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Review

Translocation or membrane disintegration? Implication of peptide–membrane interactions in pep-1 activity‡

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Abstract: The Cell membrane is impermeable for most peptides, proteins, and oligonucleotides. Moreover, some cationic peptides, the so-called cell-penetrating peptides (CPPs), are able to translocate across the membrane. This observation has attracted much attention because these peptides can be covalently coupled to different macromolecules, which are efficiently delivered inside the cell. The mechanism used by these peptides to pass across the membrane is a controversial matter of debate. It has been suggested that endocytosis is the main mechanism of internalization and this was confirmed by several studies for different peptides. Pep-1 is an exception worthy of attention for its ability to translocate cargo macromolecules without the need to be covalently attached to them. A preferential internalization by an endocytosis-independent mechanism was demonstrated both *in vitro* and *in vivo*. Pep-1 has a high affinity to lipidic membranes, it is able to insert and induce local destabilization in the lipidic bilayer, although without pore formation. No cytotoxic effects were found for pep-1 concentrations where translocation is fully operative. At much higher concentrations, membrane disintegration takes place by a detergent-like mechanism that resembles anti-microbial peptide activity. In this review, the ability of pep-1 to transverse the membrane by an endocytosis-independent mechanism, not mediated by pores as well as an ability to induce membrane disintegration at high peptide concentration, is demonstrated. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cell-penetrating peptides; peptide-membrane interactions; translocation mechanism; drug delivery; anti-microbial peptides

INTRODUCTION

The hydrophobic nature of the cell membrane is responsible for cellular integrity and is one of the limitations for the introduction of hydrophilic macromolecules in the cytoplasm. Microinjection, electroporation, liposomes and viral vectors have been used as delivery strategies to overcome membrane permeability. All these methods have drawbacks such as toxicity, poor specificity and being time consuming [1]. The observation that some cytoplasmic proteins are able to cross the membrane when added to extracellular medium, (e.g. HIV-1 transcriptional activator Tat protein [2] and the *Drosophila* antennapedia transcription protein (pAntp) [3]) originated an alternative strategy based on the basic amino acid sequences within these proteins which are translocating-enabling sequences. The observation that these basic peptides allow cellular delivery of conjugated molecules such as peptides or proteins made these molecules attractive and a new class of vectors, known as cell-penetrating peptides (CPPs), emerged [4]. This family now includes all the peptides with the ability to translocate across membranes, whether natural peptides, synthetic, or chimaeric peptides. So far, these vectors have been used to translocate a wide range of macromolecules into living cells such as proteins [5–7], peptides [8,9], oligonucleotides [10,11], peptide nucleic acids [12], and polysaccharides [13]. Nanoparticles [14] and liposomes [15] have also been internalized in cells by means of CPP.

The mechanism used by these peptides to translocate across biologic membranes has been a subject of debate and controversy in the literature (Ref. 16 and references therein). The CPP derived from pAntp (penetratin) and the one from Tat protein (TAT) are the two most intensively studied CPPs. Both peptides use endocytic pathways to reach the cytoplasm [17–23]. Moreover, even in a scenario where the endocytosis is the physiological means of CPP uptake, the escape of the CPP/cargo from endosomes into the cytoplasm is mandatory for a successful delivery of the cargo molecule. An escape from endosomes due to acidification was proposed for penetratin and TAT and confirmed [24]. A translocation dependent on a transmembrane potential was also identified *in vitro* for TAT and penetratin [25].

PEP-1 A CHIMAERIC PEPTIDE

Pep-1 (acetyl-KETWWETWWTEWSQPKKKRKV-cysteamine) is a CPP with primary amphipathicity (i.e

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BIOGRAPHY

Sónia Troeira Henriques obtained her degree in Biochemistry in 2003 and has recently finished her Ph.D. programme at the Faculty of Science, University of Lisbon, Portugal. Her research focuses on the study of the interaction of peptides, mainly cell-penetrating peptides, with model membranes and with mammalian cellular lines, using fluorescence and IR spectroscopy methodologies, surface plasmon resonance and fluorescence microscopy.

