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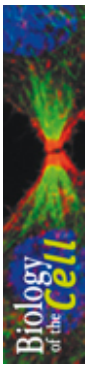
Review



Cell-penetrating peptides: from molecular mechanisms to therapeutics

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The recent discovery of new potent therapeutic molecules which do not reach the clinic due to poor delivery and low bioavailability have made the delivery of molecules a keystone in therapeutic development. Several technologies have been designed to improve cellular uptake of therapeutic molecules, including CPPs (cell-penetrating peptides), which represent a new and innovative concept to bypass the problem of bioavailability of drugs. CPPs constitute very promising tools and have been successfully applied for *in vivo*. Two CPP strategies have been described to date; the first one requires chemical linkage between the drug and the carrier for cellular drug internalization, and the second is based on the formation of stable complexes with drugs, depending on their chemical nature. The Pep and MPG families are short amphipathic peptides, which form stable nanoparticles with proteins and nucleic acids respectively. MPG- and Pep-based nanoparticles enter cells independently of the endosomal pathway and efficiently deliver cargoes, in a fully biologically active form, into a large variety of cell lines, as well as in animal models. This review focuses on the structure–function relationship of non-covalent MPG and Pep-1 strategies, and their requirement for cellular uptake of biomolecules and applications in cultured cells and animal models.

Introduction

The poor permeability of the plasma membrane of eukaryotic cells to drugs and DNA, together with the low efficiency of DNA or oligonucleotides to reach their target within cells, constitute the two major barriers for the development of therapeutic molecules. Therefore, over last 10 years, substantial progress has been made in the design of new technologies to improve cellular uptake of therapeutic compounds (Opalinska and Gewirtz, 2002; Järver and Langel, 2004; Glover et al., 2005; Torchilin, 2005; De Fougerolles et al., 2007; Kong and Mooney, 2007). This development has been directly correlated with the dramatic acceleration in the production of new therapeutic molecules. Before then, cell delivery systems were restricted by specific problems. A number of non-viral strategies have been proposed, including lipid-, polycationic-, nanoparticle- and peptide-based methods (Morris et al., 2000; Ogris and Wagner, 2002; Järver and Langel, 2004; Torchilin, 2005), but only a few of these technologies have been efficiently applied *in vivo* at either pre-clinical or clinical levels. Their major limitations include the poor stability of the complexes and the rapid degradation of the cargo, as well as its insufficient ability to reach its target. CPPs (cell-penetrating peptides) constitute one of the most promising tools for delivering biologically active molecules into cells and therefore play a key role in the future of disease treatments (Järver and Langel, 2004; Joliot and Prochiantz, 2004; Langel, 2007; Moschos et al., 2007). CPPs have been shown to efficiently improve intracellular delivery of various biomolecules, including plasmid DNA, oligonucleotides, siRNA (short interfering RNA), PNA (peptide nucleic acid), proteins and peptides, as well as liposome nanoparticles, into cells both *in vivo* and *in vitro*. Short synthetic CPPs have been designed to overcome both extracellular and intracellular limitations, and to trigger the movement of a cargo across

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Key words: cell-penetrating peptide (CPP), drug delivery, nanoparticle, non-covalent delivery system, short interfering RNA (siRNA).

Abbreviations used: CPP, cell-penetrating peptide; FTIR, Fourier-transform infrared; GAG, glycosaminoglycan; gp41, glycoprotein 41; HypNA–*p*PNA, *trans*-4-hydroxyl-L-proline–phosphonate-PNA; NLS, nuclear localization sequence; PEG, poly(ethylene glycol); PNA, peptide nucleic acid; siRNA, short interfering RNA.

the cell membrane into the cytoplasm and improve its intracellular trafficking, thereby facilitating interactions with the target (Gariepy and Kawamura, 2000; Morris et al., 2000; Järver and Langel 2004; Joliot and Prochiantz, 2004;, Deshayes et al., 2005; Snyder and Dowdy, 2005; Langel, 2007). Two major strategies have been described: (1) the covalent linkage of the cargo to the CPP, thereby forming a conjugate which is achieved by either chemical cross-linking, cloning or expression of a protein fused to the CPP (Nagahara et al., 1998; Gait, 2003; Moulton and Moulton, 2004; Zatsepin et al., 2005); and (2) the formation of a non-covalent complex between the two partners. Peptides derived from the *trans*-activating regulatory protein, TAT, of HIV (Frankel and Pabo, 1998; Fawell et al., 1994; Vives et al., 1997; Schwarze et al., 1999), the third α-helix of Antennapedia homeodomain protein (Derossi et al., 1994; Joliot and Prochiantz; 2004), the VP22 protein from herpes simplex virus (Elliott and O'Hare, 1997), the polyarginine peptide sequence (Wender et al., 2000; Futaki et al., 2001), peptides derived from calcitonin (Schmidt et al., 1998; Krauss et al., 2004) or from antimicrobial peptides buforin I and SynB (Park et al., 2000), as well as polyproline sweet-arrow peptide (Pujals et al., 2006), transportan and derivates (Pooga et al., 1998, 2001; Järver and Langel, 2004) 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 (Joliot and Prochiantz, 2004; El-Andaloussi et al., 2005; Murriel and Dowdy, 2006; Langel, 2007). Although conjugation methods offer several advantages for *in vivo* applications, including rationalization and control of the CPP–cargo, they remain limited from the chemical point of view, as they risk altering the biological activity of the cargoes. In order to offer an alternative to covalent methods, we have proposed a new potent strategy for the delivery of biomolecules into mammalian cells, on the basis of the short amphipathic peptide carriers MPG and Pep (Morris et al., 1997, 2001; Simeoni et al., 2003). MPG and Pep form stable nanoparticles with cargoes without the need for cross-linking or chemical modifications. MPG efficiently delivers nucleic acids (plasmid DNA, oligonucleotides and siRNA) and Pep improves the delivery of proteins and peptides in a fully biologically active form into a variety of cell lines and *in vivo* (Morris et al., 2001, 2007a, 2007b; Simeoni et al., 2003, 2005). This non-covalent strategy has been recently extended to other CPPs, including TAT (Meade and Dowdy, 2007), polyarginine (Kim et al., 2006; Kumar et al., 2007) and transportan (Lundberg et al., 2007). The mechanism through which MPG and Pep deliver active macromolecules does not involve the endosomal pathway and therefore allows the controlled release of the cargo into the appropriate target subcellular compartment (Deshayes et al., 2004a, 2004b).

