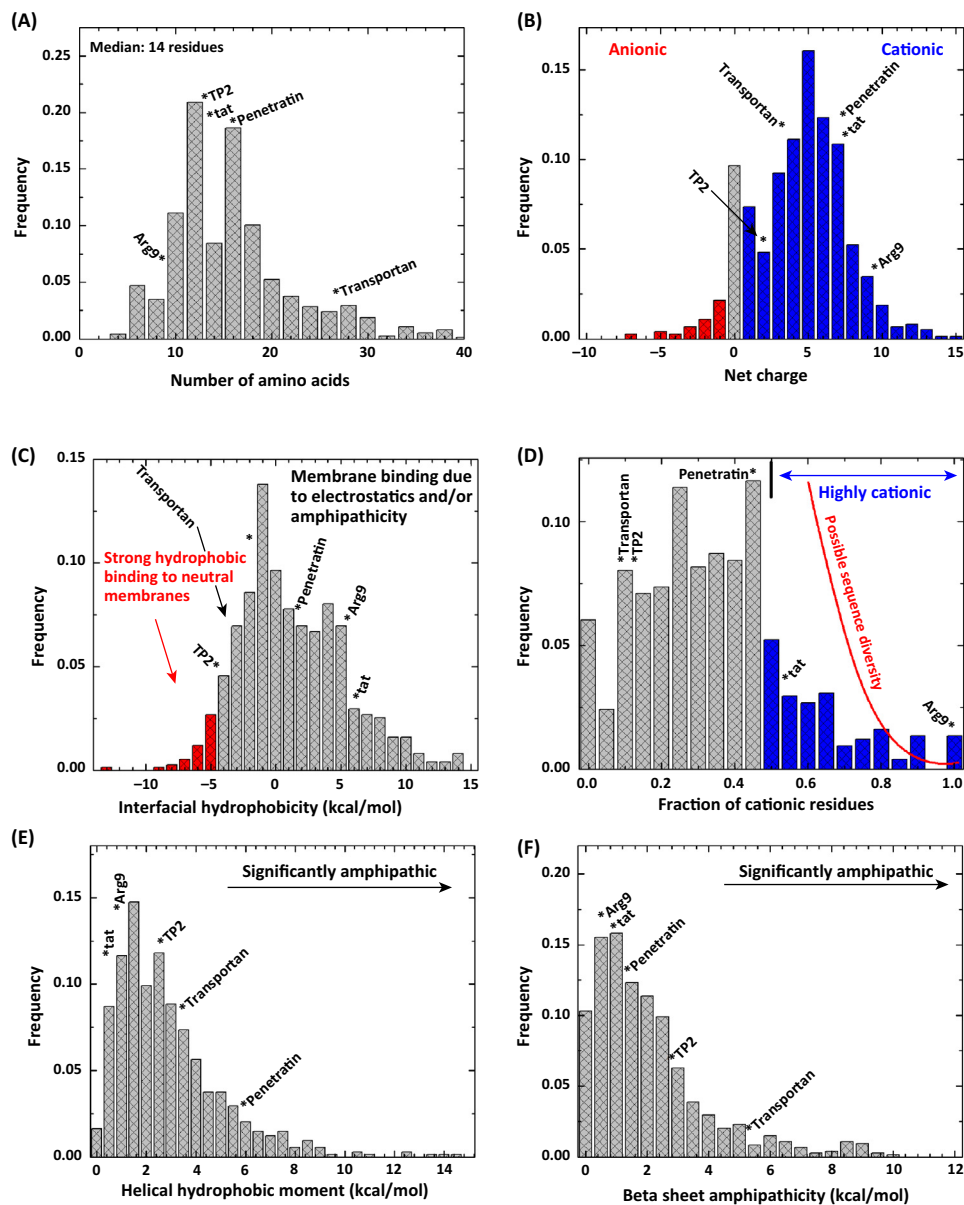
Trends in Biochemical Sciences

CellPress

Figure 2. Frequency Distributions of Various Physical Properties 747 Non-Redundant Cell Penetrating Peptides (CPPs). (A) Total peptide length. (B) Net charge assuming pH 7.4, C-terminal amidation, and no contribution from histidine. (C) Wimley–White interfacial hydrophobicity score [26] for the CPP sequences, assuming a C-terminal amide and an N-terminal amino group. Positive is unfavorable for partitioning. (D) Fraction of residues that are cationic, excluding histidine. (E) Helical hydrophobic moment [96]. (F) Absolute value of the β-sheet hydrophobic moment calculated assuming an unbroken diad repeat motif. On each histogram, we show the values for the five representative CPPs described in the text.

portion that, when mutated, causes the homeoprotein to not be internalized *in vitro*. The 16-residue third helix of pAntp (RQIKIWFQNRRMKWKK) proved to be the minimal motif for internalization, and was named penetratin [36]. Like many CPPs, the mechanisms of entry for penetratin are variable and depend on experimental conditions, especially peptide concentration [37]. Penetratin has been shown to enter cells via direct translocation and also by endocytosis

CellPress

[37,38]. Penetratin internalization was shown to be dependent on the presence of GAGs and on membrane domains created by either cholesterol depletion or ceramide formation in cell membranes [39].

Transportan

The N terminus of galanin (a porcine neuropeptide) and mastoparan (a pore-forming peptide found in wasp venom) were combined to form a chimeric CPP known as transportan (GWTLNSAGYLLGKINLKALAALAKKIL) [40]. Transportan, less cationic and more amphipathic than most CPPs, has faster kinetics of cell entry than *tat* and penetratin [65]. Transportan may have multiple mechanisms of entry at low concentration, including both energy-dependent and energy-independent internalization [41]. Interestingly, transportan was shown to enter various plant tissues in a non-endocytic manner [42]. A shortened variant, called transportan 10, or TP10 (AGYLLGKINLKALAALAKKIL), has even better activity [43].

TP2

This peptide (PLIYLRLLRGQF), which is unrelated to TP10, was selected from a peptide library screened for soluble peptides that could spontaneously translocate across synthetic vesicle bilayers without membrane permeabilization [8]. TP2 conjugated to otherwise impermeant dye cargoes also showed rapid entry into eukaryotic cells at low concentration (<2 μM) and at room temperature, when endocytosis is inhibited [33]. As discussed later, it was proposed that monomeric TP2 enters cells via spontaneous membrane translocation [8,33].

These five archetypal peptides represent most of the known structural and mechanistic diversity of the CPPs. In the sections that follow, we will refer frequently to these example peptides.

CPPs are Interfacially Active Peptides

CPPs have been studied in many synthetic bilayer systems with a wide collection of biophysical techniques. By virtue of their strong electrostatic interaction with anionic membrane surfaces and their sometimes amphipathic nature, many CPPs have 'interfacial activity' defined as the ability to bind at the bilayer–water interface and perturb membrane structure (Figure 3) [44]. Although the details vary widely, once the local concentration of an interfacially active peptide rises above a threshold, unique to each peptide, it can cooperatively disrupt the vertical segregation of hydrophobic and hydrophilic groups in a bilayer and allow the passage of polar molecules, minimally the peptide itself, across the membrane.

