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A non-covalent peptide-based strategy for protein and peptide nucleic acid transduction

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Abstract

The development of therapeutic peptides and proteins is limited by the poor permeability and the selectivity of the cell membrane. The discovery of protein transduction domains has given a new hope for administration of large proteins and peptides in vivo. We have developed a non-covalent strategy for protein transduction based on an amphipathic peptide, Pep-1, that consists of a hydrophobic domain and a hydrophilic lysine-rich domain. Pep-1 efficiently delivers a variety of fully biologically active peptides and proteins into cells, without the need for prior chemical cross-linking or chemical modifications. The mechanism through which Pep-1 delivers active macromolecules does not involve the endosomal pathway and the dissociation of the Pep-1/macromolecule particle occurs immediately after it crosses the cell membrane. Pep-1 has been successfully applied to the screening of therapeutic peptides in vivo and presents several advantages: stability in physiological buffer, lack of toxicity and of sensitivity to serum. In conclusion, Pep-1 technology could contribute significantly to the development of fundamental and therapeutic applications and be an alternative to covalent protein transduction domain-based technologies.

Keywords: Cell-penetrating peptide; Therapeutic protein; Peptide delivery; Peptide nucleic acid; Amphipathic peptide; Peptide-based nanoparticle

In order to circumvent the problems of gene therapy technology, an increasing interest is being taken in designing novel strategies that allow the delivery of peptides and full-length proteins into a large number of cells [1–5]. However, the development of peptide-drugs and therapeutic proteins remains limited by the poor permeability and the selectivity of the cell membrane for large molecules. Recently, substantial progress has been made in the development of cell-penetrating peptide-based drug delivery systems which are able to overcome both extracellular and intracellular limitations [1–7]. The cell penetrating peptide (CPP) family includes several peptide sequences: synthetic and natural cell-permeable peptides, protein transduction domains (PTDs) and membrane-translocating sequences, which are all capable of translocating the cell membrane independently of transporters or specific receptors [2]. A series of small peptides, termed protein transduction domains (PTDs), have been shown to cross biological membranes efficiently, and to promote the delivery of peptides and proteins into both cultured cells and animals [2,4–6]. Peptides derived from the trans-activating regulatory protein (TAT) of human immunodeficiency virus (HIV) [8–11], the third alpha-helix of Antennapedia homeodomain protein [2,12], VP22 protein from herpes simplex virus [13], the poly-arginine peptide sequence [14,15], peptide derived from calcitonin [16,17] or from antimicrobial peptides Buforin I and SynB [18,19] as well as Transportan and derivates [4,20] have been successfully used to improve the delivery of covalently-linked peptides or proteins into cells and have been shown to be of considerable interest for protein therapeutics [1–5]. The use of PTD-mediated transduction has proven that "protein therapy" can have a major impact on the future of therapies in a variety of viral diseases and cancers.

Protein transduction technology described so far requires the attachment of the PTD to the target peptide or protein, which is achieved by either chemical cross-linking or cloning and

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expression of a protein fused to the PTD [21,22]. Conjugation methods offer several advantages for in vivo applications including rationalization, reproducibility of the procedure together with the control of the stoichiometry of the PTD-cargo. However, the covalent PTD technology is limited from the chemical point of view, as it is mainly performed via a synthetic disulfide linkage, and risks of alterating the biological activity of the cargoes. In order to offer an alternative to the covalent PTDs technology, we have designed a new strategy for the delivery of full-length proteins and peptides into mammalian cells, based on a short amphipathic peptide carrier, Pep-1. This peptide carrier improves the delivery of several different proteins and peptides in a fully biologically active form into a variety of cell lines, without the need for prior chemical covalent coupling or denaturation steps [23]. Pep-1 technology constitutes a powerful tool for basic research and has been demonstrated to be extremely powerful for studying the role of proteins, and for targeting specific protein/protein interactions in vitro as well as in vivo [23–29]. In this review article, we describe the characteristics of the non-covalent Pep-1 strategy and its applications for protein and peptide nucleic acid (PNA) transduction both in vitro and in vivo. The internalization mechanism of Pep-1 will be discussed on the basis of the structural requirement for its cellular uptake.