In this review we will describe the characteristics and mechanism(s) of amphipathic peptide-based non-covalent strategies in the general context of the CPPs. We will also highlight the use of both MPG and Pep carriers for the delivery of nucleic acids, peptides or analogues both *in vitro* and *in vivo*.

MPG and Pep families

Design and structure of MPG and Pep-1

An amphipathic molecule can be defined, in short, as consisting of two domains: a hydrophilic (polar) domain and a hydrophobic (non-polar) domain. For peptides, the amphipathic character may arise from either the primary structure or the secondary structure. Primary amphipathic peptides can be defined as the sequential assembly of a domain of hydrophobic residues with a domain of hydrophilic residues. Secondary amphipathic peptides are generated by the conformational state which allows positioning of hydrophobic and hydrophilic residues on opposite sides of the molecule. MPG (27 residues: GALFLGFLGAAGSTMGAWSQPKKKRKV) and Pep-1 (21 residues: KETWWETWWTEWSQPKKKRKV) are primary amphipathic peptides (Figure 1A), consisting of three domains: a variable N-terminal hydrophobic motif; a hydrophilic lysine-rich domain, which, in both peptide families, is derived from the NLS (nuclear localization sequence) of SV40 (simian virus 40) large T-antigen (KKKRKV), and is required for the main interactions with nucleic acids, intracellular trafficking of the cargo and solubility of the peptide vector; and a linker domain (WSQP), separating the two domains mentioned above, that contains a proline residue, which improves the flexibility and the integrity of both the hydrophobic and the hydrophilic domains (Morris et al., 1997, 1999a; Simeoni et al., 2003). The two peptide families differ mainly in their

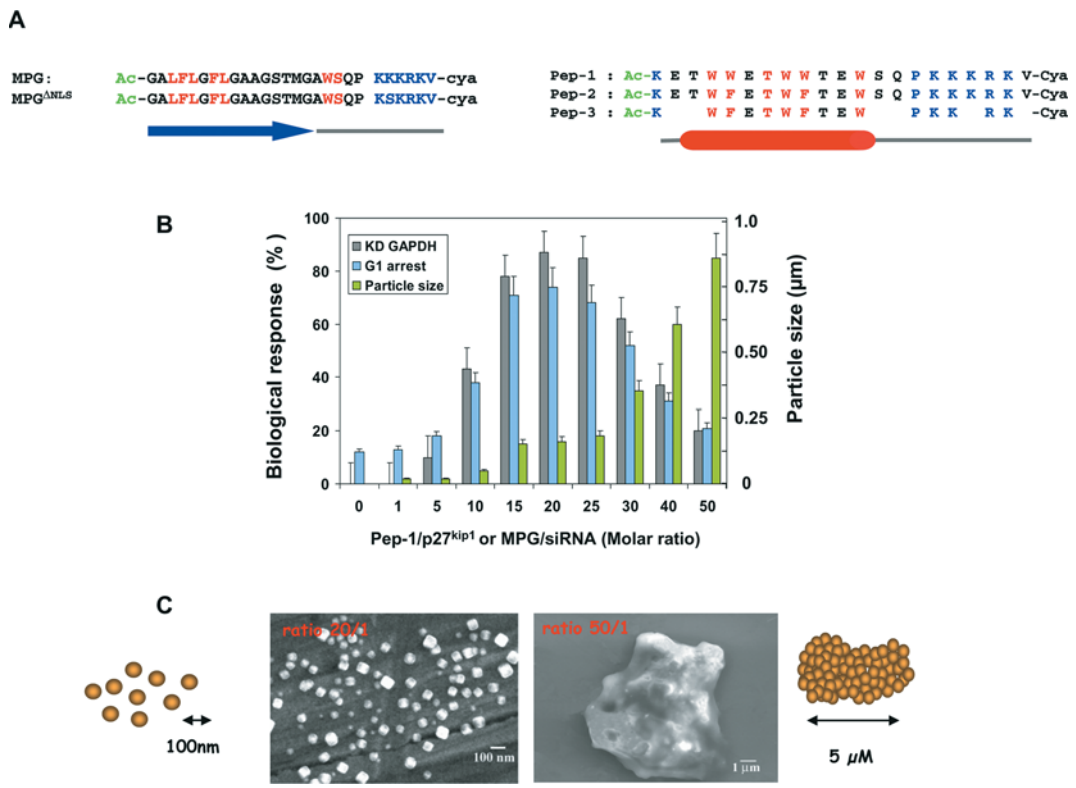
hydrophobic domain. The hydrophobic moiety of MPG (GALFLGFLGAAGSTMGA), derived from the fusion sequence of the HIV gp41 (glycoprotein 41), is required for efficient targeting to the cell membrane and cellular uptake. The hydrophobic motif of Pep-1 corresponds to a tryptophan-rich cluster (KETWWETWWTEW), which is also required for efficient targeting to the cell membrane and for forming hydrophobic interactions with proteins. Structural and mechanistic investigations have revealed that the flexibility between the two domains of MPG or Pep-1, which are maintained by the linker sequence between the fusion and the NLS motifs, is crucial for macromolecule delivery (Morris et al., 1999a; Simeoni et al., 2003; Deshayes et al., 2004a; Simeoni et al., 2005). Both peptide sequences are acetylated at their N-terminus and carry a cysteamide group at their C-terminus, both of which are essential for the stability of the peptides and their transduction mechanism. The cysteamide function is also essential for cellular uptake mechanisms and is required for stabilization of the carrier–cargo particles (Mery et al., 1993; Morris et al., 2001, 2007b; Simeoni et al., 2003). It is clearly established that for potent cell entry, the cysteamide group needs to be free and not involved in a disulfide bridge. The sequence of MPG has been modified in order to facilitate rapid release of the cargo into the cytoplasm and to allow functionalization of the carrier for selective *in vivo* targeting (Simeoni et al., 2003, Crombez et al., 2007). A single mutation on the second lysine residue of the NLS sequence to a serine ($MPG^{\Delta NLS}$; GALFLGFLGAAGSTMGAWSQPKSKRKV) abolishes the nuclear translocation property (Simeoni et al., 2003). Several modifications of Pep-1 sequences have also been proposed to stabilize the cargo–carrier complexes or to extend the potency of this strategy to other cargo molecules (Figure 1A). Pep-2 was reported to facilitate the cellular uptake of PNAs and analogues *in cellulo* (Morris et al., 2004a). A rational approach based on Pep-2 was performed to identify residues required for optimal carrier functions and parameters for the optimization of Pep sequence (Morris et al., 2007b) as follows: (1) the sequence should bear a minimum of four cationic residues within the hydrophilic domain for the initial electrostatic interactions between the peptide and cell membrane components; (2) the presence of a charge (Lys^1) at the N-terminus of the peptide and the flexibility between the hydrophobic and the hydrophilic domains associated with the presence of a proline (Pro^{14}) residue are essential; and (3) the helical structure of Pep carriers and the position of a Trp–Phe tandem on the same side of the helix are required for the interaction with the cell membrane, and to enable aromatic residues to interact with lipids and consequently favour membrane disorganization (Deshayes et al., 2004a). Taking these factors into account a new peptide, Pep-3, was designed, which tends to adopt a helical structure within membranes, forms 'nanoparticles' with charged and uncharged PNAs and improves their delivery into a large range of cell lines, as well as in animal models. Moreover, Pep-3 has been PEGylated [where PEG is poly(ethylene glycol)] at the N-terminus to improve *in vivo* delivery of biomolecules (Morris et al., 2004a, 2007b).

