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Re-evaluating the role of strongly charged sequences in amphipathic cell-penetrating peptides
A fluorescence study using Pep-1

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Abstract Cell-penetrating peptides (CPPs) are able to translocate across biological membranes and deliver bioactive proteins. Cellular uptake and intracellular distribution of CPPs is commonly evaluated with fluorescent labels, which can alter peptide properties. The effect of carboxyfluorescein label in the Lys-rich domain of the amphipathic CPP pep-1, was evaluated and compared with non-labelled pep-1 in vitro and in vivo. A reduced membrane affinity and an endosomal-dependent translocation mechanism, at variance with non-labelled pep-1, were detected. Therefore, the charged domain is not a mere enabler of peptide adsorption but has a crucial role in the translocation pathway of non-labelled pep-1.

Keywords: Cell-penetrating peptide; Drug delivery; Translocation mechanism; Transmembrane potential

1. Introduction

The discovery of various basic peptides (9–33 amino acid residues) with ability to translocate across cell membranes has attracted much interest in biomedical research [1]. These peptides, known as cell-penetrating peptides (CPPs), have been successfully used to deliver biopharmaceutical macromolecules in vivo. The use of such delivery systems is of great interest to evade the poor cellular access and bioavailability of drugs [2].

The translocation mechanism used by this group of carriers has been extensively studied in the last 10 years, but the results are frequently contradictory and disperse. A single general mechanism for all does not seem reasonable and more than one mechanism for a unique peptide is a possibility [3,4]. To evaluate the cellular uptake and intracellular distribution of CPP, fluorescent labels are commonly used. However, when these labels are large and lipophilic they may alter physicochemical properties and the cellular distribution of the peptide [5].

Pep-1 (Ac-KETWWETWWTEWSQPKKKRKV-cysteamine) is a synthetic peptide carrier forming physical assemblies with a great variety of proteins and other macromolecules, which have been successfully translocated in different cell lines [6–10]. It has been shown that pep-1 translocates across membranes by a physical process mediated by transmembrane potential, both in vitro and in vivo, in free form [11] or when complexed with a protein, with no evidence for an alternative mechanism [7]. Because the translocation is solely physically mediated, the hydrophobicity and charge distribution of the peptide and its interaction with membranes is of first importance. The amphipathicity of the carrier is probably responsible by the strong interaction with the lipidic membranes [12]. Unlike other cationic CPPs (e.g., penetratin) [13] pep-1 has a high affinity for neutral vesicles and for membranes in gel-like phase. The presence of cysteamine group in C terminal seems to play a crucial role in the delivery efficiency of cargoes into cells [12].

In the present paper, the study of a modified peptide with a carboxyfluorescein probe (pep-1CF) is compared with pep-1. In this peptide, the hydrophilic domain (KKKRKV-cysteamine) has been customized to accommodate the label. An extra Lys was introduced to link the probe and C terminal is blocked with a Ser instead of cysteamine to avoid steric constraints (KKRKVK(CF)-S). The effect of the alteration introduced in this hydrophilic domain in the translocation mechanism is presented, using both the Trp residues and CF moiety as reporters of the so-called hydrophobic and hydrophilic domains, respectively.

2. Materials and methods

Pep-1 and Pep-1CF with purity >95% were obtained from *GenScript Corporation*, New Jersey. β-Galactosidase from *Escherichia coli* (β-Gal), 4-methylumbelliferyl-galactoside (MUG), Triton X-100 (TX-100) and trypan blue (TB) were obtained from Sigma–Aldrich, MO. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-(phospho-rac-(1-glycerol)) (POPG), were from Avanti Polar-Lipids, Alabama. 5-Doxyl-stearic acid (5DS) and 16-doxyl-stearic acid (16DS) from Aldrich Chem Co., WI. Minimum essential medium Eagles with Earle's salts (MEME) and supplements were obtained from Gibco Invitrogen Corporation, CA.