1. Design and properties of Pep-1 peptide family

Pep-1 is a primary amphipathic peptide, of 21 residues, (KETWWETWWTEWSQPKKKRKV) consisting of three domains: a hydrophobic tryptophan rich motif (KETWWETWWTEW), required for efficient targeting to the cell membrane and for forming hydrophobic interactions with proteins, a hydrophilic lysine rich domain derived from the Nuclear Localization Sequence (NLS) of SV40 large T antigen (KKKRKV), required for the intracellular delivery and solubility of the peptide vector, and a linker domain (SQP), separating the two domains mentioned above, containing a proline residue, which improves the flexibility and the integrity of both the hydrophobic and the hydrophilic domains [23]. Pep-1 sequence is acetylated at its N-terminus and carries a cysteamide group at its C-terminus, both of which are essential for the stability of the peptide and its transduction mechanism [23,30]. The cysteamide function was introduced into several peptide carriers [31,32] as its offers the advantage of being compatible with Fmoc synthesis and avoids the use of cysteine, thereby protecting its side chain [33]. Since Pep-1 was described in 2001, several modifications of Pep-1 sequences have been proposed in order to stabilize the cargo/carrier complex or to extend the potency of this strategy to other cargo molecules. Recently, we have designed a new peptide carrier, Pep-2 (KETWFETWFTEWSQPKKKRKV), which is able to interact with and facilitate the cellular uptake of PNAs and derivatives such HypNA-*p*PNAs (trans-4-hydroxyl-L-proline/phosphonate-PNA) [34]. The Pep-2 sequence differs from that of Pep-1 essentially by two Phe residues at positions 5 and 9, which replace Trp residues in the hydrophobic domain [34].

Both Pep-1 and Pep-2 associate rapidly with cargoes in solution through non-covalent hydrophobic interactions and form stable complexes independently of a specific peptidyl sequence [23–30]. Binding of cargoes to Pep-1 or Pep-2 can be easily monitored by fluorescence spectroscopy using the Trp residues located in the hydrophobic domain of the peptide, as a sensitive sensor of the interaction (Fig. 1A). We have demonstrated that both Pep-1 and Pep-2 exhibit a high affinity for their cargoes, proteins or PNAs, in the nanomolar-range, and form stable peptide-based nano-particles around the cargo, of a size estimated between 100 and 200 nm diameter. The nano-particles correspond to peptide/cargo ratios of about 10/1 to 15/1 depending on the nature of the cargo (Fig. 1A and B). An important criterion for a CPP is the balance between efficiency and toxicity of the peptide, as most peptides, which interact with membranes, are toxic to a certain extent. Evaluation of the toxicity of Pep-1 and Pep-2 on different cell lines revealed that both peptides do not exhibit any toxicity up to a concentration of 100 μM, and 50% toxicity is reached for an average concentration of peptides between 0.6 and 0.8 mM. Interestingly, when Pep-carriers are associated to cargoes their toxicity is reduced by 6-fold, with CC_{50} values between 3 and 5 mM (Fig. 1C and D). This decrease in toxicity is associated with particle structure stabilization, which involves the cysteamide group, as already reported for other peptides used in the non-covalent strategy [32,35].

2. Mechanism of internalization of Pep-1

Recently, the cellular uptake mechanism of PTDs has been completely revised and shown to be essentially associated with the endosomal pathway [36–40]. This was clearly demonstrated for TAT-peptide; monitoring the cell entry of a biologically active protein, the Cre-recombinase, has revealed a lipid-raft-dependent macropinocytosis mechanism [40]. However, for most CPPs, the cellular uptake mechanism still needs to be confirmed, mainly due to the fact that very little is known concerning the active conformation of CPPs associated with membrane penetration and translocation [7,41]. There are evidences for several routes of cellular uptake of CPPs, some of which are independent of the endosomal pathway and involve the trans-membrane potential [42,44,45]. When investigating uptake mechanisms of CPPs, several factors that may affect their cellular uptake should be taken into account; including the nature and structural constraints of the peptide, the nature and size of the cargoes and cell types. Whichever the CPP considered, the common major concern is to avoid the endosomal pathway and/or to facilitate the escape of the cargoes from the early endosomes to prevent their degradation. An important criterion to be considered is the structural requirements for cellular uptake of CPPs. Only a few CPPs are able to interact with the membrane lipids, and as clearly demonstrated for Tat-PTD, the interaction with the cell surface occurs through electrostatic contacts involving heparan sulfate at the surface of the cells, without any conformational changes of the associated PTD [7,41]. In contrast, Penetratin [3,44,45], Transportan [46] or MPG [47,48] exhibit the ability to interact with membrane