Non-covalent strategy: formation of carrier–cargo complexes

MPG and Pep-1 peptides associate rapidly in solution with their respective cargo (oligonucleotide or protein/peptide) through non-covalent electrostatic or hydrophobic interactions and form stable complexes independently of specific sequences (Morris et al., 1997, 1999a, 2001). MPG and Pep-1 exhibit high affinity in the nanomolar range for their respective cargoes. MPG interacts with small single- and double-stranded oligonucleotides, as well as with large plasmid DNA. The carrier–oligonucleotide interactions are initiated by the electrostatic NLS domain, and then followed by peptide–peptide interactions through the gp41 hydrophobic domain, thus generating a peptide cage around the nucleic acid (Morris et al., 1999a; Marthinet et al., 2004). Pep-1 forms stable peptide-based nanostructures around a diverse range of cargoes, including proteins, peptides, quantum dots, viruses and uncharged hydrophobic molecules (Morris et al., 2001, 2007a; Gros et al., 2006). The interactions involve both the aromatic residues of the hydrophobic domain of Pep-1, as well as the helical structural organization of the carrier (Morris et al., 2001, 2007b; Deshayes et al., 2004a). Both MPG and Pep peptides form nanoparticles with their respective cargoes, including several peptide molecules for one molecule of cargo, which significantly improves the stability of the cargo inside the cell and significantly protects it from degradation. Basically, the molar ratio of the carrier–cargo complex is approx. 10:25, depending on the size of the nucleic

Figure 1 | MPG- and Pep-based nanoparticles for macromolecule delivery

(**A**) Sequence alignment and secondary structure of the different peptide carriers. Residues involved in cargo binding and cellular uptake are in red and blue respectively. The structure of the hydrophobic domain of Pep and MPG carrier peptides were obtained on the basis of NMR/CD and FTIR studies (Deshayes et al., 2004a, 2004b). Ac, acetyl; Cya, cysteamide. (**B**) Correlation between the associated biological response and the size of MPG–siRNA and Pep-1–protein particles. MPG–siRNA (siRNA targeting GAPDH) and Pep-1–p27^{Kip} complexes were formed at various ratios from 1:1 to 50:1 in PBS, the volume adjusted to 500 μl in DMEM (Dulbecco's modified Eagle's medium) and applied to HS68 fibroblasts. The size of particle complexes was determined by light-scattering measurement and their ability to induce a biological response; siRNA-mediated knockdown (KD) of GAPDH or p27^{Kip}-dependent G_1 arrest was determined either by Western blotting or by FACS analysis. (**C**) Pep-1 forms stable nanoparticles with protein cargoes. The p27^{Kip} protein was used as a cargo, and Pep-1–p27^{Kip} complexes were formed in water at ratios of 20:1 and 50:1, and analysed by scanning electron microscopy. The complexes obtained at a 20:1 ratio form discrete nanoparticles of approx. 100 nm diameter. In contrast, when the concentration of Pep-1 is increased to reach a Pep-1/cargo molar ratio of 50:1, large aggregates are observed from which nanoparticular buds protrude.



acid or of the peptide–protein cargo (Morris et al., 1999a; Simeoni et al., 2003; Gros et al., 2006). Active peptide–cargo complexes were identified as discrete nanoparticles, with an estimated size between 100 and 200 nm in diameter as determined by light scattering and further characterized by scanning electron microscopy (Munoz-Morris et al., 2007). Both the size and homogeneity of the nanoparticles are dependent on the carrier/cargo ratio and optimal *in vivo* biologically efficiency has been reported for a carrier/cargo ratio of approx. 10:1 to 15:1, depending on the nature of the cargo. The use of greater ratios induces precipitation or formation of larger particles that hardly enter cells (Gros et al., 2006; Munoz-Morris et al., 2007; Morris et al., 2007b) (Figure 1B).

Cellular uptake mechanism of CPPs

Major rules: artefacts or reality?

Understanding the cellular uptake mechanism of CPPs is essential for the development of appropriate strategies and optimization for therapeutic

applications *in vivo*. In the last 5 years, researchers in the CPP field have learnt much from artefacts that have been associated with fixation methods, as described by several groups (Lundberg et al., 2003; Richard et al., 2003), or the use of fluorescent probes attached to CPPs that can modify their cellular behaviour (Pichon et al., 1999). Therefore important rules for the evaluation of CPP mechanisms have been proposed and allowed the revision of several cellular uptake mechanisms, which had been shown to be essentially associated with the endosomal pathway (Richard et al., 2003, 2005; Nakase et al., 2004; Wadia et al., 2004; Fischer et al., 2005; Murriel and Dowdy, 2007). As such, evidence for several routes of cell entry have been reported for most CPPs, depending on the nature of the CPP, of the cargo and of their active concentrations, some of which are independent of the endosomal pathway and involve the transmembrane potential (Terrone et al., 2003; Thoren et al., 2003; Rothbard et al., 2004; Deshayes et al., 2005). Investigation of the mechanism of internalization requires identification of several physicochemical properties of the carrier peptides, including membrane interactions and cellular release. Visualization of CPPs inside the cell remains limited to the use of fluorescein-labelled CPPs, with the risk that fluorescent dyes alter the uptake mechanism or trigger an unusual cell entry pathway. However, it is now clearly established that artefacts associated with fixation protocols and the nature of the dyes have to be taken into account and only experiments reported in live cells are useful (Lundberg and Johansson, 2001; Lundberg et al., 2003; Richard et al., 2003). Moreover, as several routes may exist, it is essential to identify the one actually yielding a biological response. Therefore an essential rule when investigating the uptake mechanism of CPPs is to correlate the uptake pathway with a biological response associated with a specific cargo (Wadia et al., 2004; Gros et al., 2006).