Experimentally, both electrostatic and hydrophobic contacts between peptide and lipids are important for CPP–membrane interaction [45,46]. As predicted by their positive charge and unfavorable interfacial hydrophobicity (Figure 2C), CPPs typically have weak interaction with zwitterionic synthetic membranes and strong interaction with anionic membranes [47,48]. Vesicle leakage, translocation, and other experiments show that the inclusion of anionic lipids almost uniformly enhances CPP activity [49–52]. When cationic CPPs are bound to anionic bilayers, especially when the peptide-to-lipid ratio is high (P:L $\geq$ 1:50), many effects are observed. Bilayer curvature can change [53–55], membrane domain architecture can be affected [47,54], non-bilayer phases can form [56], domains of clustered lipids and peptides can form [54,57], bilayer disorder can increase [53] or decrease [58], vesicles can undergo aggregation and fusion [59], lipid flip-flop can occur [56], or entrapped contents can be released [49–51]. Which of these effects occur, if any, is dependent on the CPP sequence, the peptide-to-lipid ratio, the lipid composition, and many other experimental details.

Unlike *tat* and polyarginine, amphipathic CPPs fold into organized secondary structures in bilayers. For example, penetratin has mostly random coil secondary structure in buffer, whereas it adopts a helical structure in the presence of anionic lipids, to which it binds strongly [58,60].

CellPress
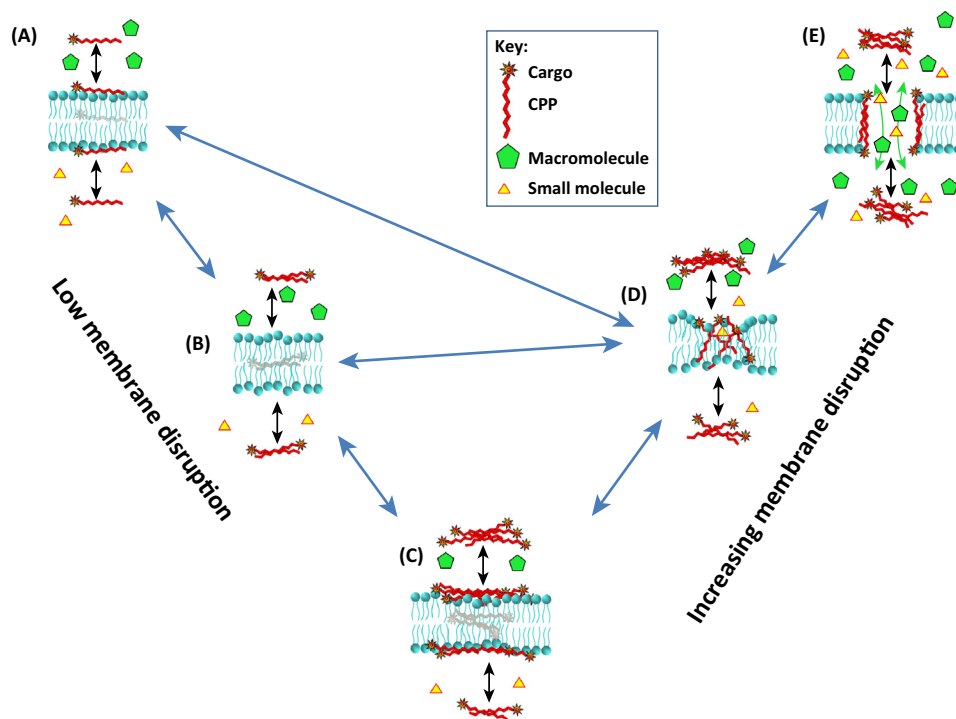
Trends in Biochemical Sciences

Figure 3. Schematic Illustration of Some of the Overlapping Mechanisms by which a Peptide May Cross a Membrane. The effect of peptide on the membrane is also indicated. (A) Spontaneous translocation of monomeric peptide that occurs without membrane disruption. (B) Translocation that requires some peptide self-assembly, but not membrane disruption. (C) Translocation that requires significant peptide aggregation and minor membrane disruption. (D) Translocation that requires peptide self-assembly and moderate (i.e., selective or short-lived) membrane disruption. (E) Translocation associated with peptide self-assembly into a structure that drives non-selective or long-lived membrane permeabilization. In this scenario, even large polar molecules pass through the membrane. This mechanism will be acutely toxic if it occurs in plasma membranes, but not if it is confined to endosomal membranes. These illustrations represent snapshots of states in a continuum of mechanisms. The arrows show some of the possible transitions between mechanisms that could arise from many experimental factors, including peptide sequence and physical chemistry, local peptide concentration, and anionic lipid content.

When at high concentration in bilayers, penetratin undergoes an additional transformation into an aggregated β-sheet structure [61], highlighting the structural and functional plasticity of CPPs. Synthetic membranes are disrupted when penetratin binds and aggregates. This can involve the formation of lipid domains, an increase of bilayer thickness and curvature, and even tubulation of lipid vesicles at high P:L ratios [57]. Similarly, circular dichroism spectra and simulations showed that transportan 10 is highly helical when bound to vesicles, and inserts its hydrophobic face into the bilayer [43,59,62–64]. After insertion into synthetic lipid vesicles, transportan interacts with lipid phosphates causing lipid rearrangement and changes in local curvature in the bilayer [25,47]. This plays a crucial role in determining the peptide orientation and insertion depth into the interfacial region of the membrane [63]. Under some conditions transportan 10 can translocate across and permeabilize synthetic bilayers that do not contain anionic lipids [34,65].

The peptide TP2, discussed in detail later, appears to behave uniquely, in that it readily translocates across synthetic bilayers. Yet, in a neutron diffraction study of bilayer structure, TP2 was shown to have little or no effect on bilayer structure, even at very high local concentration, suggesting that it is possible for a peptide to translocate without significant membrane disruption [66].

CellPress

The image that emerges from the concept of CPPs as interfacially active peptides is not a static one. A peptide's sequence and structure are not enough to define its mechanism of action under all conditions. Mechanism is also dependent on bound peptide concentration, lipid composition, and other bilayer physical properties, temperature, ionic strength, among other factors. This leads us to the concept that each CPP occupies an area or areas in a continuum of mechanisms, as illustrated in Figure 3. As is the case for interfacially active peptides in general, there is little consensus on the detailed molecular mechanisms of CPPs in synthetic membranes [44,67]. With only a few exceptions, our current knowledge provides little predictive power on the molecular mechanism or activity of specific CPPs on synthetic membranes. Furthermore, for some classes of CPPs, the mechanisms driving peptide–membrane interactions and translocation in synthetic bilayers may be different from the mechanisms that operate in the dynamic and heterogeneous environment of the cell membrane. As a result, vesicle experiments could have utility in predicting CPP function in cells only for some classes of CPPs, perhaps helping to explain why the CPP field mostly discovers new peptides fortuitously, or by trial and error. Encouragingly, there are recent exceptions including *in silico* design [68] and targeted screening [8,69,70], which suggest rational, targeted design is possible.