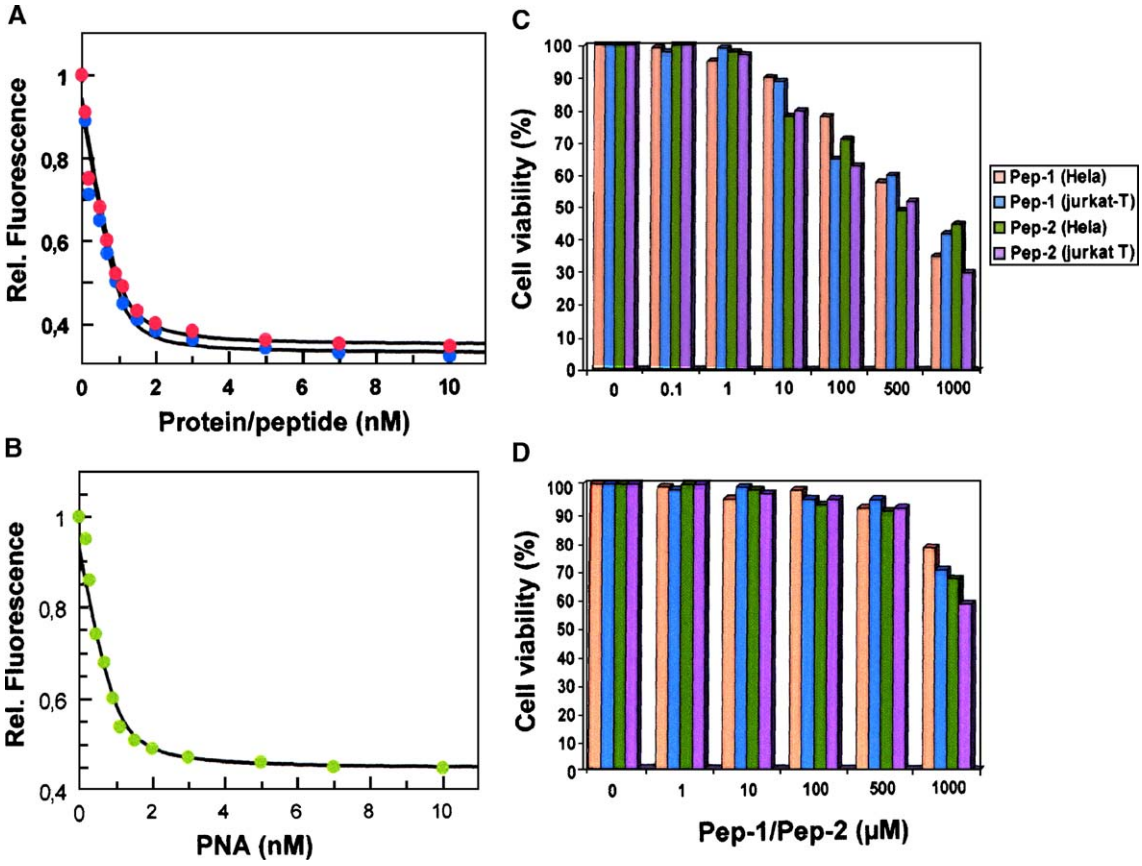

Fig. 1. Formation and toxicity of Pep-1/cargo complexes. The binding of cargoes to Pep-1 and Pep-2 was monitored by fluorescence spectroscopy in phosphate buffer. A fixed concentration of carrier (0.1 μM) was titrated by increasing concentration of cargoes. Panel A: Pep-1 was titrated with GFP-protein (red circle) and a 32-mer peptide (blue circle) as previously described [23]. Panel B: Pep-2 was titrated with a short 15-mer HypNA-pPNA [34]. Intrinsic fluorescence of Pep-1 or Pep-2 is centered at 340 nm upon excitation at 290 nm. The binding of cargo induced a quenching of intrinsic fluorescence of about 40% and dissociation constants were estimated to 12±3 nM and 14±5 nM for Pep-1/GFP and Pep-1/peptide, respectively, and to 22±4 nM for Pep-2/ HypNA-pPNA. The toxicity of Pep-1 and Pep-2 was investigated in different cell lines, including HeLa and Jurkat-T. Cells were incubated in the presence of increasing concentrations, from 0.1 μM to 1 mM of carrier associated (panel D) or not (panel C) with a cargo molecule at a molar ratio of 20/1. The cell viability was estimated using MTT staining after 24-h incubation. Results correspond to the average of 4 separate experiments and demonstrate that both carriers are not toxic on the cell lines tested, up to a concentration of 100 μM. The CC_{50} value was estimated to 0.5 mM for both peptides and is significantly increase upon association with the cargo molecule (CC_{50}: 3 mM).