Cellular uptake mechanism: involvement of the secondary structure of the CPPs

Understanding the interactions of CPPs with phospholipids constitutes a major issue. An important criterion to be considered is the structural requirements for cellular uptake of CPPs and their ability to interact with the cell surface and lipid moieties of the cell membrane. A variety of physical and spectroscopic approaches can be combined to gain insight into the structure(s) involved in the interactions of peptides with membranes, and to monitor the association of carrier–cargo complexes with lipids, and thus to characterize their mechanism of cellular internalization (Magzoub and Gräslund, 2004; Deshayes et al., 2005, 2006, 2007; Esbjokner et al., 2007). The most commonly used biophysical methods are briefly described in this section. NMR has been used for detailed identification of membrane-interacting protein and peptide structures. Most investigations have been carried out in the presence of micelles or bicelles, which give rise to resolved NMR spectra (Magzoub and Gräslund, 2004; Esbjokner et al., 2007). However, the relevance to membranes of the information obtained must be questioned, as nearly all studies indicate that peptides in the presence of micelles adopt a helical conformation, although this is not always the case (Deshayes et al., 2004a, 2004b). FTIR (Fourier-transform infrared) spectroscopy is one of the easiest methods to handle and provide information on the global conformational state, and therefore enables detection of conformational changes due to variations in the environment. FTIR also allows determination of the peptide orientation with respect to the interface (Van Mau et al., 1999; Tatulian and Tamm, 2000). CD is frequently used to identify the secondary structures of proteins or peptides under membrane-mimicking conditions. In order to obtain reliable information on the conformation of the protein or peptide, it is strongly recommended to record spectra in the far-UV region, as low as 180 nm, which precludes the use of most common buffers and salts, and can be, in some cases, a limitation for producing membrane-mimicking conditions. Fluorescence spectroscopy is a powerful method to study lipid–peptide and/or peptide–peptide interactions. The use of either intrinsic fluorescence of peptides, or of fluorescently labelled lipids or peptides, allows the determination of the thermodynamic and kinetic parameters of peptide–membrane interactions. Fluorescence quenching or FRET (fluorescence resonance energy transfer) has been used to quantify and monitor the entry of CPPs in the membrane (Deshayes et al., 2004a; Bjorklund et al., 2006). Although this method allows direct measurement of CPP–membrane interactions, the choice of the fluorescent probe is of a major importance in order to maintain the amphipathic properties of the peptide, as large

hydrophobic fluorescent dyes can have an impact on both the uptake mechanism and the kinetics of the interaction with the membrane. Mimicking bilayer membranes remains difficult to handle, therefore monomolecular films have been largely used as they constitute a good means of performing model experiments, since the thermodynamic relationship between monolayers and bilayer membranes is direct, and monomolecular films at the air–water interface overcome limitations, such as regulation of lipid lateral packing and lipid composition that occur in bilayers. This technology allows the investigation of the influence of pressure on the conformational state of peptides, measurement of the strength and the nature of peptide–lipid interactions (Chiang et al., 1996; Nordera et al., 1997), and offers the advantage of studying mixed lipid–peptide films and various membrane compositions in correlation with cell types (Verger and Pattus, 1976). Monolayers also allow identification of the conformational state of peptides and positioning by *in situ* observations, such as infrared or grazing incidence X-ray diffraction. Other techniques have also been used to monitor either the direct interaction of CPPs with lipids or the cellular uptake of CPPs, such as MS (Li et al., 2005; Burlina et al., 2007; Palm et al., 2007), atomic force microscopy (Deshayes et al., 2004c; Plenat et al., 2004) and small-angle electron diffusion (Deshayes et al., 2006; Plenat et al., 2004), which allow observation of nanometre-sized complexes and/or provide information on topographical organization of CPP within monolayers or bilayers. As these methods use either different concentrations or membrane states, it is highly recommended to describe the behaviour of lipid-interacting peptides combining several approaches to avoid misinterpretations. CPP-induced channel formation or changes in membrane conductance can be evaluated in artificial planar lipid bilayers, as well as by electrophysiological studies monitoring patch-clamped *Xenopus* oocytes, with the major advantage of using a natural membrane, and allow the investigation of the voltage-dependence of the CPP mechanism (Balali-Mood et al., 2005; Deshayes et al., 2006; Plenat et al., 2004). Combining these biophysical approaches together with molecular modelling methods constitutes a powerful means of understanding the mechanism of CPPs and to either identify new potent CPPs or optimize already described CPPs (Thomas et al., 2006).

Structure-mediated cellular uptake of MPG and Pep-1

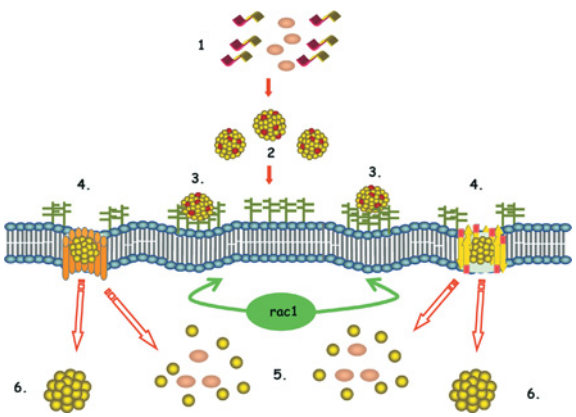
The combination of the above-mentioned technologies has allowed a better insight into cellular uptake mechanism of CPPs, in particular MPG and Pep-1. In contrast with numerous other CPPs, both MPG and Pep-1 peptides, associated or not with their respective cargoes, have been shown to strongly interact and to spontaneously penetrate lipid-phase and insert into natural membranes (Deshayes et al., 2004a, 2004b). Both peptide families form stable 1:1 complexes with lipids, as determined by MS (Li et al., 2005). The peptide–lipid interactions are initiated by their hydrophobic domain, either the gp41 fusion sequence of MPG or the tryptophan-rich motif of Pep-1, which are crucial for insertion of the peptide into the membrane, and corroborate the notion that hydrophobic interactions between fusion proteins and cell-membrane phospholipids initiate membrane perturbation in the early stages of viral fusion. Moreover, the fact that MPG and Pep-1 bind more tightly to negatively charged lipids [DLPG (dilauroylphosphatidylglycerol) or DMPG (dimyristoylphosphatidylglycerol)] than overall neutral zwitterionic ones [DLPC (dilauroylphosphatidylcholine) and DMPC (dimyristoylphosphatidylcholine)] reveals that electrostatic interactions clearly play a significant role in stabilizing peptide–lipid complexes (Li et al., 2005). Pep-1 and MPG are extremely versatile from the structural point of view; both are non-structured in water at low concentrations and their conformations are not significantly affected on the formation of particles with their cargo. In contrast, NMR, CD and FTIR analysis have revealed that the interaction of peptide carriers or of carrier–cargo complexes with phospholipids results in folding of the carrier, the hydrophobic N-terminal domain of MPG and Pep-1 fold into a β-sheet structure and an α-helix respectively, whereas the rest of the molecule (linker plus NLS) remains unstructured (Deshayes et al., 2004a, 2004b). For both peptide families, folding of the N-terminal domain is a major step in the mechanism. The helical structure of Pep-1 and derivative peptides within the lipid phase of the membrane is essential for its cellular uptake and the stabilization of the Pep–cargo particles (Morris et al., 2001; Deshayes et al., 2004a). The β-sheet structure of MPG is also a prerequisite for cellular uptake, as slight changes in the MPG sequence introduced