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Abbreviations: CPP, cell-penetrating peptide; CF, carboxyfluorescein; β-Gal, β-galactosidase; MUG, 4-methylumbelliferyl-galactoside; TX-100, Triton X-100; TB, trypan blue; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-(phospho-rac-(1-glycerol)); 5DS, 5-doxyl-stearic acid; 16DS, 16-doxyl-stearic acid; MEME, minimum essential medium Eagles; LUVs, large unilamellar vesicles; 4-MU, 4-methylumbelliferone

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2.1. Characterization of pep-1CF in aqueous solution

Pep-1CF solutions were prepared in HEPES buffer (10 mM HEPES, pH 7.4 containing 10 mM (low ionic strength) or 150 mM NaCl (the so-called physiologic ionic strength)). The assays were performed at room temperature in a UV–Vis spectrophometer Jasco V-560 and in a spectrofluorometer SLM Aminco 8100. Fluorescence intensity values were corrected for inner filter effect [14].

The absorption and fluorescence emission characteristics of the hydrophobic domain (by means of Trp fluorescence; $\lambda_{exc} = 280$ nm, $\lambda_{em} = 350$ nm) and of the hydrophilic domain (by CF group; $\lambda_{exc} = 490$ nm, $\lambda_{em} = 520$ nm), were studied. Quantum yield-dependence on peptide concentration (0–18 μM) and ionic strength (by means of NaCl concentration variation) were also evaluated. Fluorescence quenching of Trp residues by acrylamide was carried out using $\lambda_{exc} = 290$ nm to minimize the relative quencher/fluorophore light absorption ratio. Quenching data were corrected as described in [12].

2.2. Interaction of pep-1CF with model membranes

Large unilamellar vesicles (LUVs), with typical 100 nm diameter were prepared by the extrusion method described elsewhere [15] and used as model of biological membranes. Liquid-crystal phase vesicles composed by POPC or POPC:POPG (4:1 molar) were used to model the outer leaflet (neutral) and the inner leaflet (negatively charged) of mammals' biological membranes [16]. The extent and kinetics of partition and the in-depth location of pep-1CF were evaluated using the same procedure as for pep-1 [12]. Both hydrophobic and hydrophilic domains were studied.

The $DiSBAC_2(3)$ dye is a probe with a higher affinity for depolarized than polarized membranes and its apparent quantum yield being dependent on the extent of interaction with membranes [17]. These fluorescence properties were used to evaluate alterations in transmembrane potential of model membranes upon addition of pep-1CF. Vesicles in the absence and presence of a negative transmembrane potential were prepared as described in [11]; $DiSBAC_2(3)$ was added to lipidic suspension to a final concentration of 0.25 mM and fluorescence emission intensity ($\lambda_{exc} = 540$ nm, $\lambda_{em} = 558$ nm) was followed during titration of lipidic suspension with pep-1CF (576 μM stock solution) or non-labelled pep-1 (688 μM stock solution).

2.3. Translocation studies of pep-1CF in HeLa cells

Adherent human negroid cervix epitheloid carcinoma cells (HeLa) were grown in MEME supplemented with 2 mM L-Glu, 2 mM non-essential aminoacids, 10% (v/v) fetal bovine serum and 1% (v/v) streptomycin and penicillin, in a 5% CO_2 humidified atmosphere at 37 °C. Cell viability was determined by the TB exclusion assay, see [7].

The translocation of pep-1CF and it capacity to mediate the uptake of β-Gal were followed in non-fixation conditions. Cells with 90% confluence, seeded in 96-well plates, were incubated with 40 μL of 3.5 μM pep-1CF, (in free or complexed form with β-Gal (peptide/protein ratio of 320), prepared in free-serum medium), in sixplicates, during 0, 30, 60, 90, 120, 150, 180, 210 and 240 min at 4 or 37 °C.

The quencher properties of TB and it inability to enter in viable cells [18] were used to evaluate the pep-1CF translocation. This hydrophilic molecule is able to quench non-internalized particles, including the fraction adsorbed to the cell membrane, but is inaccessible to internalized fraction [19], so removal of extracellular peptide is needless. The pep-1CF fluorescence was followed, before and after addition of TB (1.9 μL of stock solution, 0.4% w/v), with excitation and emission filters at 485/20 (centre/width) and 590/35 nm, respectively, in a FL500 microplate fluorescence reader. To evaluate the effect of protein in the extension of peptide internalization, the same procedure was followed with pep-1CF complexed.