lipids through both electrostatic and hydrophobic contacts. The interaction with lipids induces a folding of the peptide into a helical or beta structure that controls its cellular uptake or escape from early endosomes directly and therefore provides an explanation for the difference in CPP uptake mechanisms [7,41]. A variety of physical and spectroscopic approaches have been combined to gain insight into the structure(s) involved and to understand the interactions of Pep-1/cargo complexes with lipids, and thus to characterize its mechanism of cellular internalization. We have demonstrated that Pep-1 strongly interacts with membrane lipids, mainly through its Trp-rich domain, that was shown to be critical for insertion of the peptide into the membrane. The direct interaction of the peptide with lipids limits its association with proteoglycans at the surface of the cell and the risk of uptake through the endosomal pathway [30]. Interestingly, the conformation of Pep-1 is not significantly affected upon formation of a particle with a peptide cargo. In contrast, both NMR and circular dichroism analysis have revealed that interaction of Pep-1 and Pep-1/cargo complexes with phospholipids results in folding of the carrier into an alpha helix [31]. We have suggested that the outer part of the "Pep-1-based nanoparticle" with the cargo is involved in interactions with the membrane and forms transient transmembrane helical structures that temporarily affect the cell membrane organization, without associated leakage or toxicity, thereby facilitating insertion into the membrane and initiation of the translocation process. The presence of the cysteamide group at the C-terminus of the peptide is essential for its cellular uptake and probably favors either stabilization of the particle and/or its interaction with lipids. Based on both structural and biophysical investigations, a four-step mechanism was proposed (Fig. 2): (a) formation of the Pep-1/cargo complexes involving hydrophobic and electrostatic interactions depending on the nature of the cargo, (b) interaction of the complex with the external side of the cell involving electrostatic contacts with the phospholipids head groups (c) insertion of the complex into the membrane, associated with conformational changes which induce membrane structure perturbation (d) release of the complex into the cytoplasm with partial "de-caging" of the cargo [31]. Although, the

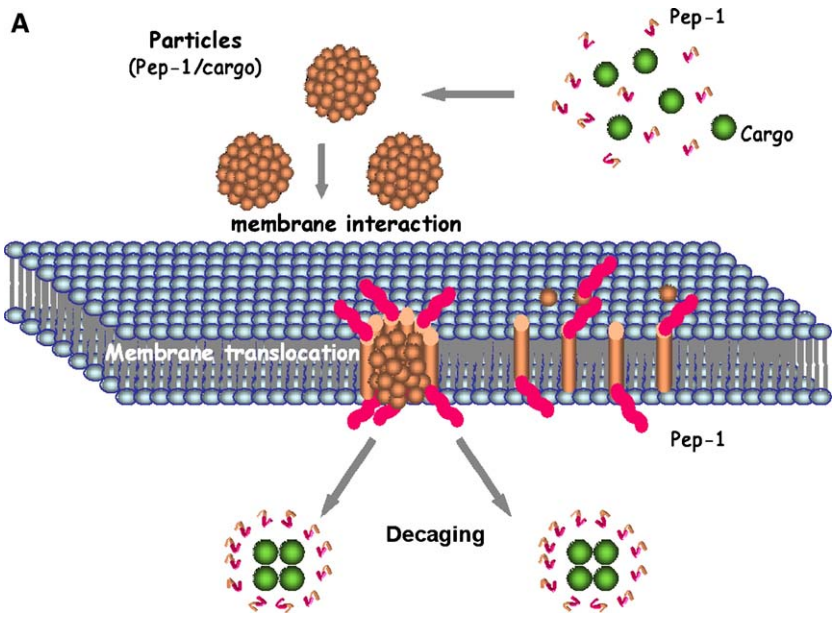

Fig. 2. Mechanism of cellular uptake of Pep-1 based on the structure and biophysical characterization. A four step models: (1) formation of the Pep-1/cargo complexes, (2) interaction of the complex with the external side of the cell involving electrostatic contacts with the phospholipids head groups (3) insertion of the complex into the membrane, associated with conformational changes inducing membrane structure perturbation (4) release of the complex into the cytoplasm followed by "de-caging" of the cargo.

overall mechanism of Pep-1 is still not fully understood, it was clearly demonstrated that "decaging" occurs rapidly in the cytoplasm, a few minutes after cellular uptake and that Pep-1 localizes in the nucleus irrespective of the nature of the cargo. This can be directly correlated to the fact that particle "undressing" is initiated in the membrane as a fraction of Pep-1 molecules remain associated to the membrane. Moreover, the kinetics of "decaging" are dependent on the nature of the cargo, on its affinity for the target and on the subcellular localization of the target.