Translocation Across Membranes

Translocation, defined as the movement of a molecule across a bilayer, has been studied for many CPPs using a variety of techniques [8,51,65,71,72]. Yet, there is no consensus about which CPPs can translocate across synthetic bilayers and which, if any, do so without simultaneous membrane disruption. Highly cationic CPPs, above a variable threshold concentration, have been reported to effectively cross synthetic anionic bilayers [52,56]. In many cases, this is likely to be attributable to disruption of the bilayer structure [7]. At low or moderate peptide concentrations, or in bilayers with low anionic lipid content, CPPs such as Arg9, *tat*, and penetratin neither translocate nor disrupt membranes [8,52,56,72]. A novel fluorescence approach [71] to measure CPP translocation across synthetic bilayers was recently applied to Arg_9, *tat*, TP2, and other CPPs. All were found to rapidly cross anionic large unilamellar vesicles without permanent disruption of bilayer structure [52,56,71], although transient disruption was not assessed. For *tat*, Arg9 and other highly cationic CPPs, the extent of translocation increases sharply with increasing peptide concentration, suggesting that the translocating species is a peptide multimer [52]. Furthermore, translocation is associated with transbilayer exchange (i.e., flip-flop) of anionic lipids, which can be triggered by small disruptions of bilayer structure, such as lipid density fluctuations, or large disruptions, such as pore formation or reverse micelle formation [56]. Finally, translocation of these highly cationic CPPs is completely dependent on anionic lipids such as phosphatidylglycerol (PG) and decreased to near zero at approximately 50% anionic lipids in a phosphatidylcholine (PC) bilayer.

By contrast, transportan 10 does not require anionic lipids for translocation. This is probably because transportan 10 is more amphipathic and more hydrophobic than, for example, *tat*, Arg_9, and penetratin (Figure 2), and has less charge (+5 for transportan 10 compared with +8 to +10 for *tat*, Arg_9, and penetratin). In a study using a unique confocal microscopy approach, transportan 10 was shown to readily translocate into giant unilamellar vesicles (GUVs) made from PC without anionic lipids [65], accompanied by a measurable influx of a charged dye molecule from solution [65]. The fact that there were no changes in the overall architecture of the GUVs suggests that transportan 10 only moderately disrupts membranes under these conditions. Interestingly, translocation and permeabilization occur on the same timescales, but are not temporally coupled. Translocation and permeabilization are not the result of a single process, although they must both be driven by related membrane activities. These studies show the power of simultaneous measurement of translocation and permeabilization (Box 1).

CellPress

Box 1. The Missing Links of CPP Experiments

Translocation or Permeabilization?

Interfacially active peptides often cause transient disruption of membranes after which peptide-disrupted bilayers relax to non-perturbed states [44]. Measurement of synthetic membrane integrity after translocation does not necessarily provide information about whether or not membrane disruption occurred during translocation. It is crucial to understanding CPP mechanism that both translocation and permeabilization of bilayers be measured at the same time, or over the same timescale and under the same conditions.

What is the Local Concentration?

There is a critical gap in knowledge of the interplay between cell surface binding, active or passive clustering, internalization, and degradation. The overall solution concentration of a peptide can be controlled, but the local concentration on a region of plasma or endosomal membrane is (i) unknown and difficult to measure; (ii) subject to rapid change as a result of simultaneous uptake, translocation, clustering, and degradation; and (iii) difficult to experimentally manipulate because it is not linearly related to solution concentration. Improved methods for measuring or controlling local concentrations of peptides on cell surfaces and in endosomes would be helpful to the field.

How Much is Too Much?

In practice, it is possible to enable detectable cell entry of almost any cationic or amphipathic peptide by simply testing it at increasing concentrations until entry is observed. Concentrations of cationic peptides greater than a few μM are likely to saturate cell surfaces, could affect cell physiology and function, and would preclude most clinical applications. The CPP field would benefit from the use of better methods for quantitating the efficiency of cargo delivery as a function of CPP concentration. This would enable the field to focus on developing CPPs that efficiently deliver useful cargoes to cells at relatively low concentrations.

Do My Eyes Deceive Me?

The CPP field relies heavily on laser scanning confocal fluorescence microscopy for assessing uptake. Although this technique is powerful and indispensable, it also has weaknesses. Specifically, imaging is often qualitative and susceptible to manipulation, automatic or manual intensity scaling (which can obscure actual concentrations), self-quenching of clustered and internalized dyes (which can mask concentrated pools of peptide), and pH and other effects (which can change dye intensity). For example, fluorescein, sometimes also called FITC, is widely used in CPP studies [22] despite the fact that its fluorescence is quenched at the pH of acidified endosomes. The convenience of fluorescent dyes as 'cargo' has caused much of the field to focus, almost exclusively, on the discovery of peptides that can deliver dyes, instead of useful cargoes. The field will be better served by diversified approaches, such as some laboratories are pursuing [97], for detecting and quantitating the delivery of useful cargoes to cells.

As an aside, we note that peptide translocation into 'giant' vesicles of diameter $\geq$10 μm is 10–100-fold faster than translocation into standard 'large' unilamellar vesicles of diameter $\sim$0.1 μm (W.C. Wimley and T. Fuselier, unpublished). Thus, direct comparisons of the two systems should be made with caution.

Recent experiments with plasma membrane-derived vesicles are beginning to bridge the critical gap between synthetic bilayers and living cells. Such vesicles are made from native plasma membranes and contain native lipids, membrane proteins, and glycoconjugates, but lack energy-dependent cellular processes, including uptake machinery, obviating the need for problematic chemical or physical inhibition of endocytic mechanisms. Using such vesicles, it has been shown that there is a direct correlation between the cellular uptake of penetratin (and some analogs) and its binding to plasma membrane-derived vesicles. Other experiments have shown that multiple CPPs, including *tat*, penetratin, transportan, and transportan 10, can accumulate inside plasma membrane-derived vesicles [73,74], although the degree of membrane disruption is unknown. Vesicle surface interactions are dynamic and driven by multiple cell surface moieties [75]. The translocation of CPPs across such native membranes favors liquid disordered membrane domains low in cholesterol and sphingomyelin [73,74].

Translocation without Membrane Disruption

There are a few peptides that, like most small molecule drugs, partition weakly into membranes and translocate 'silently' across the bilayer without peptide clustering or significant membrane disruption [8]. This mechanism represents a unique subclass of CPPs that we have termed

spontaneous membrane-translocating peptides (SMTPs) [8,33,52,56]. SMTPs have different advantages and disadvantages compared with actively internalized or membrane-disrupting CPPs. Outside of the CPP field, the direct membrane translocation capabilities of cationic peptides were considered in the context of understanding the structure and function of the voltage sensor helices of voltage-gated potassium channels. These peptides move freely across bilayers when exposed to lipids as part of an intact channel protein [76], and also translocate across synthetic bilayers as isolated peptides [72] despite the presence of four arginine residues. TP2 and related family members translocate across unilamellar and multilamellar synthetic lipid bilayers [8], without membrane disruption, even in the absence of anionic lipids [33]. Apparently, TP2 does not require aggregation or self-assembly to translocate across bilayers; it binds only weakly to membranes, causes little or no permeabilization or disruption of membrane structure [66], and readily translocates at very low concentration (P:L $\leq$ 1:1000) [8,33]. Weak membrane binding is probably a requirement for such simple passive translocation, as strong binding would drive accumulation of peptide on the bilayer, likely leading to membrane disruption.

The TP2 family of peptides was discovered in a high-throughput screen [8] that simultaneously selected for (i) solubility, (ii) rapid translocation of peptide across synthetic PC bilayers containing 10% anionic lipids at low peptide concentration, and (iii) a complete lack of membrane permeabilization in the same vesicles. The distribution of properties of the 10 368-member library screened to find TP2 very closely resembled the distribution of properties of known CPPs shown in Figure 2, including having members that are highly or completely cationic. In fact, the library contains the exact sequences of *tat*, Arg_9, and several other known CPPs, but no sequences resembling any known CPP were identified as positives in the screen [8]. Instead, a unique family of peptides was identified. Intriguingly, the TP2 family peptides contain the same hydrophobe(ϕ)–arginine motif, $\phi R\phi\phi R$, found concatenated three times in the voltage sensor helix [72]. TP2 and related family members translocate across cellular membranes under conditions where endocytosis does not occur [33]. Thus, for SMTPs, testing of rational design and engineering ideas in synthetic bilayers may be relevant to the peptide activity in cellular membranes.