Delivery efficiency of β-Gal mediated by pep-1CF was evaluated by it enzymatic activity using a non-fluorescent substrate (MUG) which is converted in a fluorescent product (4-methylumbelliferone, 4-MU), see [7] for a detailed description. Briefly, after incubation with pep-1CF/β-Gal complex (see above), cells were washed three times with phosphate buffer saline solution to eliminate non-incorporated protein and peptide. Internalized β-Gal was accessed after cell permeabilization with 0.1% (w/v) TX-100. Substrate (0.1 mM MUG) was added to the cells and incubated with the enzyme during 30 min at 37 °C. NaOH was added to stop the reaction (pH ≈ 12) and product formation (4-MU) was monitored with excitation and emission filters at 360/40 and 460/40 nm, respectively. The same procedure was performed with non-labelled peptide. Controls without pep-1 or pep-1CF were carried out.

The effect of peptide in the protein was determined by comparing it enzymatic activity in free and complexed forms (with non-labelled pep-1 or pep-1CF) in vitro (see [7]).

3. Results and discussion

3.1. Pep-1CF aggregates in aqueous solution

Similarly to non-labelled pep-1 [12], Trp residues in the hydrophobic domain of pep-1CF, have a red-edge excitation shift and an efficient fluorescence quenching by acrylamide (51.8 ± 12 and 42.1 ± 9.1 M^{-1} for low and physiologic ionic strength, respectively) with a negative deviation from linearity. These results indicate that the peptide aggregates in solution (see [12]). Nevertheless, the quantum yield and steady-state anisotropy of the CF group in hydrophilic domain is not dependent on concentration, for both ionic strengths (10 and 150 mM), which suggests that internal organization of the peptide is not affected by these factors.

3.2. Partition and in-depth location of pep-1CF in model membranes

Affinity of hydrophobic and hydrophilic domains for lipidic membranes was evaluated by titration of an aqueous suspension of the pep-1CF (5.76 μM) with lipidic vesicles; both Trp and CF fluorescence emission were monitored. Spectral alterations on fluorescence emission and anisotropy ($r = 0.05$) were not detected, either for POPC or POPC:POPG (4:1) LUVs. These results suggest that the hydrophilic domain does not strongly interact with model membranes. When Trp residues were followed, the addition of lipidic suspension led to both blue-shifted emission spectra (Fig. 1A) and an increase in the fluorophore quantum yield (Fig. 1B). The partition coefficients (determined by fluorescence emission intensity as in [12]) are $(4.2 \pm 0.8) \times 10^2$ for POPC and $(1.7 \pm 0.2) \times 10^3$ for POPC:POPG (4:1) at physiologic ionic strength. These values are significantly smaller than the ones obtained for non-labelled pep-1 ($(3.4 \pm 0.6) \times 10^3$ and $(2.8 \pm 0.4) \times 10^4$ [12]), which indicates a decrease in membrane affinity even for the hydrophobic domain. Concomitantly, partition rates decrease ($t_{1/2} = 197$ ms in POPC and 147 ms in POPC:POPG (4:1) for pep-1CF in comparison with 120 and 34 ms, respectively, for unlabelled pep-1).

An extensive fluorescence emission quenching of Trp residues by acrylamide, in the presence of lipidic membranes, is indicative of significant amounts of peptide non-inserted in the membrane, at discrepancy with pep-1 in the same conditions where no significant acrylamide quenching was detected [12].

In-depth location of the hydrophobic domain of pep-1CF was carried out by means of fluorescence quenching of Trp residues with doxyl-derivatized stearic acids. The quenching is more efficient when the quencher is closer to the Trp residues. Therefore, 5DS probes the bilayer interface while 16DS probes its core [20]. Quenching by 5DS is more efficient than by 16DS, this is true for POPC ($K_{SV,5DS} = 16.1 \pm 3.8$ M^{-1}, $K_{SV,16DS} = 5.2 \pm 0.9$ M^{-1}) and POPC:POPG (4:1) ($K_{SV,5DS} = 15.3 \pm 3.5$ M^{-1}, $K_{SV,16DS} = 4.9 \pm 1.0$ M^{-1}). This is evidence for a position of the hydrophobic region at the membrane interface in both lipidic systems studied. The same conclusion was obtained in the study of non-labelled pep-1 [12].