3. Application of Pep-1 strategy to macromolecule transduction

3.1. Pep-1-mediated transduction of peptides and large proteins

Pep-1 technology has been applied to both basic researches as well as to delivery of therapeutic peptides and proteins into plants and mammalian cell lines (Table 1). Pep-1 has been largely used for protein and peptide transduction, with high efficiency (60 to 80%), into a large number of mammalian cell lines, including non-transformed, cancer, neuronal and primary cell lines [21,49]. This carrier promotes the cellular uptake of small peptides and of large proteins, independently of the size and the nature of the polypeptide and of the cell types [23,24,28–30,49,50]. It has been shown to deliver antibodies into cells, whilst preserving their ability to recognize their target antigens, which constitutes a major interest for therapeutic application. The lack of influence of Pep-1 on the sub-cellular localization of the proteins it delivers supports the fact that the "de-caging" process namely the dissociation between Pep-1 and cargo occurs rapidly in the cytoplasm once the complexes enter the cells (Table 1). Improving the uptake of proteins and peptides into primary cell lines remains an essential challenge; several protocols have been described for the application of Pep-1 onto different primary cell lines including macrophages [51], hepatocytes [52], neural retinal [53], human stem [54] and pancreatic cells [55]. Gallo and colleagues have optimized Pep-1 protocol for peptides and proteins transduction into primary neuronal cells [25,50,56].

3.2. Mechanism of cellular uptake of Pep-1/cargo complex

Artifacts can be associated with fixation methods as described by several groups [36,37], but also with the use of fluorescent probes attached to the CPPs, that can modify their cellular behavior. Therefore, an essential rule when investigating the uptake mechanism is to correlate the uptake pathway with a biological response associated with the cargo [40]. As several routes may exist, it is essential to identify the one resulting in a biological response. A model experiment based on the ability of Pep-1 to improve the delivery of the cell cycle inhibitor protein $p27^{Kip1}$ was performed to understand its uptake mechanism. The cell cycle inhibitor $p27^{Kip1}$ binds to and inhibits Cdk/cyclin complexes involved in the G1/S transition such as Cdk2/cyclin E and Cdk2/cyclin A [57]. Due to its important role in the control of the cell cycle progression, $p27^{kip1}$ has been proposed to be a good candidate for arresting cancer cell proliferation. A covalent strategy based on a PTD-peptide has been successfully used for the delivery of $p27^{kip1}$ or derived inhibitory peptides into cultured cells and ex vivo [22,58,59]. Pep-1 can efficiently

Table 1
Pep-1 mediated transduction of proteins and peptides

Cell lines	Macromolecules	References
3T3/L1-mouse fibroblast	Antibody, Protein, Peptide	[74]
HeLa-human cervix carcinoma	Protein, Peptide, antibody, PNA	[24,75]
HEK/293-human embryonic kidney	Protein/peptide	[73]
HS-68-human foreskin fibroblast	Protein, Peptide, PNA	[23,24]
PC-12 rat pheochromocytoma	Protein, peptide	[76,84]
A549 human lung carcinoma	Antibody	[29]
CV-1 monkey kidney	Antibody	[79]
C2C12-myotubes	Antibody, protein	[86]
Primary plant protoplasts *Arabidopsis*	Protein	[28,91]
COS-7 monkey kidney	Protein, oligopeptide	[23,24,75]
WI-38 human lung fibroblast	Protein, peptide, antibody	[49,54]
WISH human placenta carcinoma	Protein	[88,89]
Primary mouse embryonic cells	Protein	[90]
Cardiomyocites	Protein	[79,80]
Primary human mesenchymal stem cells	Peptide	[49]
Macrophage	Antibody	[51]
Jurkat human T-cell leukemia	Protein, peptide, antibody	[23,24,78]
MCF-7 human breast carcinoma	Protein/PNA	[34,83]
Primary human monocyte	Protein	[81]
Primary mouse hepatocytes	Protein, PNA	[52,66]
Primary rate type II alveolar epithelial	Protein	[27]
Primary chicken neural retina cells	Protein	[53]
NRK normal rat kidney	Antibody	[82,85]
Pancreatic cells primary human	Protein	[55]
Saos-2 human osteosarcoma cells	Antibody	[90]
Primary neurons	Antibody, protein, peptide	[25,50,77,87]
Bovine and monkey zygote	Protein, antibody	[70,71]

A non-exhaustive list of the application of Pep-1 strategy.

deliver $p27^{kip1}$ in a biologically active form into non-transformed and cancer cells without the need for cross-linking, which result in a cell cycle arrest in G1 in more than 70% of the cells (Fig. 3A and B). The $p27^{kip1}$-associated response was used to investigate the uptake mechanism of Pep-1/cargo particles in cellulo. Uptake experiments were performed in the presence of several inhibitors of the endosomal pathway including Nystatin, Cytochalasin D, and Amiloride, as well as NaN_2 for energy depletion (Fig. 3C). Results demonstrated that none of the inhibitors affected the efficiency of Pep-1, with the exception of energy deprivation, which reduced by 50% the biological response associated to Pep-1-mediated $p27^{kip1}$ delivery. This result can be directly correlated to modification of membrane potential, known to be required for uptake of CPPs [43]. We therefore proposed that the uptake of the Pep-1/cargo complex leading to the biological response is independent of the endosomal pathway and is directly correlated to the size of particles and the nature of cargoes.