Miguel Castanho graduated in Biochemistry (University of Lisbon, Portugal, 1990), has a Ph.D. degree in Molecular Biophysics (Technical University of Lisbon, Portugal, 1993) and habilitation in Physical Biochemistry (University of Lisbon, Portugal, 1999). He became a group leader in the Faculty of Sciences (University of Lisbon, Portugal), where he started working on the mechanism of action of membrane active peptides at the molecular level. His work includes the development of methodologies aiming at specific functional and structural information, related to cell-penetrating, antimicrobial, viral fusion inhibitor and neuropeptides. Both *in-vitro* and *in-vivo* work is carried out, using mainly optical spectroscopic techniques. Miguel Castanho has recently been appointed full professor (Biochemistry) at the Faculty of Medicine (University of Lisbon, Portugal).

amphipathicity resulting from the aminoacid sequence itself, not from the folding structure) composed by: (i) a Trp-rich domain (KETWWETWWTEW), responsible for hydrophobic interactions with both proteins and cell membranes, (ii) a hydrophilic domain (KKKRKV) derived from a nuclear localization signal (NLS) of Simian Virus 40 (SV-40) large T-antigen, required to improve solubility, and (iii) a spacer domain (SPQ), which improves the flexibility and the integrity of the other two domains [26]. A cysteamine group is present in the *C*-terminal and an acetyl group caps the *N*-terminus. In oxidizing conditions dimmers may form due to a disulfide linking of cysteamine groups.

Pep-1 has been efficiently used to introduce several large proteins inside different cellular lines such as mammalian cells [26–29] or plant cells converted into protoplasts [30]. The efficiency of translocation, however, can vary depending on the cell type and the cargo molecule.

PEP-1 INTERACTION WITH MEMBRANES

Pep-1 is intrinsically fluorescent, which overcomes the necessity to couple a fluorescent dye in fluorescence spectroscopy studies. Trp fluorescence emission is environmental-sensitive: when Trp residues are totally exposed to aqueous environment its fluorescence emission has a spectral maximum at ~350 nm; at variance, in a more hydrophobic environment there is a blue shift in fluorescence emission spectrum with a concomitant increase in quantum yield (Figure 1(A)). The pep-1 extent of interaction with lipid membranes was quantified by means of molar ratio partition coefficient, K_P ($K_P = [Pep-1]_{Lipid}/[Pep-1]_{Aqueous}$). The increase in the fluorescence intensity (I) with lipid concentration was used to determine K_P ($I = (I_W + K_p\gamma_L[L]I_L)/(1 + K_P\gamma_L[L])$; where I_W and I_L are the fluorescence intensities in the absence of lipid and limit value for increasing lipid concentrations, respectively, γ_L is the molar volume of lipid and $[L]$ is the lipid concentration – for more details see Ref. 31). Pep-1 has high affinity for neutral membranes vesicles. The peptide insertion kinetics is fast and the interaction is highly enhanced in the presence of negatively-charged phospholipids (Figure 1(B)) [32]. This suggests that the highly charged hydrophilic domain, should be responsible for the first contact with the membrane owing to the electrostatic interactions between the polar headgroup of phospholipids and the positive charges of pep-1. This was further confirmed by the effect of ionic strength on peptide–membrane interaction [33]. The hydrophobic domain, containing five Trp residues, inserts in the membrane with a shallow positioning [32]. Together, the dehydration at membrane surface by the hydrophilic domain and the insertion of the hydrophobic domain promote membrane destabilization. Membrane destabilization was confirmed by aggregation (Figure 2(A)) and fusion (Figure 2(B)) of vesicles in the presence of pep-1 [33,34]. Moreover, segregation of anionic phospholipids induced by the presence of pep-1 was also detected [34]. However, pore formation was not detected [34–37]. At variance, other study [38] proposed a pore formation by a barrel-stave-like mechanism. This conclusion was based on changes in the membrane conductance in voltage-clamped oocytes, when a transmembrane potential was applied [38].

In reducing conditions the peptide decreases its affinity for the membrane [32]. Under these conditions the disulfide link in between two peptide molecules is reduced. Therefore, the loss in affinity can result from an alteration in peptide conformation due to the cleavage of the disulfide bond. The importance of the cysteamine group was confirmed with peptide molecules without this group (non-capped peptide [35] or peptide with an amide group [35] or a fluorophore [39]) at *C*-terminal). The modified peptides have a decreased capacity to translocate into cells.