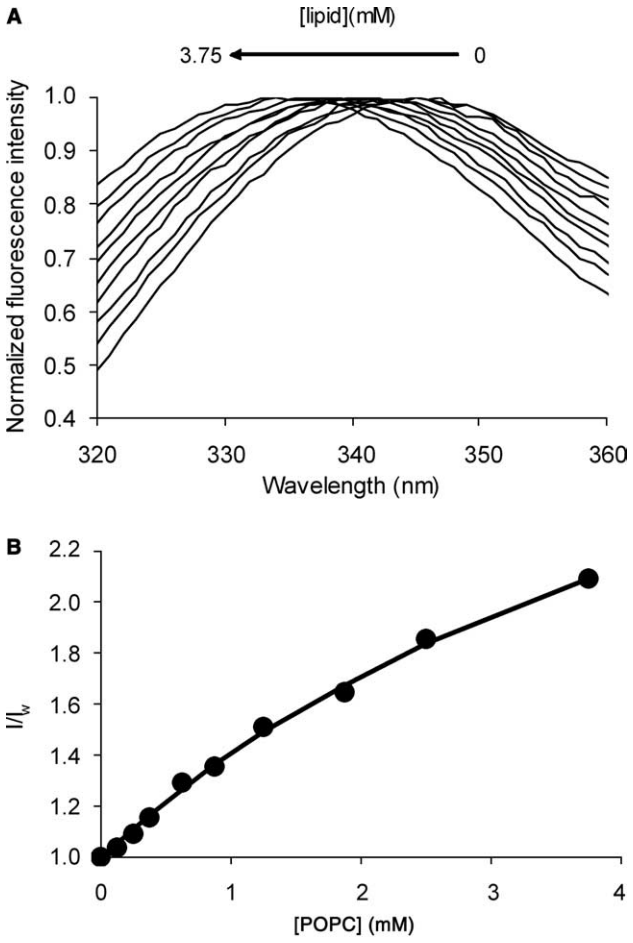

Fig. 1. Partition of Pep-1CF-hydrophobic domain in POPC LUVs (λ_{exc} = 280 nm). (A) shifting of pep-1CF fluorescence emission spectra with lipidic concentration (0–3.75 mM). (B) Fluorescence intensity emission maximum dependence on lipidic concentration.

Quenching of CF group by 5DS and 16DS was negligible, supporting the hypothesis that the hydrophilic domain does not insert in the membrane.

3.3. Translocation of pep-1 and pep-1CF in LUVs is dependent on transmembrane potential

To evaluate pep-1CF translocation in model membranes, the fluorescence of $DiSBAC_2(3)$ was followed. The quantum yield of the probe increases with depolarization of membranes.

Titration of POPC LUVs (in absence (1) or presence (2) of transmembrane potential) with pep-1CF is presented in Fig. 2. The variation of probe fluorescence emission differs for the two situations (with/without potential). When transmembrane potential exists the addition of pep-1CF induces an increase in fluorescence intensity, so membrane is depolarized. In the absence of a transmembrane potential a decrease quantum yield was detected, which indicates a polarization of membrane.

These results suggest that in the absence of transmembrane potential the peptide accumulates in the outer leaflet of membrane, without translocation. The positive global charge of pep-1CF is responsible by membrane polarization.

In the presence of a transmembrane potential (negative inside) pep-1CF is able to translocate across membrane, reducing the negative transmembrane potential, with an increase in quantum yield of $DiSBAC_2(3)$.

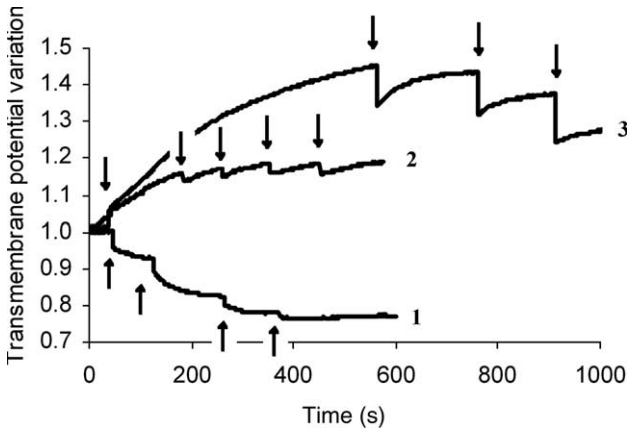

Fig. 2. Variation of transmembrane potential in POPC LUVs by titration with pep-1CF in absence (1) or presence of transmembrane potential (2) or with non-labelled pep-1 in presence of transmembrane potential (3). Monitored with $DiSBAC_2(3)$ fluorescence emission (λ_{exc} = 540 nm, λ_{em} = 558 nm) (inserts indicate addition of 5μL from pep-1CF or pep-1 stock solution).