Arginine was preferentially selected over lysine in the screen for TP2 [8], and is abundant in CPPs, overall (Figure 2D) [22]. The surprisingly low energetic cost for arginine partitioning into membranes [77,78] is probably attributable to its especially favorable interactions with lipid phosphates [77]. This interaction has been proposed to be important in the spontaneous membrane translocation of arginine-containing hydrophobic sequences, like the voltage sensor domain, TP2 family sequences, and some other CPPs. Movement of SMTPs across membranes may be aided by bidentate interactions of the positively charged guanidinium group with the negatively charged phospholipid phosphate [79–81], allowing for chaperoned diffusion [8,33] of neutralized peptide–lipid complexes across the bilayer. It was recently shown that fatty acids in combination with a transmembrane pH gradient can also chaperone arginine-rich peptides across bilayers [82], further supporting the idea that anionic amphiphiles such as lipid phosphates can do the same.

A Model of CPP Activity in Cells

Based on the interfacial activity of CPPs observed in synthetic bilayers, and on many cell studies, it is possible to relate the taxonomy of mechanisms in Figures 1 and 3 with mechanistic scenarios for CPP activities in cell membranes, illustrated in Figure 4. Classical, highly cationic CPPs probably interact with cell surface glycoconjugates, anionic lipids, embedded membrane proteins, and sometimes the plasma membrane interfacial zone (Figure 5D). Above a threshold concentration for energy-dependent internalization that is likely unique for each peptide–cargo complex, polyvalent cell surface binding can trigger clustering of CPPs, possibly by a membrane damage response mechanism [83], and subsequent uptake by endocytosis[13,17,37]. In
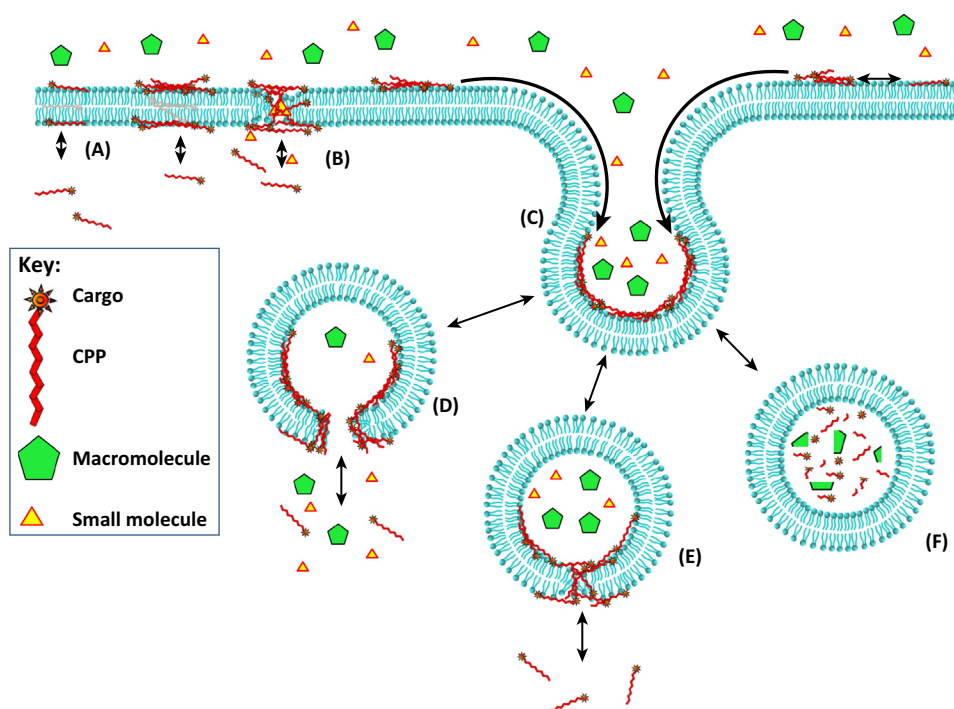
CellPress

Figure 4. Schematic Illustration of Some of the Various Mechanisms by which a Cell Penetrating Peptide (CPP) and Attached Cargo May be Internalized into a Cell. The fate of an unattached small molecule and macromolecule are also shown. (A) Spontaneous membrane translocation across the plasma membrane, which occurs without peptide self-assembly or membrane disruption (Figure 5B,E). (B) Transient plasma membrane permeabilization. (C) Endocytosis of membrane-bound peptide–cargo complex, along with unattached small and large molecule cargoes. (D) Endosomal membrane lysis, or large-scale disruption, releases the CPP–cargo conjugate and all co-encapsulated cargoes. (E) Translocation across the endosomal membrane delivers CPP and attached cargo, but not co-encapsulated cargo. (F) Degradation or recycling of CPP and all cargoes will occur rapidly if the other mechanisms do not enable delivery to the cytosol (Figure 5A,C). The mechanisms depicted are not mutually exclusive; they can happen concurrently.

Figure 5A,C, example confocal microscopy images of cells incubated with dye-labeled Arg$_9$ show that it is efficiently endocytosed. Once concentrated and internalized, CPPs may translocate across endosomal membranes or may reach the local membrane-bound concentration necessary for endosomal membrane disruption, resulting in at least partial peptide and cargo release into the cytosol. Endosomal membrane translocation or disruption may be triggered or aided by the dramatic changes in lipid composition and endosomal membrane stability that occur during endosome maturation [84,85], or by other endosome-specific environmental factors such as the acidic luminal pH and the transmembrane pH gradient [46].

As shown in the illustration of Figure 4 and the experimental images in Figure 5A,D, it is also possible for CPP and cargo to remain trapped in endosomes and never be delivered to the cytosol. This is exacerbated by the fact that L-amino acid CPPs, which are the most commonly studied by far, are subject to proteolytic degradation after uptake [86–88]. Peptide degradation can begin shortly after uptake and can be mostly complete in ~1–2 hours [86–88]. If the conditions required for endosomal translocation or disruption are not reached during that window, the peptide and possibly the cargo will be degraded or recycled without ever reaching the cytosol.