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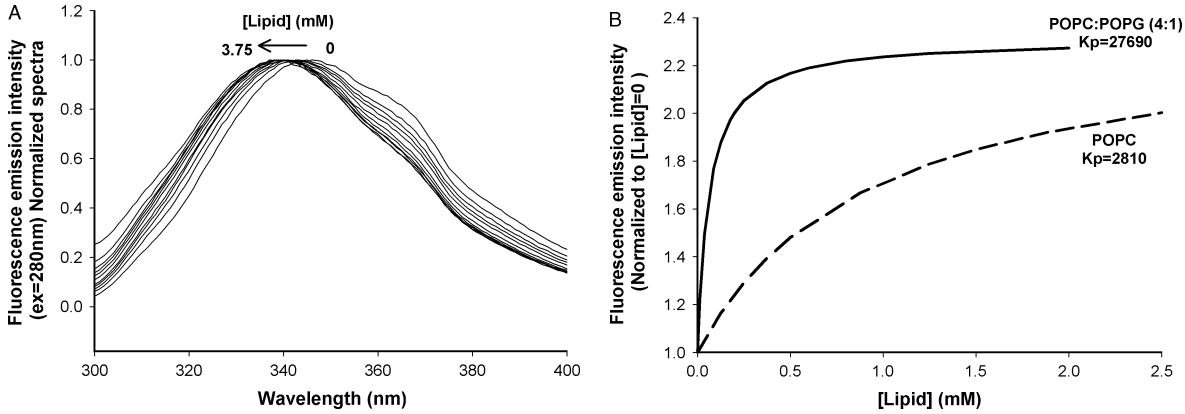

Figure 1 Pep-1 interaction with phospholipid bilayers, reported by Trp fluorescence emission (excitation at 280 nm). Titration of 6.88 μM pep-1 with large unilamellar vesicles (LUVs) of POPC, followed by normalized Trp fluorescence emission spectra (**A**) and total Trp fluorescence emission (**B**). With lipid addition there is a blue shift in the emission spectra and an increase in fluorescence intensity. The pep-1 affinity for lipidic membranes can be quantified by means of partition coefficient, K_P, which was calculated by fluorescence emission intensity and obtained by non-linear regression fit (data points omitted for the sake of clarity) – see text and Refs. 31,32 for further information. Neutral bilayers (POPC) and negatively-charged membranes [POPC : POPG (4 : 1)] are compared. A more extensive partition is obtained with negatively-charged vesicles (for further details see Ref. 32).

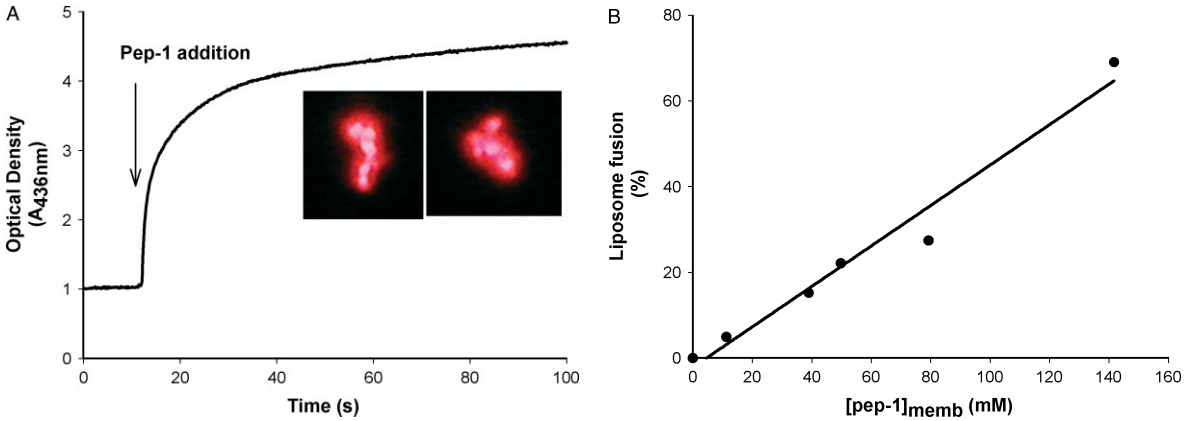

Figure 2 Vesicle aggregation and fusion induced by pep-1. (**A**) Aggregation of LUVs [25 μM POPC : POPG (4 : 1)] induced by 6.88 μM pep-1, followed by optical density and confirmed by fluorescence microscopy (inserts). (**B**) Fusion percentage in POPC and POPC/POPG LUVs. All data were obtained in the presence of 6.88 μM pep-1 total concentration; however, effective concentration in membranes vary (Ref. 34). Fusion extension and effective concentration in membranes are linearly correlated.