to favour a helical conformation have been reported to strengthen interactions with the lipid moiety, but also to reduce cellular uptake and trigger the endosomal pathway (Deshayes et al., 2004b; Veldhoen et al., 2007). A potential-induced reorientation of MPG and Pep peptides has been identified following membrane insertion. Once the hydrogen-binding pattern has been completed, the peptides are not folded in aqueous medium, implying that the peptide bonds are not engaged in hydrogen bonds, thereby precluding their insertion into the hydrophobic layer of a membrane for energetic reasons (Wimley, 1996; Deshayes et al., 2004a, 2004b; Plenat et al., 2004). Due to the ability of Pep-1 and MPG to fold into α-helical and β-sheet structures respectively, on interaction with phospholipids their folding allows their insertion into the membrane associated with a potential-induced transmembrane orientation of the folded hydrophobic domains. Although these peptides are highly positively charged similar to other CPPs, such as TAT or oligo-arginines, which do not fold and therefore accumulate at the membrane surface, the existence of a folded hydrophobic domain precludes such an accumulation and favours membrane insertion. The driving force which dictates the internalization pathway (via endosomes or not) is governed by the affinity of the carrier peptide for phospholipids. The outer part of the 'carrier-based nanoparticle', together with the cargo, plays a key role in the interactions with the membrane and forms transient transmembrane α-helical or β-sheet structures, depending on the carrier, which temporarily affect the cell-membrane organization, without associated leakage or toxicity, thereby facilitating insertion into the membrane and initiation of the translocation process. The potency of the peptide to directly interact with lipids limits their association with proteoglycans at the surface of the cell, speeds up cellular entry and therefore reduces the risk of uptake through the endosomal pathway (Deshayes et al., 2004a; Gerbal-Chaloin et al., 2007).

On the basis of both structural and biophysical investigations, a four-step mechanism has been proposed (Figure 2): (a) formation of the MPG–cargo or 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, which involves electrostatic contacts with the phospholipid head groups; (c) insertion of the complex into the membrane, which is associated with conformational changes that induce membrane structure perturbations; (d) nuclear targeting of the MPG–cargo complex or release of the Pep-1–cargo complex into the cytoplasm with partial 'de-caging' of the cargo.

Figure 2 | Mechanism of cellular uptake of MPG–cargo or Pep-1–cargo complexes

The MPG-/Pep-mediated cellular uptake mechanism can be divided into six major steps. (1) Formation of the carrier—cargo complexes through electrostatic and hydrophobic interactions; (2) interaction of the carrier–cargo nanoparticles with the cell surface involving electrostatic contacts with proteoglycans (3), then with the phospholipid head groups; (4) the direct interaction of the peptide with the lipid phase of the cell membrane, together with the Rac1-associated membrane dynamics, allows the insertion of the complex into the membrane, followed by formation of transient transmembrane β-sheet or α-helical structures; (5) finally, the Pep-1–cargo or MPG–cargo (siRNA) complexes is released into the cytoplasm or (6) is targeted to the nucleus or to specific organelles.



The gates of cells: a biological point of view

Role of proteoglycans

Proteoglycans play an essential role in the regulation of cell-surface microdomains, and evidence for direct relationships between cytoskeletal organization and activation of small GTPases has been clearly established (Conner and Schmid, 2003; Eitzen, 2003). HSPGs (heparan sulfate proteoglycans) and syndecans, which are the major components of the extracellular matrix, act as anchors for many external molecules and pathogens at the host cell surface

(Esclatine et al., 2001; Yoneda and Couchman., 2003). Their clustering triggers cytoskeletal remodelling upon activation of PKC (protein kinase C) and Rho/Rac GTPases, which control the dynamics of cholesterol-rich 'raft' microdomains, and therefore ligand binding and cellular uptake pathways (Dehio et al., 1998; Saoncella et al., 1999; Couchman, 2003; Beauvais and Rapraeger, 2004). The first contacts between the CPPs and the cell surface take place through proteoglycans. Cell-surface proteoglycan clustering and remodelling of the actin network serve as a 'capture platform', thereby triggering the 'onset' of internalization of polycationic carriers, such as polycationic peptides, cationic lipids and polyethylenimide (Rusnati et al., 1999; Belting and Peterson, 1999; Wiethoff et al., 2001). One of the major differences between CPPs is their mode of interaction with the cellular surface components. The interaction of peptides, such as TAT (Console et al., 2003; Richard et al., 2003; Wadai et al., 2004; Murriel and Dowdy, 2006) polyarginine and penetratin (Nakase et al., 2004, 2007), with the extracellular matrix has been reported to be primarily electrostatic and to trigger uptake through the endosomal pathway (Rusnati et al., 1999; Murriel and Dowdy, 2006). As most probably the case for all CPPs, the uptake mechanism of MPG and Pep-1 is also initiated by highly dynamic electrostatic interactions with the extracellular matrix. The binding of MPG or the MPG–cargo complex to the GAG (glycosaminoglycan) platform is followed by a selective activation of the GTPase Rac1, which allows for remodelling of the actin network. GTPase activation and actin remodelling constitute the 'onset' of the internalization mechanism and have a major impact on membrane fluidity, thereby promoting entry of MPG or the MPG–DNA complexes into cells (Gerbal-Chaloin et al., 2007). Although the overall uptake mechanism is dependent on the inherent properties of the peptide, we and other groups have demonstrated a direct involvement of small GTPases, mainly Rac1, in controlling or enhancing the rate of the initial step of the uptake mechanism (Gerbal-Chaloin et al., 2007; Nakase et al., 2007).