Similar results were obtained with non-labelled pep-1 using this (Fig. 2, curve (3)) and other methodologies [11], but the effect of a negative transmembrane potential is more pronounced, which suggest that pep-1 has a more efficient translocation than pep-1CF. This is expected considering the lower affinity of pep-1CF for phospholipids membranes.

3.4. Translocation kinetic of pep-1CF and pep-1CF/β-Gal in HeLa cells

Translocation of pep-1CF in HeLa cells was evaluated at 4 and 37 °C (Fig. 3) by the use of TB quenching properties. Its capacity to interact with the CF dye and to quench its fluorescence emission was used to distinguish internalized from surface-bound pep-1CF [18]. The quenching extent is decreased when the peptide translocates across cell membranes and become inaccessible to the quencher.

At 37 °C, we observed fluorescence recovery up to 240 min incubation at variance with results at 4 °C where the fluorescence intensity remained unchanged during the same time (Fig. 3A). This suggest that translocation of pep-1CF occurs via endocytosis. Recently, it has been found that two N-terminally CF-labelled CPPs are internalized by HeLa cells via raft-mediated endocytosis [21].

Significant differences in the kinetics of translocation, for free (Fig. 3A) or complexed (Fig. 3B) forms, of Pep-1CF were not detected.

The uptake of the β-Gal cargo itself, at 4 and 37 °C, was evaluated by following the enzymatic hydrolysis, of a non-fluorescent substrate (MUG) to a fluorescent product (4-MU). The hydrolysis step is carried out at 37 °C after the translocation incubation step. We observed that the translocated protein was active and that the uptake of protein was much more efficient at 37 °C than at 4 °C (180 min after incubation the fluorescence emission intensity of 4-MU was found to be 14.5 times higher at 37 °C relative to 4 °C).

In order to investigate the effect of CF-derivatization in peptide translocation, we compared the efficiency of β-Gal internalization mediated by pep-1 vs. pep-1CF (Fig. 4). When the

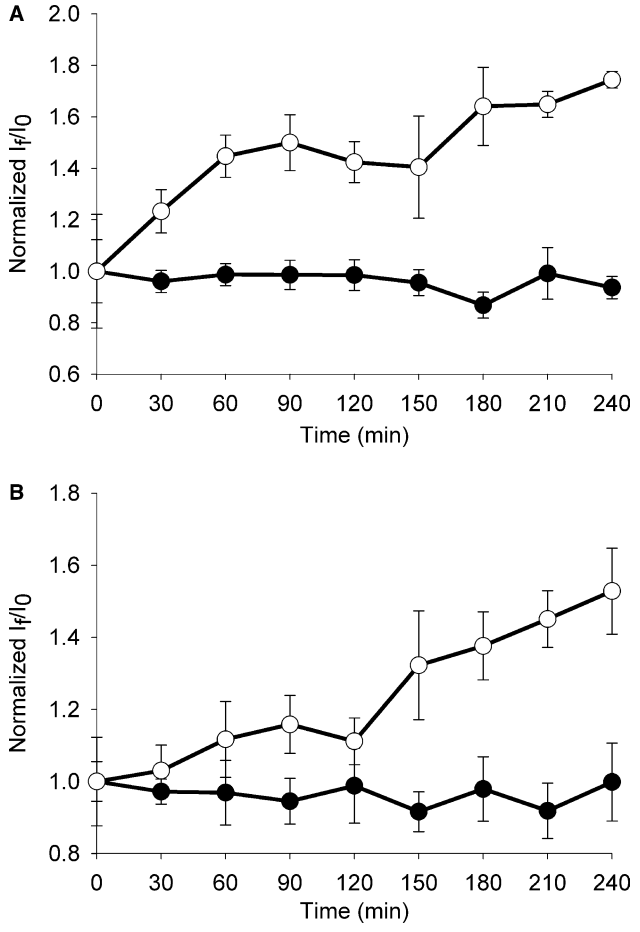

Fig. 3. Kinetics of Pep-1CF uptake in HeLa cells: (A) in free form or (B) complexed with β-Gal at 37 °C (white circles) or 4 °C (black circles). Internalization was followed by means of fluorescence emission (I_f) dequenching of CF. Peptide internalization into cells leads to fluorescence recovery due to inaccessibility to TB (quencher). The relative fluorescence intensity, I_f/I_0 (I_0 is the fluorescence intensity in the absence of TB) was normalized at $t = 0$.