Pep-1 interaction with membranes is associated with a conformational alteration [35,36,38]. In aqueous environment the peptide is mainly unstructured but with a tendency to aggregate forming inter-molecular β-sheet aggregates [36]. In the presence of lipidic membranes, a structural alteration from random coil to α-helix conformation, was detected. NMR studies show that the part of the molecule that undergoes structural alteration is the hydrophobic domain [35,38]. This domain is known to easily insert in the membrane [32].

PEP-1 TRANSLOCATION ACROSS CELL MEMBRANES

Pep-1 has been efficiently used in different cellular lines, with several proteins and cargoes. Although, *in vitro* studies with model membranes revealed a high affinity for lipidic bilayers and capacity to perturb the membrane mainly in the presence of anionic phospholipids [34], peptide translocation *in vitro* was only detected in the presence of a negative transmembrane potential (negative inside) [34]. In the absence of transmembrane potential, the peptide inserts only in the outer layer [32]. An excess of negative charges inside the liposome promotes the passage of the peptide from the outer layer to the inner layer (Figure 3(A)) [34]. The transmembrane potential is also essential for the translocation to occur in *vivo*. When HeLa cells were depolarized the uptake of Beta-Gal mediated by the pep-1 was severely reduced (Figure 3(B)).

The pep-1 uptake by endocytosis was tested by means of different methodologies. Delivery efficiency of a cargo molecule attached to pep-1 was compared

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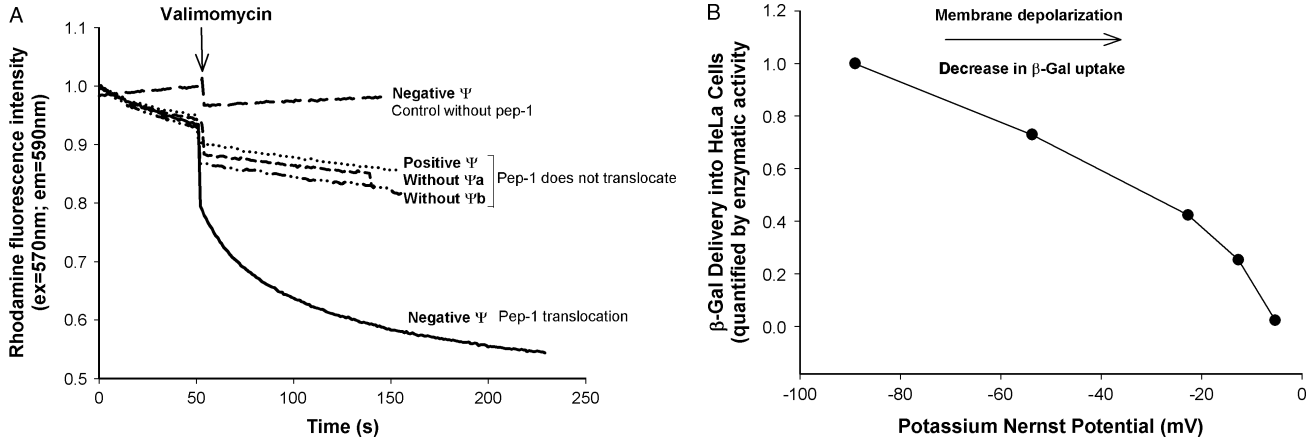

Figure 3 Pep-1 passes through membranes by a mechanism dependent on negative transmembrane potential, Ψ, (inside). (**A**) Pep-1 translocation *in vitro* followed by rhodamine (Rh) quenching [POPC : POPG (4 : 1) LUVs doped with 1% of Rh-labelled phospholipid] induced by the pep-1. In the absence of a Ψ pep-1 is able to quench Rh fluorophores in the outer layer. In the presence of a negative Ψ (created by the addition of valinomycin to liposomes loaded with K^+ and dispersed in Na^+) pep-1 translocates and a drop in Rh fluorescence is clear. With a positive Ψ (liposomes loaded with Na^+ and dispersed in K^+) pep-1 does not translocate. Controls without pep-1 and where a Ψ is not established in the presence of valinomycin (**a** – liposomes loaded with Na^+ and dispersed in Na^+; **b** – liposomes loaded with K^+ and dispersed in K^+) (Ref. 34 for further details) are also represented. (**B**) β-Gal delivery into HeLa cells, mediated by pep-1. Pep-1/β-Gal complex was incubated with HeLa cells, for 30 min at 37 °C. Cell polarization was decreased by increasing external K^+ concentrations, and maintaining the ionic strength constant ($[K^+] + [Na^+] = 150$ mM). The relative level of β-Gal uptake was determined by its enzymatic activity (Ref. 13). Depolarization of cells severely reduces the level of β-Gal uptake.