Above a threshold concentration that is distinct from, and usually higher than, the active internalization threshold, some CPPs may be able to reach a sufficiently high local concentration

Trends in Biochemical Sciences

CellPress
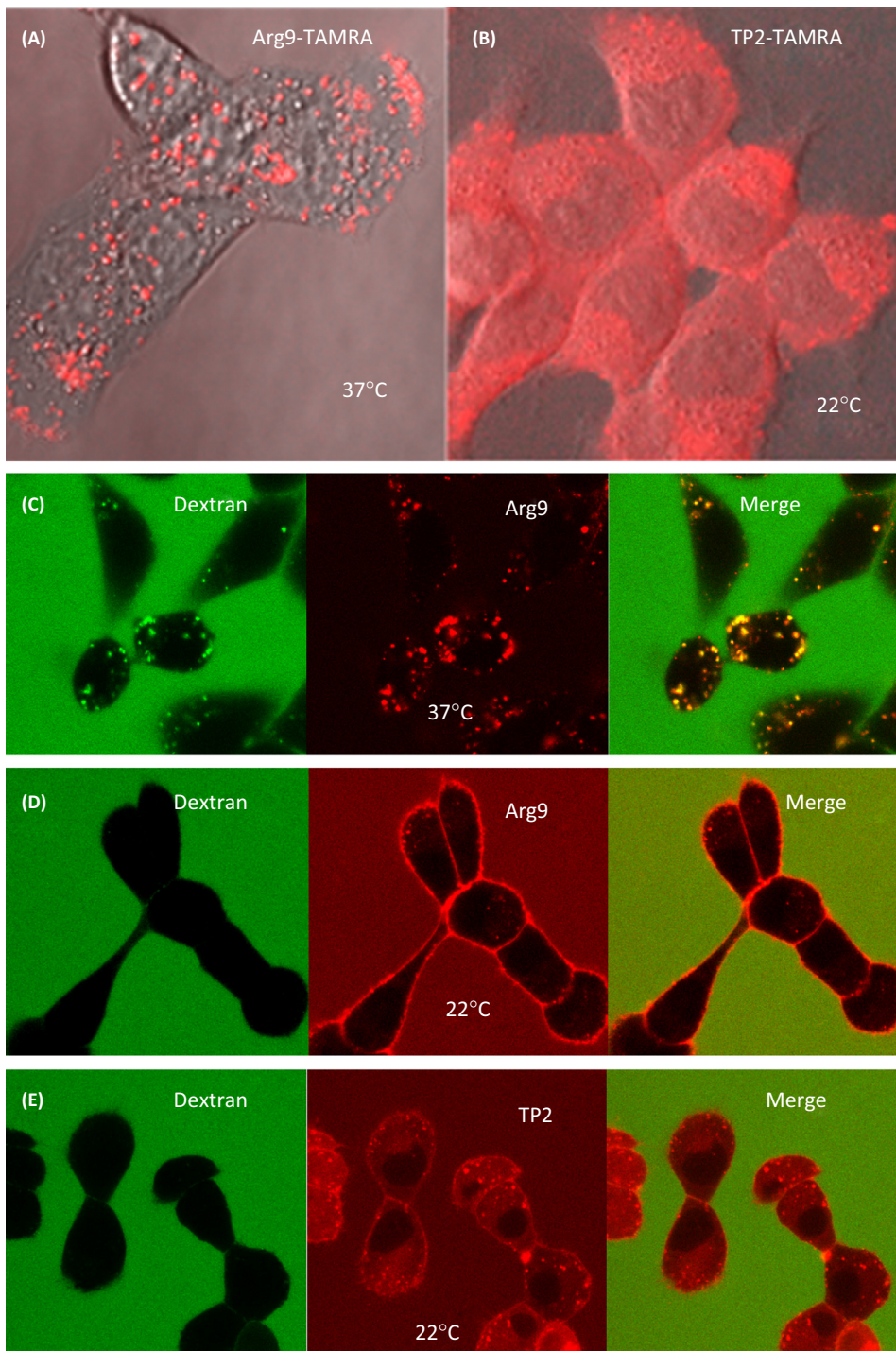
Trends in Biochemical Sciences

Figure 5. Example Confocal Microscopy Images of Fates of Dye-Labeled Cell Penetrating Peptides (CPPs) in Chinese Hamster Ovary Cells under Various Conditions. Here we show behaviors of a classical, highly cationic CPP, Arg$_9$, and a spontaneous membrane-translocating peptide (SMTP), TP2. Both peptides, which are described in the text, are labeled with the red dye tetramethylrhodamine (TAMRA). In some images, the cell media contains AlexaFluor488-labeled dextran (green), an aqueous phase probe that is passively entrapped in endosomes. (A) Endosome entrapment. Cells incubated at 37°C for 2 h with 2 μM Arg$_9$–TAMRA show punctate red intensity that is always associated with endosomes.

CellPress

on the plasma membrane to form peptide-rich domains [14,89] that can transiently disrupt the membrane [7] or promote peptide translocation and delivery of peptide and cargo into the cell cytosol. This is called transient plasma membrane permeabilization in Figure 1. For example, in a recent paper Arg$_9$ labeled with the dye tetramethylrhodamine (TAMRA) was shown to efficiently enter cells directly through the plasma membrane at concentrations above 10 µM, but not at 5 µM or below [34]. Intriguingly, in at least this case, transient plasma membrane permeabilization was reportedly inhibited by depletion of cellular ATP and by depletion of intracellular calcium [34], suggesting that the cell, through an unknown mechanism, could be an active participant in transient plasma membrane permeabilization.

The threshold local concentrations for CPP uptake or translocation across the plasma membrane are presumably highly variable, and depend on the peptide sequence, cargo, cell type, and other factors. Both processes can occur simultaneously. These concentrations are also very difficult to measure or control (Box 1). Currently one of the most significant roadblocks to CPP utility is that many CPPs only efficiently deliver cargo to cells at relatively high concentrations (≥10 µM peptide), which saturates cell surfaces with peptide [14,34,89,90] and can effect cell physiology [34,83]. At those conditions, multiple mechanisms may be at play simultaneously. In one recent study, a cyclic *tat* peptide was able to deliver green fluorescent protein to the cell cytosol *in vitro* [91]. Every example of macromolecular delivery is an important advance in the field; however, in this case, concentrations of 50–150 µM peptide–protein conjugate were needed to achieve delivery [91]. Such high threshold concentrations may be achievable in the laboratory, but they are somewhat impractical. Further, they prohibit clinical utility, a critical long-term goal of the field.

Unlike these well-known CPPs that cross the plasma membrane at high concentration as a result of transient plasma membrane permeabilization, SMTPs can cross cell membranes at low concentration, presumably in a way that causes little or no disruption of cell physiology. For example, TP2 and related peptides readily enter the cell cytosol at 1–2 µM peptide (Figure 5A,D) [33], which is useful, although such peptides are unlikely to be capable of delivering macromolecules.

Whether by transient plasma membrane disruption or by spontaneous membrane translocation, direct delivery across the plasma membrane bypasses the degradative environment of endosomal pathways. This is a critical factor for some cargo types. For example, the cytosolic delivery of bioactive peptides is becoming an especially important problem to solve in the rapidly developing field of inhibitors of protein–protein interactions in cells, which some authors have suggested will be the next great transformation in drug development [92,93]. Effective small molecule inhibitors of protein–protein interactions have proven difficult to identify, whereas peptides that block protein–protein interactions can often be readily identified using available sequence and structure information [93]. Yet, bioactive peptides, once identified, are frequently too polar to efficiently access the cell cytosol and they are very sensitive to endosomal

---

At concentrations less than 5 µM Arg$_9$, escape into the cytosol is not significant, and the entrapment of the TAMRA cargo does not change even after 24 h. (B) Spontaneous membrane translocation. Cells incubated with 2 µM TP2–TAMRA at room temperature for as little as 10 min show diffuse cytosolic fluorescence, indicating plasma membrane translocation. (C) Active uptake. Cells simultaneously incubated with 2 µM Arg$_9$–TAMRA and labeled dextran at 37°C show that dextran fluorescence often overlaps with TAMRA fluorescence in intracellular organelles. This observation demonstrates that Arg$_9$, at low concentration, enters cells only through endocytosis. (D) No uptake. Cells simultaneously incubated with 2 µM Arg$_9$–TAMRA and labeled dextran at room temperature, which inhibits endocytosis, show that neither Arg$_9$ nor dextran enter cells appreciably under these conditions. However, it is clear that Arg$_9$ binds strongly to the plasma membrane. Above 10 µM Arg$_9$, by contrast, delivery of TAMRA to the cytosol and nucleus by Arg$_9$ is significant [34]. (E) Spontaneous membrane translocation. Incubation of cells with TP2–TAMRA and labeled dextran at room temperature show that TP2 is internalized into cells without simultaneous entry of dextran. This observation, and others [33], indicate that endocytosis is not required for TP2 internalization.