3.3. Pep-1 mediated transduction of biologically active molecules

Pep-1 strategy has been successfully applied to introduce a large variety of proteins, antibodies and peptides into different cells lines (Fig. 4A–C). This strategy has been extended to the delivery of other uncharged and charged cargoes, including siRNA [60], DNA–protein complexes [23], replication-deficient viruses [61], PNAs [34] and semiconductor quantum dots [62]. PNAs have been proposed as an attractive alternative to classical antisense oligonucleotides and efficient delivery using covalent PTD-based strategies [21,63,64]. Pep-2 technology has been used to significantly improve the delivery of both uncharged PNAs and derivatives such HypNA-*p*PNAs (trans-4-hydroxyl-L-proline/phosphonate-PNA) [65] into several cell lines, this technology has been applied to target essential protein in different cellular pathways [34,66]. We have proven Pep-2

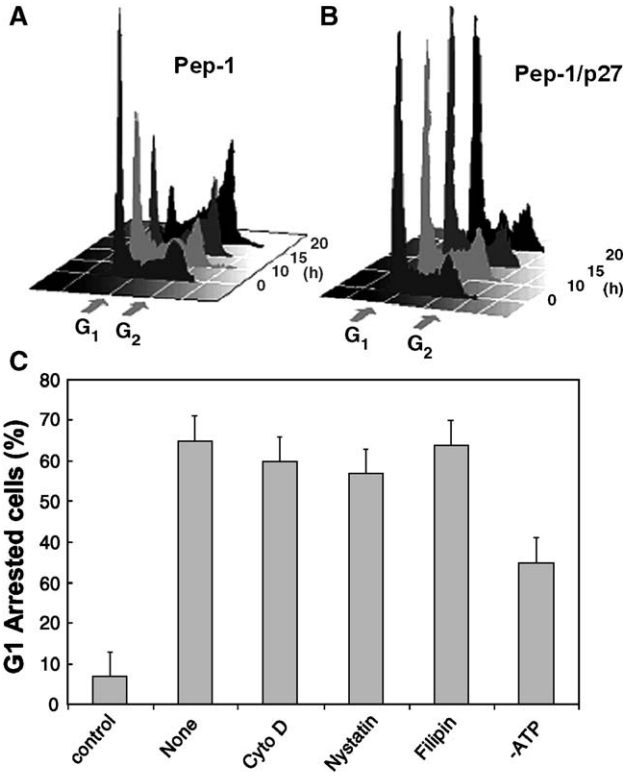

Fig. 3. Cellular uptake mechanism of the active form of Pep-1/cargo complex. The cell cycle inhibitory protein 27^{kip1} (1 μM / 2μM) was formulated with Pep-1 at a molar ratio 1/20, in phosphate buffer. Human fibroblast HS-68 cells were first synchronized by serum deprivation for 40 h, and then restimulated to enter the cycle by addition of fresh medium supplemented with 20% FCS for 4 h and overlaid with preformed Pep1/$p27^{kip1}$ complexes. Cells treated with Pep-1 or $p27^{kip1}$ (panel A) or with Pep-1/$p27^{kip1}$ (panel B) were analyzed by flow cytometry 10, 15 and 20 h after release from synchrony. Panel C: Experiments were performed in the absence (Mock) or presence of Cytochalasine D (10 μM), Nystatin (50 μg/ml), Amiloride (5 mM) and Sodium Azide (0.1%). Cells were incubated in the presence of the inhibitor 1 h prior addition of Pep-1/$p27^{kip1}$ complexes and inhibitors were maintained for 1 h after transfection. After 1 h were then treated with trypsin and resuspended in fresh medium. The level of cell arrested in G1 was analyzed by flow cytometry, 24 h after release.