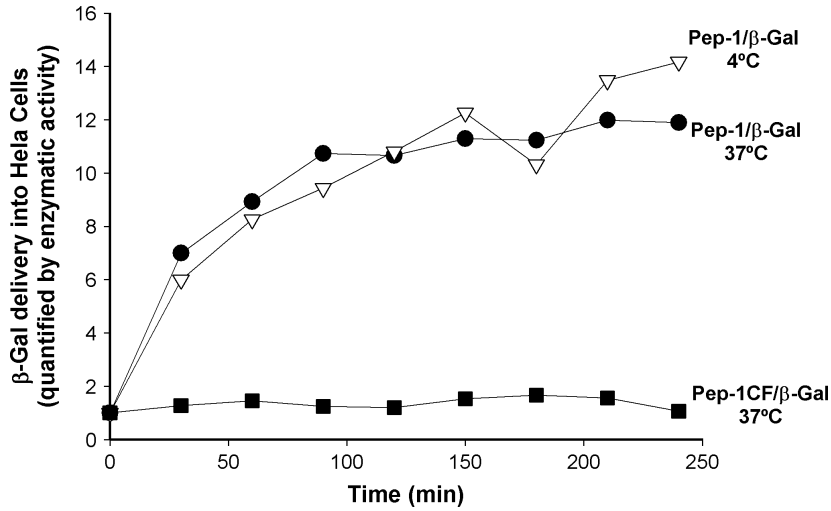

Figure 4 β-Gal delivery into HeLa cells mediated by pep-1 or pep-1 CF, followed by β-Gal enzymatic activity. HeLa cells were incubated during different intervals with Pep-1/β–Gal complex or pep-1CF/β-Gal (molar ratio 320) at 37 or 4 °C. No fixation procedures were used in this protocol. Pep-1 is able to internalize β-Gal in HeLa cells maintaining its enzymatic activity. No differences were detected for incubations at 4 or 37 °C, which suggests an endocytosis-independent internalization. β-Gal internalization is decreased when pep-1CF is used instead of pep-1 (Ref. 39 for further details).

at 4 and 37 °C [13,26] and no differences in delivery efficiency were observed (Figure 4). These results were confirmed not only by imaging methods following the protein by immunofluorescence [13,26] but also by protein activity (measurement of β-Gal enzymatic activity) (Figure 4) [13,26,39]. Procedures used to quantify the protein activity inside the cells exclude the possibility of artefacts associated with fixation procedures. Moreover co-localization of β-Gal internalized by the pep-1 with different endocytotic markers (Dextran, EEA1, Caveolin-1, and cathepsin D) followed by confocal microscopy revealed that this protein is inside cells and does not co-localize with any of these endocytic markers [13] (in the case of translocation by an endocytic pathway a co-localization with at least one of these endocytic markers would be expected when the incubation of cells with the complex pep-1/protein is performed at 37 °C).

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At variance, Weller *et al.* proposed an endocytosis-mediated entrance based on the internalization of pep-1/Thioredoxin (TRX) in the presence/absence of endocytic inhibitors. In these experiments 0.1% NaN_3 and 50 mM deoxy-D-glucose were added to the cells to inhibit ATP production. When pep-1/TRX was incubated with cells (still in the presence of NaN_3 and 50 mM deoxy-D-glucose) the TRX uptake was reduced [35]. As has been previously verified, pep-1 is able to interact with dextran molecules with 10 kDa (i.e. about 55 glucose monomers per dextran molecule) and introduce these molecules inside cells [13]. A decrease in the TRX uptake mediated by the pep-1 could thus result from the interference of glucose (which was present in a high concentration, 50 mM), decreasing pep-1/TRX complex formation and/or pep-1 capacity to deliver TRX inside cells.