ARTICLE IN PRESS

Trends in Biochemical Sciences

CellPress

degradation [86]. Thus, an obvious route for delivery of bioactive peptides is directly through the plasma membrane, a mechanism that some CPPs are known to follow.

Mechanism Matters: Closing Thoughts and Concluding Remarks

The CPP field has advanced significantly since the discovery of *tat* and penetratin peptides in the early 1990s. For example, one of the first investigational human drugs built around a CPP-like sequence (RT001, a topically applied Botulinum toxin) has performed well in human clinical trials [94]. Yet, in some ways, the field is still in a developmental stage, with much effort being made toward the discovery of novel CPPs that can deliver dye molecules, and much effort being made toward the *ex post facto* characterization of their mechanism (see Outstanding Questions). Still, our current mechanistic knowledge is almost purely observational, with little useful predictive power. Perhaps more importantly, the literature contains a surprising abundance of papers that come to different conclusions on the mechanism of action of the same CPPs. This may be attributable, in part, to the phenomenon described here in which a CPP can function across a continuum of mechanisms that is sensitive to a variety of experimental details. The mechanism of a CPP is not only an inherent characteristic of its sequence. This leads to problems in the commonly used peptide-centered approach to new CPP discovery. In that approach, dye-labeled peptides are first tested or screened for effective cell entry, followed by mechanistic studies to determine the route(s) by which the peptide enters cells or crosses bilayers. Finally, sometimes, the types of cargoes that can be delivered to cells by the peptide are determined.

The future of the CPP field is translational. Unquestionably, it will require a transition to a cargo-centered approach that begins with the identification of a cargo of genuine utility. Then, CPPs with mechanisms appropriate for the delivery of that cargo will be iteratively tested, designed, or engineered to deliver it. Finally, mechanistic studies will be done on successes and failures, to provide feedback for future CPP selection and design. Although our fundamental knowledge of the sequence–function relationships in CPPs is not yet sufficient for routine application of this approach, it may be sufficient to enable synthetic molecular evolution (i.e., rational, iterative library design, and targeted screening [95]) of CPPs to identify those that can deliver particular cargoes to cells in the laboratory. Ultimately, the far future of the field lies in the delivery of therapeutically useful cargoes targeted specifically to the appropriate cells and tissues in human patients.

Resources
i http://crdd.osdd.net/raghava/cppsite/

References
1. Lin, R. *et al.* (2015) Dual peptide conjugation strategy for improved cellular uptake and mitochondria targeting. *Bioconjug. Chem.* 26, 71–77
2. Räägel, H. *et al.* (2013) Cell-penetrating peptide secures an efficient endosomal escape of an intact cargo upon a brief photo-induction. *Cell. Mol. Life Sci.* 70, 4825–4839
3. Appelbaum, J.S. *et al.* (2012) Arginine topology controls escape of minimally cationic proteins from early endosomes to the cytoplasm. *Chem. Biol.* 19, 819–830
4. Rydberg, H. *et al.* (2012) Effects of tryptophan content and backbone spacing on the uptake efficiency of cell-penetrating peptides. *Biochemistry* 51, 5531–5539
5. Qian, Z. *et al.* (2014) Early endosomal escape of a cyclic cell-penetrating peptide allows effective cytosolic cargo delivery. *Biochemistry* 53, 4034–4046
6. LaRochelle, J.R. *et al.* (2015) Fluorescence correlation spectroscopy reveals highly efficient cytosolic delivery of certain Penta-Arg proteins and stapled peptides. *J. Am. Chem. Soc.* 137, 2536–2541
7. Herce, H.D. *et al.* (2009) Arginine-rich peptides destabilize the plasma membrane, consistent with a pore formation translocation mechanism of cell-penetrating peptides. *Biophys. J.* 97, 1917–1925
8. Marks, J.R. *et al.* (2011) Spontaneous membrane-translocating peptides by orthogonal high-throughput screening. *J. Am. Chem. Soc.* 133, 8995–9004
9. Mukherjee, S. *et al.* (1997) Endocytosis. *Physiol. Rev.* 77, 759–803
10. Mayor, S. *et al.* (2014) Clathrin-independent pathways of endocytosis. *Cold Spring Harb. Perspect. Biol.* 6, a016758
11. Gruenberg, J. and van der Goot, F.G. (2006) Mechanisms of pathogen entry through the endosomal compartments. *Nat. Rev. Mol. Cell Biol.* 7, 495–504
12. Kaplan, I.M. *et al.* (2005) Cationic TAT peptide transduction domain enters cells by macropinocytosis. *J. Control. Release* 102, 247–253
13. Fittipaldi, A. *et al.* (2003) Cell membrane lipid rafts mediate caveolar endocytosis of HIV-1 Tat fusion proteins. *J. Biol. Chem.* 278, 34141–34149
14. Duchardt, F. *et al.* (2007) A comprehensive model for the cellular uptake of cationic cell-penetrating peptides. *Traffic* 8, 848–866

Outstanding Questions

Is there a mechanistic class of CPP, such as SMTPs, that is most suitable for systemic delivery of small molecule drugs? Can we test this idea by creating a database of bioactive small molecules that have failed as drugs resulting from poor cell entry?

Is there a mechanistic class of CPP, such as endosome lytic peptides, that is most suitable for the delivery of proteins and other macromolecules to cells in culture? Can the routine delivery of macromolecules be achieved at low peptide concentration?

How can we best assess the constraints on cargo that can be delivered by particular CPPs? Can we establish 'gold standards' throughout the field of CPP research to uniformly assess the efficiency and mechanism of cell entry?

How can we improve synthetic model systems to better mimic the complex environment of the cell?

How can we improve computational approaches to predict the mechanism of CPP entry?

How might we use knowledge gained from mechanistic studies of CPPs to overcome other biological barriers such as the blood–brain barrier or the placental barrier?

How can we rationally design CPPs to enter specific cell types and tissues for targeted therapies?