Cell entry and trafficking pathways

Following binding to the GAG platform, which facilitates accumulation of the CPP and CPP–cargo complexes at the cell surface, different cell entry gates have been reported for different CPPs. Correlation of cellular uptake with a cargo-associated biological response is a major requirement to validate the efficiency of a CPP, as originally established for TAT (Wadia et al., 2004) and now extended to series of well-known CPPs (Nakase et al., 2004; Padari et al., 2005). Internalization mechanisms involving macropinocytosis (Wadia et al., 2004; Kaplan et al., 2005), and clathrin- and caveolin-dependent endocytosis have been described (Richard et al., 2005; Ziegler et al., 2005). Although these latter investigations clearly indicate that endocytosis is involved in the internalization of CPPs, it appears that different mechanisms may occur simultaneously. In the cases of penetratin and transportan, both membrane translocation and endocytosis have been suggested to occur simultaneously (Letoha et al., 2003; Säälik et al., 2004).

The ability of MPG and Pep peptides to interact with the lipid moiety of the membrane has been shown to play an essential role in their cellular uptake route. Investigations of the ability of MPG to transfect plasmids encoding reporter genes or siRNAs in the presence of several inhibitors of the endosomal pathway demonstrates that uptake of the MPG–DNA complex that generates a biological response is independent of the endosomal pathway, and mediated by the membrane potential and dependent on the size of the MPG–DNA particles (Simeoni et al., 2003; Deshayes et al., 2004b; Munoz-Morris et al., 2007). The NLS motif of MPG is required for both electrostatic interactions with DNA and nuclear targeting translocation of the nucleic acid. After crossing the cell membrane, MPG–DNA particles are able to interact with the nuclear import machinery and the presence of the NLS domain promotes rapid delivery of the plasmid into the nucleus (Morris et al., 1999a; Simeoni et al., 2003).

For Pep peptides, as for MPGs, we have shown that delivery of active molecules is not associated with the endosomal pathway and that most cargoes are rapidly released within the cytoplasm (Morris et al., 2001; Gros et al., 2006). Pep-1-mediated delivery of the cell-cycle inhibitor protein $p27^{Kip1}$ and the associated biological response was used to investigate the uptake mechanism of Pep-1–cargo particles *in cellulo*. Our studies demonstrate that none of the inhibitors of the endosomal pathway affect the efficiency of Pep-1, with the exception of energy deprivation which can

be directly correlated to modifications of membrane potential, which is known to be required for uptake of CPPs (Terrone et al., 2003). Therefore uptake of the Pep-1–cargo complex leading to a biological response is independent of the endosomal pathway and is directly correlated to the size of particles and the nature of cargoes (Gros et al., 2006; Munoz-Morris et al., 2007).

Application of MPG and Pep strategies to the delivery of therapeutic molecules

MPG-mediated delivery for nucleic acids

In the last decade, a number of CPP-based gene delivery systems have been proposed to overcome both extracellular and intracellular limitations (Morris et al., 2000; Niidome and Huang, 2002; Järver and Langel, 2004; Moulton and Moulton, 2004; El-Andaloussi et al., 2005). In order to improve nuclear delivery of cargoes (drugs or DNA), synthetic peptides containing NLSs have been used extensively (Cartier and Reszka, 2002; Escriou et al., 2003). Several strategies have been designed either to avoid the endosomal pathway and/or to facilitate the escape of cargoes from early endosomes to prevent their degradation. Peptide carriers that combine DNA-binding and membrane-destabilizing properties have been developed to facilitate gene transfer into cultured cells and living animals (Morris et al., 2000; Niidome and Huang, 2002; Glover et al., 2005). CPPs have also been used to improve the delivery of DNA (Branden et al., 1999; Tung et al., 2002; Ignatovich et al., 2003; Rudolph et al., 2003) or oligonucleotides (Moulton and Moulton, 2004; Zatsepin et al., 2004; El-Andaloussi et al., 2005) and have been combined with other lipid-based non-viral methods (Torchilin, 2005). Several CPPs have been successfully applied for the delivery of small oligonucleotides *in vivo* through covalent coupling (Gait et al., 2003; Moulton and Moulton, 2004; Zatsepin et al., 2005). In contrast, only a few CPPs have been validated *in vivo* for gene delivery. So far, the secondary amphipathic peptide PPTG1 constitutes one of the only examples reporting a significant *in vivo* gene expression response following intravenous injection (Rittner et al., 2002).

MPG technology has been applied to both plasmid DNA and oligonucleotide delivery with high efficiency into a large number of cells in suspension and adherent cell lines (Morris et al., 2007b). The ability of MPG to improve the nuclear translocation of nucleic acids without requiring nuclear membrane breakdown during mitosis has been reported in several protocols for gene and oligonucleotide delivery on primary cell lines and non-dividing cells (Simeoni et al., 2005; Morris et al., 2007). As the cellular uptake mechanism and therefore the efficiency of MPG are directly correlated to the MPG–cargo particle size, the procedure to prepare the MPG–cargo complexes and the molar ratio of these complexes are crucial parameters. In particular, the carrier/cargo ratio should be maintained between 20:1 and 40:1 to avoid any aggregation, as well as endosomal uptake, and to yield an optimal associated biological response. MPG technology has been reported to be a potent method for the delivery of unmodified antisense oligonucleotides and full-length antisense constructs targeting the cell-cycle regulatory protein Cdc25C (Morris et al., 1997, 1999a), locked nucleic acids, phosphorothioate oligonucleotides targeting the promoter of the MDR-1 (multidrug resistance 1) promoter in human CEM leukaemia cells (Marthinet et al., 2004; Labialle et al., 2006) and thio-phosphoramidate telomerase template antagonists in cancer cells (Asai et al., 2003; Gryaznov et al., 2003).