Under *in vitro* conditions, pep-1 translocates through a mechanism mediated by the transmembrane potential. This hypothesis was also tested *in vivo*. Cellular depolarization inhibited β-Gal uptake mediated by pep-1 (Figure 3(B)) [13]. Considering the overall results we conclude that the endocytic pathway is not the main internalization pathway used by this peptide to introduce proteins inside cells.

A modified pep-1, in which a carboxyfluorescein (CF) probe was added at the *C*-terminus, lost the ability to internalize β-Gal (Figure 4) [39]. This was emcompassed by a decrease in the affinity for lipidic bilayers [39]. The loss in membrane affinity with a decrease in uptake suggests that translocation efficiency and partition of pep-1 in lipidic membranes are strongly correlated. However, comparing incubations at 4 and 37 °C it was possible to identify a slight internalization of pep-1 CF by an endocytosis-dependent uptake (at 4 °C internalization was inhibited) [39]. This small uptake by endocytosis seems to operate only when the membrane affinity is lost, suggesting that membrane affinity and the capacity to destabilize it, dictate the extent to which the peptide enters the cell by a physical mechanism (a process faster than the endocytosis) to the detriment of the endocytosis itself.

CAN PEP-1 WORK AS ANTI-MICROBIAL PEPTIDE?

Like CPPs, anti-microbial peptides (AMPs) are short and cationic peptides with high affinity for membranes. These peptides are characterized by an efficient killing of several species of bacteria with the ability to preserve host-cell integrity. The main target of these peptides is the bacterial membrane, provoking membrane lysis, membrane permeabilization or other forms of bilayers disruption [40].

A pep-1 translocation by pore formation was recently suggested [38] but this suggestion was not confirmed by experiments where the capacity of pep-1 to induce leakage was tested [34–37]. For high peptide/lipid ratios, pronounced membrane damage takes place in lipidic vesicles. Pore formation is not the course of the damage. A detergent-like mechanism seems to operate [36]. This explains the toxicity when pep-1 is present at high concentration [26,35].

Pep-1 anti-microbial activity was tested for different bacterial strains. The minimal inhibitory concentration (MIC) is dependent on the strain [37]. Although pep-1 is not as efficient as mellitin to kill bacteria, it is able to efficiently kill *bacillus subtilus* at a low concentration, and to kill other strains at higher concentrations. The capacity to kill bacteria was truly improved and comparable to the one observed for mellitin when Glu residues were replaced by Lys. The capacity of Lys-modified-pep-1 to kill bacteria is not related with the capacity to induce leakage [37], this further implying that pep-1 translocation and vector activity cannot be explained by a pore formation mechanism.

Considering these results we can conclude that pep-1 has the capacity to work as a CPP or as an AMP. The threshold between these two properties relies on the peptide concentration, the composition of the membrane, and the final peptide/lipid ratio.

CONCLUSION

Pep-1 translocates and is able to work as a vector to introduce proteins or other cargo molecules inside cells. This peptide is able to strongly interact with the lipid bilayer causing local pertubation, and is also able to cross the membrane by a physical mediated mechanism promoted by the transmembrane potential and not involving pore formation. For many CPPs endocytosis uptake may be the main mechanism of uptake but sound evidence show that pep-1 translocates by a mechanism mediated by physical peptide–membrane interactions when a favourable transmembrane potential is present. This does not exclude a possible internalization by an endocytic route in all situations. Nevertheless, the time required for the physical mechanism to be completed is lesser than that for the endocytic uptake. Therefore, if both mechanisms are operative the non-endocytic route is dominant. Differences between pep-1 and other CPPs can be related to the affinity for membrane lipids. Peptides with higher affinity have a greater propensity to be internalized by a non-endocytic mechanism. Lower affinity for membranes can favour endocytic uptake.

REFERENCES

1. Wadia JS, Becker-Hapak M, Dowdy SF. Protein transport. In *Cell-Penetrating Peptides, Processes and Applications*, Langel U (ed.). CRC Press: New York, 2002; 365–375.
2. Frankel AD, Pabo CO. Cellular uptake of the tat protein from human immunodeficiency virus. *Cell* 1988; **55**: 1189–1193.