Trends in Biochemical Sciences

CellPress

15. Maiolo, J.R. *et al.* (2005) Effects of cargo molecules on the cellular uptake of arginine-rich cell-penetrating peptides. *Biochim. Biophys. Acta Biomembr.* 1712, 161–172

16. Thoran, P.E.G. *et al.* (2004) Membrane binding and translocation of cell-penetrating peptides. *Biochemistry* 43, 3471–3489

17. Vives, E. (2003) Cellular uptake of the Tat peptide: an endocytosis mechanism following ionic interactions. *J. Mol. Recognit.* 16, 265–271

18. Chen, C.J. *et al.* (2015) A heparan sulfate-binding cell penetrating peptide for tumor targeting and migration inhibition. *Biomed. Res. Int.* 2015, 237969

19. Tyagi, M. *et al.* (2001) Internalization of HIV-1 tat requires cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* 276, 3254–3261

20. Zollmann, T. *et al.* (2015) Single liposome analysis of peptide translocation by the ABC transporter TAPL. *Proc. Natl. Acad. Sci. U.S.A.* 112, 2046–2051

21. Behr, J.P. (1997) The proton sponge: a trick to enter cells the viruses did not exploit. *CHIMIA Int. J. Chem.* 51, 34–36

22. Gautam, A. *et al.* (2012) CPPsite: a curated database of cell penetrating peptides. *Database (Oxford)* 2012, bas015

23. Nielsen, P.E. and Shiraishi, T. (2011) Peptide nucleic acid (PNA) cell penetrating peptide (CPP) conjugates as carriers for cellular delivery of antisense oligomers. *Artific. DNA PNA XNA* 2, 90–99

24. Wimley, W.C. and White, S.H. (1996) Experimentally determined hydrophobicity scale for proteins at membrane interfaces. *Nat. Struct. Biol.* 3, 842–848

25. Almeida, P.F. and Pokorny, A. (2009) Mechanisms of antimicrobial, cytolytic, and cell-penetrating peptides: from kinetics to thermodynamics. *Biochemistry* 48, 8083–8093

26. White, S.H. and Wimley, W.C. (1999) Membrane protein folding and stability: physical principles. *Annu. Rev. Biophys. Biomol. Struct.* 28, 319–365

27. Green, M. *et al.* (1989) Mutational analysis of HIV-1 Tat minimal domain peptides: identification of trans-dominant mutants that suppress HIV-LTR-driven gene expression. *Cell* 58, 215–223

28. Vives, E. *et al.* (1997) A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J. Biol. Chem.* 272, 16010–16017

29. Richard, J.P. *et al.* (2003) Cell-penetrating peptides: a reevaluation of the mechanism of cellular uptake. *J. Biol. Chem.* 278, 585–590

30. Mitchell, D.J. *et al.* (2000) Polyarginine enters cells more efficiently than other polycationic homopolymers. *J. Pept. Res.* 56, 318–325

31. Tunnemann, G. *et al.* (2008) Live-cell analysis of cell penetration ability and toxicity of oligo-arginines. *J. Pept. Sci.* 14, 469–476

32. Rothbard, J.B. *et al.* (2002) Arginine-rich molecular transporters for drug delivery: role of backbone spacing in cellular uptake. *J. Med. Chem.* 45, 3612–3618

33. He, J. *et al.* (2013) Direct cytosolic delivery of polar cargo to cells by spontaneous membrane-translocating peptides. *J. Biol. Chem.* 288, 29974–29986

34. Melikov, K. *et al.* (2015) Efficient entry of cell-penetrating peptide nona-arginine into adherent cells involves a transient increase in intracellular calcium. *Biochem. J.* 471, 221–230

35. Derossi, D. *et al.* (1994) The third helix of the Antennapedia homeodomain translocates through biological membranes. *J. Biol. Chem.* 269, 10444–10450

36. Dupont, E. *et al.* (2011) Penetratin story: an overview. *Methods Mol. Biol.* 683, 21–29

37. Jiao, C.Y. *et al.* (2009) Translocation and endocytosis for cell-penetrating peptide internalization. *J. Biol. Chem.* 284, 33957–33965

38. Alves, I.D. *et al.* (2011) Relationships between membrane binding, affinity and cell internalization efficacy of a cell-penetrating peptide: penetratin as a case study. *PLoS ONE* 6, e24096

39. Bechara, C.R. *et al.* (2014) Massive glycosaminoglycan-dependent entry of Trp-containing cell-penetrating peptides induced by exogenous sphingomyelinase or cholesterol depletion. *Cell. Mol. Life Sci.* 72, 809–820

40. Pooga, M. *et al.* (1998) Cell penetration by transportan. *FASEB J.* 12, 67–77

41. Hällbrink, M. *et al.* (2001) Cargo delivery kinetics of cell-penetrating peptides. *Biochim. Biophys. Acta Biomembr.* 1515, 101–109

42. Chugh, A. and Eudes, F. (2008) Cellular uptake of cell-penetrating peptides pVEC and transportan in plants. *J. Pept. Sci.* 14, 477–481

43. Vasconcelos, L. *et al.* (2014) Effects of cargo molecules on membrane perturbation caused by transportan 10 based cell-penetrating peptides. *Biochim. Biophys. Acta Biomembr.* 1838, 3118–3129

44. Wimley, W.C. (2010) Describing the mechanism of antimicrobial peptide action with the interfacial activity model. *ACS Chem. Biol.* 5, 905–917

45. Walrant, A. *et al.* (2012) Membrane interactions of two arginine-rich peptides with different cell internalization capacities. *Biochim. Biophys. Acta Biomembr.* 1818, 1755–1763

46. Madani, F. *et al.* (2013) Modeling the endosomal escape of cell-penetrating peptides using a transmembrane pH gradient. *Biochim. Biophys. Acta Biomembr.* 1828, 1198–1204

47. Joanne, P. *et al.* (2009) Lipid reorganization induced by membrane-active peptides probed using differential scanning calorimetry. *Biochim. Biophys. Acta Biomembr.* 1788, 1772–1781

48. Witte, K. *et al.* (2013) Structure and dynamics of the two amphipathic arginine-rich peptides RW9 and RL9 in a lipid environment investigated by solid-state NMR and MD simulations. *Biochim. Biophys. Acta Biomembr.* 1828, 824–833

49. Yandek, L.E. *et al.* (2008) Small changes in the primary structure of transportan 10 alter the thermodynamics and kinetics of its interaction with phospholipid vesicles. *Biochemistry* 47, 3051–3060

50. Jobin, M.L. *et al.* (2013) The enhanced membrane interaction and perturbation of a cell penetrating peptide in the presence of anionic lipids: toward an understanding of its selectivity for cancer cells. *Biochim. Biophys. Acta Biomembr.* 1828, 1457–1470

51. Walrant, A. *et al.* (2013) Direct translocation of cell-penetrating peptides in liposomes: a combined mass spectrometry quantification and fluorescence detection study. *Anal. Biochem.* 438, 1–10

52. Di Pisa, M. *et al.* (2015) Translocation mechanism(s) of cell-penetrating peptides: biophysical studies using artificial membrane bilayers. *Biochemistry* 54, 194–207

53. Choi, D. *et al.* (2012) Insertion mechanism of cell-penetrating peptides into supported phospholipid membranes revealed by X-ray and neutron reflection. *Soft Matter* 8, 8294

54. Maniti, O. *et al.* (2014) Basic cell penetrating peptides induce plasma membrane positive curvature, lipid domain separation and protein redistribution. *Int. J. Biochem. Cell Biol.* 50, 73–81

55. Schmidt, N. *et al.* (2010) Arginine-rich cell-penetrating peptides. *FEBS Lett.* 584, 1806–1813

56. Swiecicki, J.M. *et al.* (2014) The efficacies of cell-penetrating peptides in accumulating in large unilamellar vesicles depend on their ability to form inverted micelles. *ChemBioChem* 15, 884–891

57. Lamaziere, A. *et al.* (2010) Lipid domain separation, bilayer thickening and pearling induced by the cell penetrating peptide penetratin. *Biochim. Biophys. Acta Biomembr.* 1798, 2223–2230

