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Review

Cell biology meets biophysics to unveil the different mechanisms of penetratin internalization in cells

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ABSTRACT

Although cell-penetrating peptides are widely used as molecular devices to cross membranes and transport molecules or nanoparticles inside cells, the underlying internalization mechanism for such behavior is still studied and discussed. One of the reasons for such a debate is the wide panel of chemically different cell-penetrating peptides or cargo that is used. Indeed the intrinsic physico-chemical properties of CPP and conjugates strongly affect the cell membrane recognition and therefore the internalization pathways. Altogether, the mechanisms described so far should be shared between two general pathways: endocytosis and direct translocation. As it is established now that one cell-penetrating peptide can internalize at the same time by these two different pathways, the balance between the two pathways relies on the binding of the cell-penetrating peptide or conjugate to specific cell membrane components (carbohydrates, lipids). Like endocytosis which includes clathrin- and caveolae-dependent processes and macropinocytosis, different translocation mechanisms could co-exist, an idea that emerges from recent studies. In this review, we will focus solely on penetratin membrane interactions and internalization mechanisms.

Contents

Abbreviations: Bzp, benzophenone; CHO, Chinese hamster ovary cells; CPP, cell-penetrating peptide; DHPC, dihexanoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleylphosphatidylcholine; DSPG, distearoylphosphatidylglycerol; ESR, electron spin resonance; GAG, glycosaminoglycans; GUV, giant unilamellar vesicle; LUV, large unilamellar vesicle; MDS, molecular dynamics simulation; MLV, multilamellar vesicle; NMR, nuclear magnetic resonance; POPG, palmitoylphosphatidylglycerol; DSC, differential scanning calorimetry; MALDI MS, matrix-assisted laser desorption/ionization mass spectrometry; PWR, plasmon waveguide resonance; SAXS, small angle X-ray scattering; SM, sphingomyelin; SPR, surface plasmon resonance; SUV, small unilamellar vesicle

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1. Introduction

Cell-penetrating peptides have been widely used to transport various types of cargoes inside cells for biological or therapeutical applications [1–3]. Additionally, some CPPs including penetratin have been shown to possess antimicrobial activity [4–6].

Transported cargoes are of wide size (peptide and antibody) and with various physico-chemical profiles (oligonucleotide, protein and nanoparticles).

The cargo is usually covalently conjugated to a cell-penetrating peptide, often through a disulfide bridge in the case of protein/peptide but, non-covalent strategies are now greatly expanding [1,7]. Once inside cells, non-covalent CPP–cargo conjugates can dissociate from each other and CPP–cargo disulfide conjugates are separated in the reducing cytosol medium. Thus, once inside the cell, the cargo is theoretically free to interact with intracellular targets to evoke/induce biological activity. However, one crucial point in the field which remains is the question of the final localization of the cell-penetrating peptide and the cargo. The intracellular cell-penetrating or cargo localization (obtained from fluorescence imaging) without any associated biological activity often led to conflicting data and possible misinterpretations regarding the internalization mechanisms. For example, it has been shown in hippocampal neurons that (8–80 nM) penetratin–SiRNA–FITC conjugates internalized rapidly and could be found essentially as extensive punctate cytoplasmic fluorescence, suggesting the presence of the SiRNA in vesicles, while a cytosolic SiRNA activity could be measured [8].

Therefore, many strategies have emerged now to favor the escape of CPP–cargo conjugates from endosomal entrapping. Among those, the use of fusogenic peptides containing acidic residues [9], acidification inhibitors [10], and direct [11] or indirect photosensitizers [12] has been described as efficient methods to release a cargo from endosomal vesicles.

One consensus point in the CPP field is that the physico-chemical properties of the cargo [13,14], and the cell-penetrating peptide [15] have an impact in the intracellular delivery pathways of the conjugate. Therefore, it is obvious that the internalization pathways and the final localization of conjugates within cells can hardly be anticipated.