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3. Joliot A, Pernelle C, Deagostini-Bazin H, Prochiantz A. Antennapedia homeobox peptide regulates neural morphogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 1991; **88**: 1864–1868.
4. Lindgren M, Hallbrink M, Prochiantz A, Langel U. Cell-penetrating peptides. *Trends Pharmacol. Sci.* 2000; **21**: 99–103.
5. Ezhevsky SA, Nagahara H, Vocero-Akbani AM, Gius DR, Wei MC, Dowdy SF. Hypo-phosphorylation of the retinoblastoma protein (pRb) by cyclin D : Cdk4/6 complexes results in active pRb. *Proc. Natl. Acad. Sci. U.S.A.* 1997; **94**: 10699–10704.
6. Fawell S, Seery J, Daikh Y, Moore C, Chen LL, Pepinsky B, Barsoum J. Tat-mediated delivery of heterologous proteins into cells. *Proc. Natl. Acad. Sci. U.S.A.* 1994; **91**: 664–668.
7. Rojas M, Donahue JP, Tan Z, Lin YZ. Genetic engineering of proteins with cell membrane permeability. *Nat. Biotechnol.* 1998; **16**: 370–375.
8. Rojas M, Yao S, Donahue JP, Lin YZ. An alternative to phosphotyrosine-containing motifs for binding to an SH2 domain. *Biochem. Biophys. Res. Commun.* 1997; **234**: 675–680.
9. Theodore L, Derossi D, Chassaing G, Llirbat B, Kubes M, Jordan P, Chneiweiss H, Godement P, Prochiantz A. Intraneuronal delivery of protein kinase C pseudosubstrate leads to growth cone collapse. *J. Neurosci.* 1995; **15**: 7158–7167.
10. Allinquant B, Hantraye P, Mailleux P, Moya K, Bouillot C, Prochiantz A. Downregulation of amyloid precursor protein inhibits neurite outgrowth in vitro. *J. Cell Biol.* 1995; **128**: 919–927.
11. Morris MC, Vidal P, Chaloin L, Heitz F, Divita G. A new peptide vector for efficient delivery of oligonucleotides into mammalian cells. *Nucleic Acids Res.* 1997; **25**: 2730–2736.
12. Pooga M, Soomets U, Hallbrink M, Valkna A, Saar K, Rezaei K, Kahl U, Hao JX, Xu XJ, Wiesenfeld-Hallin Z, Hokfelt T, Bartfai T, Langel U. Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission in vivo. *Nat. Biotechnol.* 1998; **16**: 857–861.
13. Henriques ST, Costa J, Castanho MA. Translocation of beta-galactosidase mediated by the cell-penetrating peptide pep-1 into lipid vesicles and human HeLa cells is driven by membrane electrostatic potential. *Biochemistry* 2005; **44**: 10189–10198.
14. Lewin M, Carlesso N, Tung CH, Tang XW, Cory D, Scadden DT, Weissleder R. Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells. *Nat. Biotechnol.* 2000; **18**: 410–414.
15. Torchilin VP, Rammohan R, Weissig V, Levchenko TS. TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 2001; **98**: 8786–8791.
16. Magzoub M, Graslund A. Cell-penetrating peptides: from inception to application. *Q. Rev. Biophys.* 2004; **37**: 147–195.
17. Console S, Marty C, Garcia-Echeverria C, Schwendener R, Ballmer-Hofer K. Antennapedia and HIV transactivator of transcription (TAT) "protein transduction domains" promote endocytosis of high molecular weight cargo upon binding to cell surface glycosaminoglycans. *J. Biol. Chem.* 2003; **278**: 35109–35114.
18. Drin G, Cottin S, Blanc E, Rees AR, Temsamani J. Studies on the internalization mechanism of cationic cell-penetrating peptides. *J. Biol. Chem.* 2003; **278**: 31192–31201.
19. Fittipaldi A, Ferrari A, Zoppe M, Arcangeli C, Pellegrini V, Beltram F, Giacca M. Cell membrane lipid rafts mediate caveolar endocytosis of HIV-1 Tat fusion proteins. *J. Biol. Chem.* 2003; **278**: 34141–34149.
20. Lundberg M, Wikstrom S, Johansson M. Cell surface adherence and endocytosis of protein transduction domains. *Mol. Ther.* 2003; **8**: 143–150.
21. Potocky TB, Menon AK, Gellman SH. Cytoplasmic and nuclear delivery of a TAT-derived peptide and a beta-peptide after endocytic uptake into HeLa cells. *J. Biol. Chem.* 2003; **278**: 50188–50194.