58. Kwon, B. *et al.* (2013) A 2H solid-state NMR study of lipid clustering by cationic antimicrobial and cell-penetrating peptides in model bacterial membranes. *Biophys. J.* 105, 2333–2342

59. Wadhwani, P. *et al.* (2012) Antimicrobial and cell-penetrating peptides induce lipid vesicle fusion by folding and aggregation. *Eur. Biophys. J.* 41, 177–187

60. Bahnsen, J.S.B. *et al.* (2013) Antimicrobial and cell-penetrating properties of penetratin analogs: effect of sequence and secondary structure. *Biochim. Biophys. Acta Biomembr.* 1828, 223–232

61. Lee, C.C. *et al.* (2010) Membrane-mediated peptide conformation change from alpha-monomers to beta-aggregates. *Biophys. J.* 98, 2236–2245

62. McKeown, A.N. *et al.* (2011) A thermodynamic approach to the mechanism of cell-penetrating peptides in model membranes. *Biochemistry* 50, 654–662

63. Dunkin, C.M. *et al.* (2011) Molecular dynamics studies of transportan 10 (Tp10) interacting with a POPC lipid bilayer. *J. Phys. Chem. B* 115, 1188–1198

64. Fanghanel, S. *et al.* (2014) Structure analysis and conformational transitions of the cell penetrating peptide transportan 10 in the membrane-bound state. *PLoS ONE* 9, e99653

65. Wheaten, S. *et al.* (2013) Translocation of cationic amphipathic peptides across the membranes of pure phospholipid giant vesicles. *J. Am. Chem. Soc.* 135, 16517–16525

66. Cruz, J. *et al.* (2013) A membrane translocating peptide penetrates into bilayers without significant bilayer perturbations. *Biophys. J.* 104, 2419–2428

67. Wimley, W.C. and Hristova, K. (2011) Antimicrobial peptides: successes, challenges and unanswered questions. *J. Membr. Biol.* 239, 27–34

68. Gautam, A. *et al.* (2013) In silico approaches for designing highly effective cell penetrating peptides. *J. Transl. Med.* 11, 74

69. Kondo, E. *et al.* (2012) Tumour lineage-homing cell-penetrating peptides as anticancer molecular delivery systems. *Nat. Commun.* 3, 951

70. Kamide, K. *et al.* (2010) Isolation of novel cell-penetrating peptides from a random peptide library using in vitro virus and their modifications. *Int. J. Mol. Med.* 25, 41–51

71. Swiecicki, J.M. *et al.* (2015) Accumulation of cell-penetrating peptides in large unilamellar vesicles – a straightforward screening assay for investigating the internalization mechanism. *Biopolymers* 104, 533–543

72. He, J. *et al.* (2012) A highly charged voltage sensor helix translocates spontaneously across membranes. *Angew. Chem. Int. Ed.* 51, 7150–7153

73. Pae, J. *et al.* (2014) Translocation of cell-penetrating peptides across the plasma membrane is controlled by cholesterol and microenvironment created by membranous proteins. *J. Control. Release* 192, 103–113

74. Säälik, P. *et al.* (2011) Penetration without cells: membrane translocation of cell-penetrating peptides in the model giant plasma membrane vesicles. *J. Control. Release* 153, 117–125

75. Amand, H.L. *et al.* (2011) Binding of cell-penetrating penetratin peptides to plasma membrane vesicles correlates directly with cellular uptake. *Biochim. Biophys. Acta Biomembr.* 1808, 1860–1867

76. Jiang, Y. *et al.* (2003) The principle of gating charge movement in a voltage-dependent K$^+$ channel. *Nature (London)* 423, 42–48

77. Hristova, K. and Wimley, W.C. (2010) A look at arginine in membranes. *J. Membr. Biol.* 239, 49–56

78. Freites, J.A. *et al.* (2006) A voltage-sensor water pore. *Biophys. J.* 91, L90–L92

79. Su, Y. *et al.* (2010) Membrane-bound dynamic structure of an arginine-rich cell-penetrating peptide, the protein transduction domain of HIV tat, from solid-state NMR. *Biochemistry* 49, 6009–6020

80. Rothbard, J.B. *et al.* (2005) Adaptive translocation: the role of hydrogen bonding and membrane potential in the uptake of guanidinium-rich transporters into cells. *Adv. Drug Deliv. Rev.* 57, 495–504

81. Freites, J.A. *et al.* (2005) Interface connections of a transmembrane voltage sensor. *Proc. Natl. Acad. Sci. U.S.A.* 102, 15059–15064

82. Herce, H.D. *et al.* (2014) Fundamental molecular mechanism for the cellular uptake of guanidinium-rich molecules. *J. Am. Chem. Soc.* 136, 17459–17467

83. Palm-Apergi, C. *et al.* (2009) The membrane repair response masks membrane disturbances caused by cell-penetrating peptide uptake. *FASEB J.* 23, 214–223

84. Yang, S.T. *et al.* (2010) Cell-penetrating peptide induces leaky fusion of liposomes containing late endosome-specific anionic lipid. *Biophys. J.* 99, 2525–2533

85. Yang, S.T. *et al.* (2010) Fusion between intraluminal vesicles of late endosomes as a possible mechanism of endosomal escape by cell-penetrating peptides. *Biophys. J.* 98, 672A

86. Palm, C. *et al.* (2007) Peptide degradation is a critical determinant for cell-penetrating peptide uptake. *Biochim. Biophys. Acta Biomembr.* 1768, 1769–1776

87. Lee, Y.J. *et al.* (2008) Real-time fluorescence detection of protein transduction into live cells. *J. Am. Chem. Soc.* 130, 2398–2399

88. Sagan, S. *et al.* (2015) Study of CPP mechanism by mass spectrometry. *Methods Mol. Biol.* 1324, 107–111

89. Tunnemann, G. *et al.* (2006) Cargo-dependent mode of uptake and bioavailability of TAT-containing proteins and peptides in living cells. *FASEB J.* 20, 1775–1784

90. Hällbrink, M. *et al.* (2004) Uptake of cell-penetrating peptides is dependent on peptide-to-cell ratio rather than on peptide concentration. *Biochim. Biophys. Acta* 1667, 222–228

91. Nischan, N. *et al.* (2015) Covalent attachment of cyclic TAT peptides to GFP results in protein delivery into live cells with immediate bioavailability. *Angew. Chem. Int. Ed. Engl.* 54, 1950–1953

92. Hopkins, A.L. and Groom, C.R. (2002) The druggable genome. *Nat. Rev. Drug Discov.* 1, 727–730

93. Craik, D.J. *et al.* (2013) The future of peptide-based drugs. *Chem. Biol. Drug Des.* 81, 136–147

94. Lee, J. *et al.* (2015) Experiences with CPP-based self assembling peptide systems for topical delivery of Botulinum toxin. *Methods Mol. Biol.* 1324, 397–415

95. Krauson, A.J. *et al.* (2013) Synthetic molecular evolution of pore-forming peptides by iterative combinatorial library screening. *ACS Chem. Biol.* 8, 823–831

96. Snider, C. *et al.* (2009) MPEx: a tool for exploring membrane proteins. *Protein Sci.* 18, 2624–2628

97. Holub, J.M. *et al.* (2013) Improved assays for determining the cytosolic access of peptides, proteins, and their mimetics. *Biochemistry* 52, 9036–9046