β-Gal uptake was mediated by pep-1 there were no significant differences between 4 and 37 °C [7]. However, efficiency was different for the labelled and unlabelled peptides (Fig. 4),

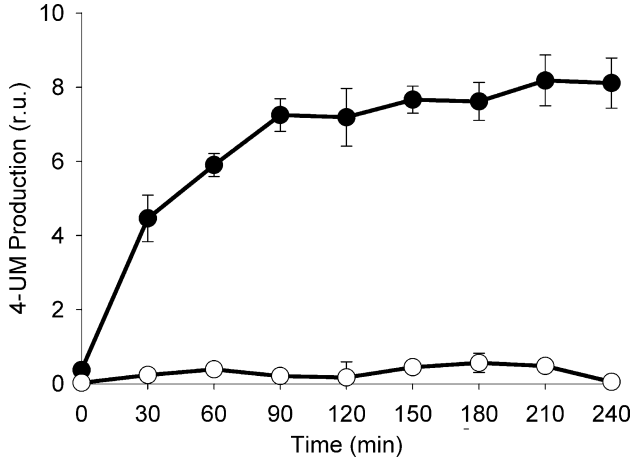

Fig. 4. Delivery of β-Gal into HeLa cells at 37 °C, followed by enzymatic production of 4-UM, mediated by pep-1CF (white circles) or by pep-1 (black circles).

revealing that the hydrophilic domain of the peptide is of critical importance for translocation.

We analysed the enzyme activity, in vitro, in free and in complexed forms. The determined v_0 of β-Gal are: 19.18 ± 0.24 μM/min for free form; 11.60 ± 0.53 μM/min when complexed with unlabelled-pep-1 and 19.61 ± 0.38 μM/min in the complex with pep-1CF. The presence of pep-1 reduces the activity of the enzyme (in agreement with [7]), but this does not hold for pep-1CF. This suggests that the pep-1CF does not interact as strongly with the protein, which is an additional effect induced by the presence of CF in the Lys-rich domain. This supports the hypothesis that pep-1 interacts with the cargo both by means of hydrophobic and electrostatic interactions [7].

The mechanism of translocation of pep-1CF is different from the one identified previously for pep-1. A simple physical mechanism mediated by electrostatic interaction between pep-1 and membrane is supported by different sets of experimental data [7,11]. However, Pep-1CF follows a different pathway, dependent on temperature, i.e., endocytic.

It should be stressed that pep-1CF has translocation ability via physical (non-endocytic) processes (Fig. 2), however, such ability is clearly decreased compared to unlabelled pep-1 (Fig. 2), in agreement with its smaller partition into membranes. At 4 °C, the low cell membrane fluidity also contributes to inhibit physical translocation of pep-1CF. It is only at 37 °C that the endocytic pathway becomes an alternative to the physical process and translocation occurs. A small contribution of the endocytic pathway for translocation of unlabelled pep-1 at 37° cannot be discarded, although a physical process is clearly dominant [7,11].

A reduced protein-uptake mediated by pep-1CF is due, not only, to a decrease in translocation efficiency but also to a diminished interaction of the pep-1CF with β-Gal.

3.5. Conclusion

Translocation of pep-1 is mediated by a physical process governed by electrostatic interactions [7,11]. Modification of the hydrophilic domain, with a CF group, extensively decreases the affinity of pep-1 for phospholipid membranes and for the cargo macromolecule, which affects the translocation of the peptide alone as well as its capacity as delivery agent. It was recently proposed that labelling CPPs influence the final location inside the cell [5]. We now generalize this difference to a decrease in the extension of internalization and to a change in the main mechanism of translocation. A small chemical modification of these sequences may modify the translocation efficiency and even its pathway. It is worth mention that oligo-Arg peptides have translocation ability on their own [22] with no need for "hydrophobic" sequences.

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