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The role of tryptophans on the cellular uptake and membrane interaction of arginine-rich cell penetrating peptides

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ABSTRACT

Cell-penetrating peptides (CPP) are able to efficiently transport cargos across cell membranes without being cytotoxic to cells, thus present a great potential in drug delivery and diagnosis. While the role of cationic residues in CPPs has been well studied, that of Trp is still not clear. Herein 7 peptide analogs of RW9 (RRWWRRWRR, an efficient CPP) were synthesized in which Trp were systematically replaced by Phe residues. Quantification of cellular uptake reveals that substitution of Trp by Phe strongly reduces the internalization of all peptides despite the fact that they strongly accumulate in the cell membrane. Cellular internalization and biophysical studies show that not only the number of Trp residues but also their positioning in the helix and the size of the hydrophobic face they form are important for their internalization efficacy, the highest uptake occurring for the analog with 3 Trp residues. Using CD and ATR-FTIR spectroscopy we observe that all peptides became structured in contact with lipids, mainly in α-helix. Intrinsic tryptophan fluorescence studies indicate that all peptides partition in the membrane in about the same manner (Kp ~ 10^5) and that they are located just below the lipid headgroups (~10 Å) with slightly different insertion depths for the different analogs. Plasmon Waveguide Resonance studies reveal a direct correlation between the number of Trp residues and the reversibility of the interaction following membrane washing. Thus a more interfacial location of the CPP renders the interaction with the membrane more adjustable and transitory enhancing its internalization ability.

1. Introduction

One of the major obstacles to the use of large therapeutic molecules or imaging agents having intracellular targets is their low permeability through biomembranes. One of the breakthroughs of the last 20 years is the discovery of cell-penetrating peptides (CPP) as molecules capable of internalizing into cells in a receptor- and energy-independent way and without being toxic to cells. Their great potential relies on the fact that they can transport a great variety of cargoes into cells both in terms of size and nature and some CPPs are even already used as drug delivery vector (for a review, see [1]). Green & Loewenstein and Frankel & Pabo discovered the first CPP almost simultaneously in 1988 [2,3]. They found that the Tat protein from HIV-1 was internalized into cells and Vives et al., found in 1997 the minimal sequence that was responsible for the protein internalization [4]. Following this finding, penetratin was discovered in 1994 by the group of Alain Prochiantz [5]. This is a peptide derived from the homeodomain of the Drosophila homeobox *Antennapedia* and was shown to possess a good internalization efficacy. Since then a large number of Structure/Activity Relationship studies have been performed both to study their membrane-translocating capabilities and to design novel sequences with greater efficacy and better selectivity. Since the Tat peptide possesses a large number of basic residues (6 Arg and 2 Lys on 13 residues) Wender and Rothbard discovered that a polyarginine comporting 9 residues is an efficient CPP [6]. Futaki's group then synthesized oligoarginines of different lengths (R_n with $6 < n < 12$) and studied their internalization efficiency [7]. They could determine that 8 Arg residues were sufficient to confer the polyarginine cell penetrating properties. Different derivatives of penetratin have also been synthetized and it was observed that the internalization was based neither on the chirality of the peptide, nor its amphiphilicity or its secondary structure [5,8]. Regarding the mechanisms implicated in their cellular internalization, it has been generally accepted that both endocytosis and direct translocation through the membrane are implicated in their uptake. The balance between the two mechanisms depends on a great variety of aspects

Abbreviations: AMP, Anti-microbial Peptide; ATR-FTIR, Attenuated Total Reflectance-Fourier Transform Infrared; CPP, cell-penetrating peptide; CD, Circular dichroism; CHO, Chinese hamster ovary; DOPC, Dioleoylphosphatidylcholine; DOPG, Dioleoylphosphatidylglycerol; DLS, Dynamic light scattering; GAG, Glycosaminoglycan; HSPG, Heparan sulfate proteoglycans; LUV, Large unilamellar vesicle; MALDI, Matrix-Assisted Laser Desorption/Ionization; MLV, Multi lamellar vesicle; MS, Mass spectrometry; PC, Phosphatidylcholine; PG, Phosphatidylglycerol; PWR, Plasmon Waveguide Resonance; SAR, Structure/Activity Relationship; SUV, Small Unilamellar Vesicles