MPG-mediated delivery of siRNAs *in vitro* and *in vivo*

siRNAs constitute a powerful tool to silence gene expression post-transcriptionally (Hannon, 2002; Elbashir et al., 2001; Dorsett and Tuschl, 2004; De Fougerolles et al., 2007). However, the major limitation of siRNA applications, similar to most antisense- or nucleic-acid-based strategies, is their poor cellular uptake associated with the poor permeability of the cell membrane to nucleic acids. Several viral and non-viral strategies have been proposed to improve the delivery of either siRNA-expressing vectors or synthetic siRNAs both in cultured cells and *in vivo* (McManus and Sharp, 2002; Brummelkamp et al., 2002; Xia et al., 2002; Hommel et al., 2003; Song et al., 2003, 2005; Takeshita et al., 2005). The delivery of siRNAs *in vivo* constitutes a major challenge and, to date, there are no universal approaches (De Fougerolles et al., 2007) The most efficient method for *in vivo* applications remains the non-viral 'hydrodynamic' tail-vein injection of mice with high doses

of natural and modified siRNAs (McCaffrey et al., 2002; Song et al., 2003; Lewis et al., 2002; Fountaine et al., 2005; Soutscek et al., 2004). A strategy using CPPs has been used for the delivery of siRNAs into cultured cells, and siRNA covalently linked to transportan (Muratovska and Eccles, 2004) and penetratin (Davidson et al., 2004) have been associated with a silencing response. However, non-covalent strategies appear to be more appropriate for siRNA delivery and yield a significant associated biological response (Simeoni et al., 2003; Kim et al., 2006; Crombez et al., 2007; Meade and Dowdy, 2007; Lundberg et al., 2007; Kumar et al., 2007).

MPG forms stable non-covalent complexes with siRNAs, increases their stability, promotes their cellular uptake without the need for prior chemical covalent coupling and enables robust down-regulation of target mRNAs (Simeoni et al., 2003, 2005; Morris et al., 2007a). A variant of MPG with a single mutation in the NLS ($MPG^{\Delta NLS}$) was designed to favour rapid release of the siRNA into the cytoplasm, thereby allowing for a more significant biological response. Both peptide carriers can deliver siRNA into cultured cells with high efficiency, with a final subcellular localization of the siRNA dependent on the MPG carrier used. A fluorescently labelled siRNA localized rapidly to the nucleus when transfected with MPG, but remained mostly in the cytoplasm with $MPG^{\Delta NLS}$. These experiments reveal that the efficiency of MPG-like peptides is similar to that of Oligofectamine™, suggesting that MPG is able to release the siRNA rapidly without affecting its biological effect upon or following cellular internalization. The MPG strategy has been used for the delivery of siRNAs into a large range of cell lines, including adherent cell lines, cells in suspension, cancer and primary cell lines, which cannot be transfected using other non-viral approaches (Morris et al., 2004b; Langlois et al., 2005; Simeoni et al., 2005; Pastore et al., 2006; Nguyen et al., 2006). siRNA targets localize both to the cytoplasm and to the nucleus, depending on the mechanism involved. Tampering with the NLS sequence of MPG allows discrimination between delivery to the nucleus and the cytoplasm, and to control the release of the siRNA into the appropriate subcellular compartment (cytoplasm or nucleus). MPG can efficiently deliver promoter-directed siRNA into the nucleus, thereby inhibiting transcription (Morris et al., 2004; Langlois et al., 2005). In contrast $MPG^{\Delta NLS}$ rapidly releases the cargo into the cytoplasm. Moreover, MPG has been successfully applied to the delivery of siRNAs into animal models by topical intra-tumoral or systemic intravenous injections (M.C. Morris, L. Crombez and G. Divita, unpublished data), as well as for the delivery of siRNAs into mouse blastocytes (Zeineddine et al., 2006). This technology has been applied to target an essential cell-cycle protein, cyclin B1; intravenous injection of MPG–cyclin B1 siRNA particles has been shown to efficiently block tumour growth (Crombez et al., 2007; Morris et al., 2007b). MPG forms highly stable nanoparticles with siRNAs with a slow degradation rate that can be easily used for specific targeting; these are the major advantages for *in vivo* siRNA delivery over covalent CPP technologies. We have recently demonstrated that cholesterol- and PEG-functionalized MPG nanoparticles dramatically improve the efficiency of siRNA delivery *in vivo* (M.C. Morris, L. Crombez and G. Divita, unpublished data).

Pep-based strategy for transduction of macromolecules

In order to circumvent problems associated with 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 (Järver and Langel, 2004; El-Andaloussi et al., 2005; Snyder and Dowdy, 2006; Langel, 2007; Moschos et al., 2007). CPPs have been successfully used for the administration of large proteins and peptides that exhibit a therapeutic potential *in vivo*. Several CPP-based covalent strategies have been or are currently evaluated in clinical trials, proving that 'protein therapy' can have a major impact on the future of therapies in a variety of viral diseases and cancers (Snyder and Dowdy, 2006; Langel 2007).

Pep-1-mediated transduction of peptides and proteins *in vitro* and *in vivo*

Pep-1 technology has been applied to basic research, as well as to delivery of therapeutic peptides and proteins, with high efficiency, into a large number of mammalian cell lines, including non-transformed, cancer, neuronal and primary cell lines (Morris et al., 2001, 2006; Gallo et al., 2003). Several protocols for

Pep-1-based peptide and protein delivery have been described and key parameters standardized, including the Pep-1/cargo ratio, which is directly associated with the size of the particle and therefore the cellular uptake mechanism. This ratio should be maintained between 15:1 and 20:1 to avoid aggregation and precipitation, and to obtain an optimal associated biological response (Munoz-Morris et al., 2007). Pep-1 promotes the cellular uptake of small peptides and of large proteins, independently of the size and nature of the polypeptide and of the cell type (Gros et al., 2006; Morris et al., 2007b). The rapid release of the cargo into the cytoplasm allows for a rapid biological response and limits alterations in the biological function of the cargo. Pep-1 has been shown to deliver antibodies into cells, while preserving their ability to recognize their target antigens, which constitutes a major interest for therapeutic applications (Morris et al., 2007b). Pep-1 also improves the uptake of proteins and peptides into challenging primary cell lines, including neurons (Gallo, 2003), macrophages (Garnon et al., 2005), hepatocytes (Badag-Gorce et al., 2003; Tang et al., 2007), neural retinal cells (Gehler et al., 2004), human stem cells (Chan et al., 2005) and pancreatic cells (Pratt and Kinch, 2002; Pandey et al., 2003).