22. Richard JP, Melikov K, Vives E, Ramos C, Verbeure B, Gait MJ, Chernomordik LV, Lebleu B. Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. *J. Biol. Chem.* 2003; **278**: 585–590.
23. Thoren PE, Persson D, Isakson P, Goksor M, Onfelt A, Norden B. Uptake of analogs of penetratin, Tat(48–60) and oligoarginine in live cells. *Biochem. Biophys. Res. Commun.* 2003; **307**: 100–107.
24. Magzoub M, Pramanik A, Graslund A. Modeling the endosomal escape of cell-penetrating peptides: transmembrane pH gradient driven translocation across phospholipid bilayers. *Biochemistry* 2005; **44**: 14890–14897.
25. Terrone D, Sang SL, Roudaia L, Silvius JR. Penetratin and related cell-penetrating cationic peptides can translocate across lipid bilayers in the presence of a transbilayer potential. *Biochemistry* 2003; **42**: 13787–13799.
26. Morris MC, Depollier J, Mery J, Heitz F, Divita G. A peptide carrier for the delivery of biologically active proteins into mammalian cells. *Nat. Biotechnol.* 2001; **19**: 1173–1176.
27. Coulpier M, Anders J, Ibanez CF. Coordinated activation of autophosphorylation sites in the RET receptor tyrosine kinase: importance of tyrosine 1062 for GDNF mediated neuronal differentiation and survival. *J. Biol. Chem.* 2002; **277**: 1991–1999.
28. Ikari A, Nakano M, Kawano K, Suketa Y. Up-regulation of sodium-dependent glucose transporter by interaction with heat shock protein 70. *J. Biol. Chem.* 2002; **277**: 33338–33343.
29. Zhou J, Hsieh JT. The inhibitory role of DOC-2/DAB2 in growth factor receptor-mediated signal cascade. DOC-2/DAB2-mediated inhibition of ERK phosphorylation via binding to Grb2. *J. Biol. Chem.* 2001; **276**: 27793–27798.
30. Wu Y, Wood MD, Tao Y, Katagiri F. Direct delivery of bacterial avirulence proteins into resistant Arabidopsis protoplasts leads to hypersensitive cell death. *Plant J.* 2003; **33**: 131–137.
31. Santos NC, Prieto M, Castanho MA. Quantifying molecular partition into model systems of biomembranes: an emphasis on optical spectroscopic methods. *Biochim. Biophys. Acta* 2003; **1612**: 123–135.
32. Henriques ST, Castanho MA. Environmental factors that enhance the action of the cell penetrating peptide pep-1 A spectroscopic study using lipidic vesicles. *Biochim. Biophys. Acta* 2005; **1669**: 75–86.
33. Sharonov A, Hochstrasser RM. Single-molecule imaging of the association of the Cell-penetrating peptide Pep-1 to model membranes. *Biochemistry* 2007; **46**: 7963–7972.
34. Henriques ST, Castanho MA. Consequences of nonlytic membrane perturbation to the translocation of the cell penetrating peptide pep-1 in lipidic vesicles. *Biochemistry* 2004; **43**: 9716–9724.
35. Weller K, Lauber S, Lerch M, Renaud A, Merkle HP, Zerbe O. Biophysical and biological studies of end-group-modified derivatives of Pep-1. *Biochemistry* 2005; **44**: 15799–15811.
36. Henriques ST, Quintas A, Bagatolli LA, Homble F, Castanho MA. Energy-independent translocation of cell-penetrating peptides occurs without formation of pores. A biophysical study with pep-1. *Mol. Membr. Biol.* 2007; **24**: 282–293.
37. Zhu WL, Lan H, Park IS, Kim JI, Jin HZ, Hahm KS, Shin SY. Design and mechanism of action of a novel bacteria-selective antimicrobial peptide from the cell-penetrating peptide Pep-1. *Biochem. Biophys. Res. Commun.* 2006; **349**: 769–774.
38. Deshayes S, Heitz A, Morris MC, Charnet P, Divita G, Heitz F. Insight into the mechanism of internalization of the cell-penetrating carrier peptide Pep-1 through conformational analysis. *Biochemistry* 2004; **43**: 1449–1457.
39. Henriques ST, Costa J, Castanho MA. Re-evaluating the role of strongly charged sequences in amphipathic cell-penetrating peptides: a fluorescence study using Pep-1. *FEBS Lett.* 2005; **579**: 4498–4502.
40. Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. *Pharmacol. Rev.* 2003; **55**: 27–55.