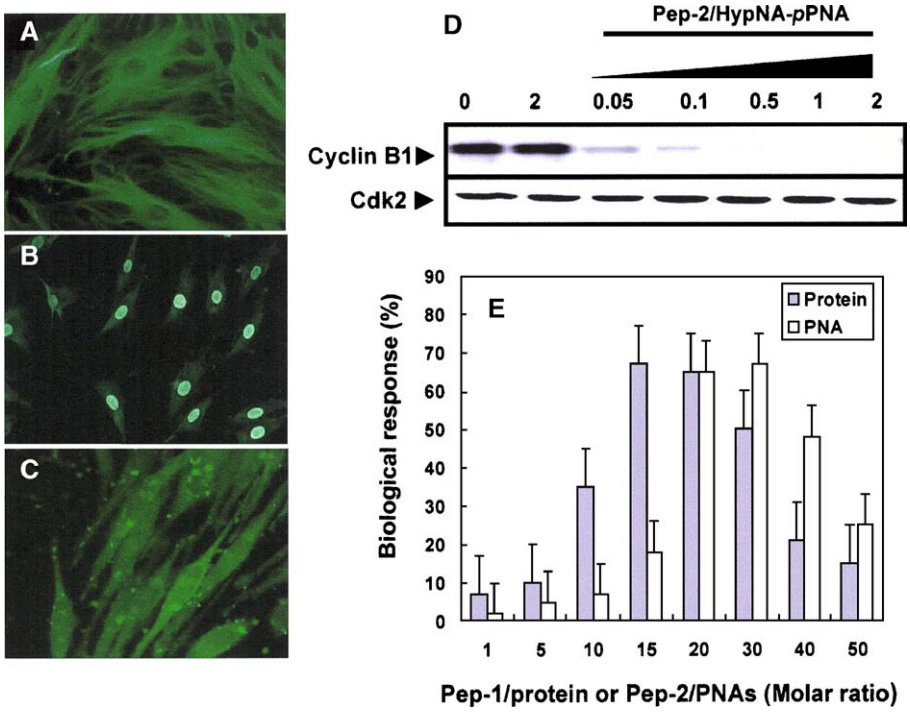

Fig. 4. Pep-1 mediated delivery of macromolecules into mammalian cells. A concentration of 0.1 μM (0.7 μg) of FITC-labeled monoclonal anti-β-actin (panel A) or 51-mer peptides (0.1 μg) (panel B) was associated with Pep-1 at molar ratio 20/1. A concentration of 0.1 μM of FITC-labeled PNA (Panel C) was associated with Pep-2 at a molar ratio 10/1. Cells were incubated with complexes for 1 h and then extensively washed prior to observation by confocal microscopy. Pep-2-mediated delivery of PNA and HypNA-pPNA inhibits cyclin B1 expression. Increasing concentrations of HypNA-*p*PNA were incubated with Pep-2 at a molar ratio of 1:25 at 37 °C for 1 h, then overlaid onto cultured cells, as previously described [34]. Cyclin B1 protein levels were analyzed by Western blotting after 24 h. Cdk2 protein was used as a control to normalize protein levels (panel D). Panel E: Concentration-dependent Pep-1 and Pep-2 mediated cellular delivery of protein and PNA analogs. Fixed concentrations of $p27^{kip1}$ (0.5 μM/1 μg) and of an antisense HypNA-*p*PNA targeting Cyclin B1 (0.1 μM/0.2 μg) were associated with either Pep-1 or Pep-2, at different molar ratios (from 1/1 to 50/1). The complexes were overlaid onto cultured HS-68 cells and the biological response being, either the level of G1-arrested cells induced by $p27^{kip1}$ or the decrease in Cyclin B1 protein levels associated to antisense HypNA-*p*PNA, was monitored 24 h after transduction.

potential by demonstrating that we could specifically reduce cyclin B1 protein (Fig. 4D), and efficiently block cancer cell proliferation at very low concentrations of an antisense HypNA-*p*PNA designed to target cyclin B1 [34]. We were able to reduce both the doses and the time required for them to induce a specific and robust biological response significantly, thereby limiting non-specific cytotoxic effects described upon treatment with high concentrations of PNAs.

Pep-1 and Pep-2 are manufactured under the name of Chariot™ I and II by Active Motif inc. (http://www.activemotif.com) and an update of the published work on the Chariot strategy is available on the website of the company. Pep-1 protocols have been optimized according to the cell type or the nature of the cargo and important parameters that need to be taken into account have been defined; including reagent concentration, cell confluence, toxicity of the cargo and exposure time of cells to the Pep1/macromolecule complexes [24]. The formation of Pep-1/Protein complexes is a critical step which is directly associated to the ratio of Pep-1/cargo and to the particle and therefore to the cellular uptake mechanism. As a general rule, this ratio should be maintained between 15/1 and 20/1 to avoid aggregation/precipitation and to obtain an optimal associated biological response (Fig. 4E). However, the nature and size of specific cargo may affect the stability of the Pep-1/cargoes particle and/or its dissociation within the cell. As Pep-1 establishes mainly hydrophobic interactions with cargoes, complex assembly depends on the structure, hydrophobic pattern and biophysical properties of both components and complex formation can therefore vary between different combinations of Pep-1 and macromolecules [23]. Moreover, a large implication of the electrostatic domain of Pep-1, which is required for cellular uptake, in the interaction with the cargo may reduce its efficiency. Finally, the size of the protein or peptide may also affect the "decaging" step, and low efficiency may be observed for mainly electrostatic peptides shorter than 15 residues [23,24].