In that regard, unveiling all possible internalization pathways of CPP–cargo conjugate is important to further rationalize the physico-chemical parameters determinant for the control of the final intracellular localization of conjugates. Thus, the aim of the review is to summarize studies on that subject using biophysics, biochemistry and cell biology approaches. We shall focus more particularly on penetratin derived from the homeoprotein Antennapedia [16] which has been extensively studied.

Using mass spectrometry to quantify internalized peptides [17,18] it was possible to study the internalization pathways of penetratin and other cell-penetrating peptides. Briefly, the method relies on the quantification of the internalized (^{1}H) cell-penetrating peptide with a known amount of the same peptide isotopically-labeled (^{2}H) used as an internal standard. Indeed, using the ion signal intensity directly does not make mass spectrometry quantitative. Thus, the two peptides will be chemically equivalent but separated on the mass spectra by the difference between the number of deuterium and hydrogen in the two peptides. The absolute quantification of the internalized ^{1}H-peptide is determined from the areas of the ^{1}H-peptide and ^{2}H-peptide [M+H]+ signals. Using this method, we have previously shown that internalized and membrane-bound peptides could be distinguished [17,19].

Therefore in the first part, recent *in cellulo* studies of the internalization pathways of penetratin according to four important parameters: incubation time, temperature, peptide concentration, membrane potential and membrane affinity will be summarized. In the second part, we will focus on membrane model systems used to examine the specific phospholipids interaction with penetratin, a prerequisite for internalization through direct translocation. A summarized list of biochemical and biophysical methods used to learn about penetratin mode of action, and discussed herein, are presented in Table 1.

2. Penetratin internalization pathways: where do we stand?

2.1. Kinetics of internalization

Very few studies have reported kinetics of internalization at 37 °C and even less at 4 °C. It should be noticed that in all these studies CPPs linked to a cargo rather than free CPPs were used. Indeed, the cell-

Table 1
Summary of methods/techniques and the corresponding information obtained to learn about the mode of action of CPPs.

Method/technique	Information provided	References[a]
Circular dichroism	Secondary peptide structure	[23,49–51]
Infra red	Secondary peptide structure	[52,53]
NMR (solution and solid)	Effect of the peptide on lipid supramolecular organization, positioning and depth of the peptide relative to the membrane, peptide 3D structure	[54–57,60,65,68,69]
Trp fluorescence	Depth of peptide insertion into lipids	[58,59]
Differential scanning calorimetry (DSC)	Effect of the peptide on lipid phase transitions (temperature phase transition, cooperativity, etc)	[51,55–79]
Isothermal titration calorimetry (ITC)	Affinity between the peptide and lipids/sugars	[48,63]
Electron spin resonance (ESR)	Interaction of peptide with cell membrane components at the molecular level	[62]
Plasmon waveguide resonance (PWR)	Binding affinity, effect of peptide on lipid organization (mass and structural changes, anisotropy, bilayer thickness)	[54,69,73,74]
Turbidity/dynamic light scattering	Effect of peptide on liposome organization (precipitation, fusion); average size of objects	[51,52,70]
Videomicroscopy	Effect of peptide on lipid organization at the macroscopic level (GUV)	[68,70]
Confocal microscopy	Distribution of the peptide in cells	[29,59]
X-ray scattering	Effect of peptide on bilayer thickness, organization (lipid phases)	[68,70]
Molecular dynamics simulations (MDS)	Snapshots of the peptide and lipid organization at molecular level	[71,72]
Electron microscopy	Membrane structures induced by the peptide and peptide distribution in cells	[80]
Flow cytometry	Amount of the cellular association of the peptide	[59,80]
Radioactivity	Indirect quantitative measure of cargo or CPP internalization	[30,81]
Mass spectrometry	Quantitative measure of intracellular or membrane cargo or CPP. In cell CPP proteolysis study	[17,39,82,83]

This is a summarized list of methods that have been used for penetratin studies. Therefore the list is not exhaustive, alternative methods have been used for other cell-penetrating peptides.

[a] The list of reference is not exhaustive, few examples are provided to illustrate, most of them refer to penetratin studies.