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such as the nature and size of the CPP and its cargo, the nature of the link between the two, the temperature at which internalization experiments are conducted, the cell lines used, among other parameters [9–12]. Electrostatic interactions between the positive charges in the peptide and negative charges in the cell membrane surface have been shown to be essential during the first stage of interaction with the membrane. The presence of basic amino acids in the sequence has been well studied and Arg residues have been reported to be especially important for cellular internalization. It was shown that the uptake efficiency is attributed to the type of bond formed between Arg and the lipid headgroups rather than the charges presented to the membrane. Indeed Arg can form bidentate hydrogen bonds that interact simultaneously with phosphate moieties on multiple lipid headgroups while Lys residues can only form monodentate hydrogen bonds that interact with the phosphate moiety on a single headgroup [13,14]. Guanidinium-rich peptides also establish strong electrostatic interactions with negatively charged heparan sulfate proteoglycans (HSPG) on the cell surface, important as a first recognition and their accumulation in the membrane [15,16]. The presence of hydrophobic residues for internalization has also been investigated. The substitution of the Trp^{48} and Trp^{56} by Phe abolished totally the internalization of penetratin [17]. From these studies, the Arg-rich CPP RW9 (RRWWRRWRR) was designed and determined to possess very high cellular uptake efficiency [18]. Biological studies on RW9 have revealed an important decrease in internalization in GAG-deficient cells evidencing that proteoglycans at the membrane surface are important for its cellular internalization [19]. Biophysical studies have shown its preferential interaction with anionic model membranes corroborating biological studies on the importance of electrostatic interactions between peptide and membranes. Previous studies on RL9 (RRLLRRLRR), an analog of RW9 where Trp residues were replaced by Leu residues have shown that this peptide was not internalized into cells despite the fact that it accumulated in the membrane [19,20]. At the same time, oligoarginine peptide (R_9) possessing no aromatic residues internalizes very well in eukaryotic cells at approximately the same level as RW9 [19]. The main question addressed here is to understand the role of the Trp in membrane translocation regarding the RW9 sequence. Therefore, we synthesized 7 peptides in which Phe systematically replaced Trp residues (RX9) (Table 1). The strategy was to keep the hydrophobicity but specially the aromaticity of the hydrophobic residues, because previous NMR studies on RW9 evidenced the existence of π–cation interactions between certain Arg and Trp residues [19].

Q4 Quantification of the total amount of internalized peptides (all intracellular compartments included) shows that the replacement of all Trp residues almost completely abolished the peptide internalization while the substitution of 1 or 2 Trp strongly decreases their internalization efficiency. To understand these differences in cellular internalization we have decided to investigate the interaction of these peptides with lipid membranes and therefore shed some light into their membrane crossing and translocation. It should be noted that direct translocation through the cell membrane is just one of the many mechanisms used by CPPs to internalize, nonetheless a good understanding of CPP interaction with lipids is important. For that we have used lipid model systems and different biophysical approaches in an attempt to correlate their cellular uptake and membrane direct translocation with bilayer interaction. Since electrostatic interactions were found to be important for the membrane interaction of Arg-rich peptides with cellular membranes [see [21] for a review] we have included anionic lipids in the model membranes used. Even though anionic lipids are very weakly present in eukaryotic cell membrane, especially in the outer leaflet, the few anionic lipids present (~2%) can have their potential enhanced by assembling into domains, a property reported to be induced by certain CPPs [22]. Although, the outer leaflet of healthy eukaryotic membranes possesses almost no anionic lipids, important electrostatic interactions between the CPPs and GAG can be established. Often biophysicists have employed anionic lipids just to mimic the overall anionic character of the cell membrane surface, which is also our approach here. Additionally it should be noted that during certain cellular dysfunctions such as when cells become tumoral or enter apoptosis, the amount of anionic lipids in their outer leaflet (mostly phosphatidylserine) increases up to 9%, rendering the cellular membrane significantly more anionic [23–26]. CD and ATR-FTIR were used to investigate the secondary structure of the peptides in contact with model membranes to define if there was a correlation between their tendency to adopt a secondary structure in the presence of lipids and their internalization capacities. No direct correlation was found. Their cytotoxicity on cells and effect on model membranes were explored. The replacement of Trp by Phe induced no cytotoxicity or dye leakage although the peptides bind and slightly perturb the membrane. The affinity and insertion depth of the peptides were studied by Plasmon Waveguide Resonance and Trp fluorescence and a correlation with their internalization capacities was established.