3.4. Pep-1 mediated PNA and protein transduction in vivo

Several Pep-1-based formulations for in vivo applications have been described including intra-venous, intra-tumoral, intra-tracheal injections as well as transduction into oocytes or sprays for skin or nasal delivery, suggesting that Pep-1 technology can be a potent technology for the delivery of therapeutic proteins in vivo [27,29,67–69]. Interestingly, Pep-1 has be shown to be an excellent technology for protein and antibody transduction in primary neurons and to be able to cross the blood–brain barrier [26,50], as already reported for several other CPPs [3,11]. Recently Pep-1 strategy has been applied in vivo to the delivery of caspase 3 into the lung of mice to

produce alveolar wall apoptosis [27], or to repair a defective step in a cellular signaling pathway in vivo. Maron et al. [29] have used Pep-1 for the delivery of PKA into distal lung epithelial cells of rat in order to correct the defect in that protein kinase. Pep-1 strategy was also applied for the evaluation of the antitumoral activity of peptide inhibitors of protein kinase or of antisense PNAs targeting cyclin B1, via intratumoral injections [68,69] as well as for the delivery of antibodies and protein into immature bovine and mouse oocytes, which offers a potent tool for studying early embryonic development [70,71].

4. Conclusions

Over the past 10 years, substantial progress has been made in the design of new technologies to improve cellular uptake of therapeutic compounds [1–5]. This evolution is directly correlated with the dramatic acceleration in the production of new therapeutic molecules, whilst cell delivery systems described until then were restricted by very specific issues. CPPs constitute one of the most promising generations of tools for delivering biologically active molecules into cells and can thereby have a major impact on future of treatments [1–7]. They have been shown to efficiently introduce drugs, antisense DNAs, PNAs, oligonucleotides and small proteins into cells both in vitro and in vivo. Peptide carriers present several advantages, in that they are modulatable, lack immunogenicity, are easy to produce and can incorporate a number of specific attributes required for efficient cargo delivery and for controlling their cellular behavior. Moreover, in addition to their usefulness in the laboratory, they are promising reagents for therapeutic applications [1–7].

Most of the CPPs described so far need a cross-linking step for delivery of the protein, which may be a limitation to their applications. In order to offer an alternative to covalent strategies, we have proposed a new potent strategy for the delivery of full-length proteins, peptides and PNAs into mammalian cells, based on a short amphipathic peptide carrier, Pep-1, which can form stable nano-particles with cargoes without the need for cross-linking or chemical modifications [23]. Large varieties of proteins, antibodies and peptides have been successfully introduced into different cell lines using Pep-1 strategy and have been shown to retain their full biological activity (Table 1). Pep-1 technology has been successfully applied to the delivery of different cargoes (PNAs, peptides and proteins) in different animal models [27,29,67–72]. This peptide-based protein delivery strategy presents several advantages including rapid delivery of proteins into cells with very high efficiency, stability in physiological buffers, lack of toxicity and lack of sensitivity to serum. The lack of prerequisite for covalent coupling to formation of Pep-1/macromolecule particles favors rapid release of peptides or proteins into the cytoplasm as soon as the cell membrane has been crossed. The final localization of the delivered macromolecule is then determined by its inherent intracellular targeting properties [23,24].

A major concern with the cellular uptake of CPPs is to avoid the endosomal pathway or to favor escape of the cargo from early endosomes. To this aim a domain-based strategy was recently proposed for the Tat-peptide, which combines the PTD sequence with a membranolytic peptide sequence, the HA2 fusogenic peptide of influenza to improve the release from the endosomes [40,72]. Pep-1 behaves significantly differently from other similarly designed cell-penetrating peptides. Although we cannot exclude that Pep-1 uptake follows several routes, the major Pep-1 cell translocation mechanism is independent of the endosomal pathway and involves transient membrane disorganization associated with folding of Pep-1 into a helical structure within the phospholipids membrane. Given that the mechanism through which Pep-1 delivers macromolecules does not involve the endosomal pathway, the degradation of macromolecules delivered is significantly limited and rapid dissociation of the Pep-1/macromolecule particle is favored as soon as it has crossed the cell membrane.

In conclusion, although, it is clear than Pep-1 technology is still in its early days and needs to be optimized for systematic in vivo applications, it is already a powerful tool for basic research, for studying the role of proteins and for targeting specific protein/protein interactions in vitro and in vivo, as well as in a therapeutic context for screening potential therapeutic proteins and peptides. We believe that Pep-1 technology constitutes a great alternative to covalent strategies and can have a major impact on the use of proteins and peptides for future therapies.

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