t1.1 **Table 1**
t Q2 Amino-acid sequences of the RX9 peptides used in this study.

t1.3		Peptide sequence	MW (Da)	Charges (at pH 7)
t1.4	RW9	Biotin(O_2)-GGGG-RRWWRRWRR-NH_2	1999	6
t1.5	RFFF9	Biotin(O_2)-GGGG-RRFFRRFRR-NH_2	1882	6
t1.6	RFFW9	Biotin(O_2)-GGGG -RRFFRRWRR-NH_2	1921	6
t1.7	RWFF9	Biotin(O_2)-GGGG-RRWFRRFRR-NH_2	1921	6
t1.8	RFWF9	Biotin(O_2)-GGGG-RRFWRRFRR-NH_2	1921	6
t1.9	RFWW9	Biotin(O_2)-GGGG-RRFWRRWRR-NH_2	1960	6
t1.10	RWWF9	Biotin(O_2)-GGGG-RRWWRRFRR-NH_2	1960	6
t1.11	RWFW9	Biotin(O_2)-GGGG-RRWFRRWRR-NH_2	1960	6

2. Materials & methods

2.1. Materials

All lipids were obtained from Avanti Polar Lipids (Alabaster, AL, USA). The calcein and acrylamide were obtained from Sigma Aldrich. Biotin(O_2)-($[^1H]$-G)$_4$-RRFFRRFRR-NH_2(RF_9), Biotin(O_2)-($[^1H]$-G)$_4$-RRFFRRWRR-NH_2($RFFW_9$), Biotin(O_2)-($[^1H]$-G)$_4$-RRWFRRFRR-NH_2($RWFF_9$), Biotin(O_2)-($[^1H]$-G)$_4$-RRFWRRFRR-NH_2 ($RFWF_9$), Biotin(O_2)-($[^1H]$-G)$_4$-RRFWRRWRR-NH_2 ($RFWW_9$), Biotin(O_2)-($[^1H]$-G)$_4$-RRWWRRFRR-NH_2($RWWF_9$), Biotin(O_2)-($[^1H]$-G)$_{44}$-RRWFRRWRR-NH_2($RWFW_9$) and Biotin(O_2)-($[^2H]$-G)$_4$-RRFFRRFRR-NH_2, Biotin(O_2)-($[^2H]$-G)$_4$-RRFFRRWRR-NH_2, Biotin(O_2)-($[^2H]$-G)$_4$-RRWFRRFRR-NH_2, Biotin(O_2)-($[^2H]$-G)$_4$-RRFWRRFRR-NH_2, Biotin(O_2)-($[^2H]$-G)$_4$-RRFWRRWRR-NH_2, Biotin(O_2)-($[^2H]$-G)$_4$-RRWWRRFRR-NH_2, Biotin(O_2)-($[^2H]$-G)$_4$-RRWFRRWRR-NH_2 were synthesized using the Fmoc solid-phase strategy ($[^1H]$-G and $[^2H]$-G correspond to non-deuterated and bi-deuterated glycine, respectively). The oxidation protocol of the biotin was as follows: 10 g of biotin was dissolved in 40 mL of H_2O_2 (30% in H_2O) and 120 mL of AcOH was added. The mixture was stirred at room temperature for few hours and a precipitate was formed. The precipitate was filtered, washed with Et_2O and dried under vacuum. Oxidation efficiency of the biotin was checked by liquid-state NMR. Biotin sulfone was coupled to the peptide under the same conditions used for the amino-acid coupling. Peptides were purified by High Performance Liquid Chromatography (HPLC), in a reverse phase column (RP) C18 using H_2O/CH_3CN/TFA gradient. MALDI-TOF mass spectrometry was used to characterize the peptides. To efficiently remove the TFA counter-ion a simple method was used that consists in lyophilizing the sample 3 times in the presence of 10 mM HCl directly replacing TFA counter-ions with chloride ions [27]. A low concentration of HCl was used to prevent peptide degradation. The removal of TFA was followed by ^{19}F-NMR.

2.2. Cell culture

Wild type Chinese hamster ovary CHO-K1 (WT) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with

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