Pep-1 technology has been shown to be a potent strategy for the delivery of therapeutic proteins *in vivo* and can cross the blood–brain barrier (Gallo et al., 2002; Aoshiba et al., 2003; Payne et al., 2003; Rawe et al., 2004; Eum et al., 2004; Maron et al., 2005; Gros et al., 2006; Jevsek et al., 2006). Several Pep-1-based formulations for *in vivo* applications have been described, including intra-venous, intra-tumoral and intra-tracheal injections, as well as transduction into oocytes, sprays for nasal delivery or direct penetration through the skin. Pep-1 strategy has been applied *in vivo* to the delivery of proteins into the lungs of mice to produce alveolar wall apoptosis or to correct defects in PKA (protein kinase A) (Aoshiba et al., 2003; Maron et al., 2005). Pep-1 strategy was also applied for the evaluation of the anti-tumoral activity of peptide inhibitors of protein kinases or to repair a defective step in a cellular signalling pathway *in vivo* (Eum et al., 2004; Gros et al., 2006), as well as for the delivery of proteins into bovine and mouse oocytes, thereby providing an efficient means of studying early embryonic development (Payne et al., 2003; Rawe et al., 2004).

Pep-1-mediated transduction of biologically active molecules

Pep-1 strategy has been extended to the delivery of other uncharged and charged cargoes, including siRNAs (Arita et al., 2005), DNA–protein complexes (Morris et al., 2007b), replication-deficient viruses (Kowolik et al., 2003), PNAs (Morris et al., 2004a), DNA mimics (Morris et al., 2007b) and semiconductor quantum dots (Mattheakis et al., 2004). Pep-2 and Pep-3 carrier peptides have been used to improve the delivery of both uncharged PNAs and derivatives, such as HypNA-*p*PNAs (*trans*-4-hydroxyl-L-proline–phosphonate-PNA) (Efimov et al., 2001), into several cell lines and *in vivo* (Morris et al., 2004a, 2007b). Pep-2 strategy was applied to target essential proteins in different cellular pathways in several cell lines (Morris et al., 2004a, Nan et al., 2005). Pep-3 exhibits far less toxicity than other delivery systems and is appropriate for suspension and primary cell lines (Morris et al., 2007b). *In vivo*, bioavailability of DNA mimics constitutes a major limitation in therapeutics. Several CPP-based covalent approaches have been reported for the delivery of antisense PNA (Koppelhus and Neilsen, 2003), however, only a few have been used *in vivo* and until recently none of them were reported to be active at submicromolar concentrations (Opalinska and Gewirtz, 2002; Gait et al., 2003; Abes et al., 2007). Pep-3–antisense HypNA-*p*PNA particles targeting the cell-cycle regulatory protein cyclin B1 were successfully applied *in vivo* through intra-tumoral and intra-venous administration, and were found to inhibit tumour growth as efficiently as proven siRNA molecules (Morris et al., 2007b). PEGylation of the carrier improves the efficacy of the response by stabilizing the complexes. The study by Morris et al. (2007b) shows that such a modification significantly improves Pep-3 for *in vivo* systemic administration, allowing us to reduce the dose required to induce a specific and robust biological response, thereby limiting non-specific cytotoxic effects described upon treatment with high concentrations of PNAs. Together, these data reveal that Pep-3 constitutes an excellent candidate for *in vivo* delivery of charged PNA and DNA mimics.

Conclusions and perspectives

The dramatic acceleration in the discovery of new and highly potent therapeutic molecules which do not,

however, make it into the clinic due to poor delivery, low bioavailability and lack of rational targeting has made it clear that delivery was a keystone to therapeutic development (Kong and Moorey, 2007). To circumvent these problems studies have been focused on improving the chemistry of these molecules, and several delivery systems have recently been developed. A suitable drug carrier should (1) be biodegradable and biocompatible, (2) lack intrinsic toxicity and antigenicity, (3) show no accumulation in the body, (4) bear adequate functional groups for chemical fixation, (5) retain the original specificity for the target and (6) maintain the original activity of the delivered drug until it reaches the site of action. Accordingly, carrier peptides represent a new and innovative concept to bypass the problem of bioavailability of certain drugs, such as peptides, proteins and nucleic acids, which are currently rarely considered as therapeutics due to the above-mentioned limitations.

A large number of CPPs have been described that can be covalently attached to biomolecules and improve their delivery both into cultured cells and animal models. In order to offer an alternative to covalent strategies, we have proposed a new potent strategy for the delivery of cargoes into mammalian cells, on the basis of a short amphipathic peptide carrier, which can form stable nanoparticles with cargoes without the need for cross-linking or chemical modifications (Morris et al., 2006; Gros et al., 2006; Munoz-Morris et al., 2007). MPG and Pep technologies have been successfully applied to the delivery of different cargoes (siRNA and peptides) in primary cell lines and *in vivo.* These peptide-based strategies present several advantages, including rapid delivery of cargoes into cells with very high efficiency, stability in physiological buffers, lack of toxicity and of sensitivity to serum. Moreover, the lack of prerequisites for covalent coupling upon formation of carrier–macromolecule particles favours the intracellular trafficking of the cargo and enables its controlled release into the target cellular compartment. The final localization of the delivered macromolecule is then determined by its inherent intracellular targeting properties. A major concern with the cellular uptake of CPPs is to avoid the endosomal pathway or to favour escape of the cargo from early endosomes. In many cases, the secondary structure of peptides is critical and is directly correlated to their mode of membrane interaction. Key parameters need to be taken into account in the design of a non-covalent peptide-based delivery strategy, including the secondary structure of the carrier, the presence of a critical number of charged and aromatic residues, as well as the size and the stability of the carrier–cargo particles. In particular, for non-covalent strategies, there is a direct correlation between biological efficiency, carrier–cargo affinity, complex stability, particle size and homogeneity. MPG and Pep-1 behave significantly differently from other similarly designed CPPs. Although we cannot exclude the possibility that the uptake of MPG or Pep-1 follows several routes, we have shown that the major cell translocation mechanism is independent of the endosomal pathway and involves transient membrane disorganization associated with folding of the carrier into either an α-helical or β-sheet structure within the phospholipid membrane.

In conclusion, both MPG and Pep-1 technologies constitute an excellent alternative to covalent strategies and will have a major impact on the application of siRNAs, proteins and peptides for future therapies. These technologies are powerful tools for basic research and for targeting specific cellular events both *in vitro* and *in vivo*, as well as in a therapeutic context for screening potential therapeutic molecules. Moreover, functionalization of MPG- and Pep-1-based nanoparticles will be of major interest for rational targeting and systemic application of therapeutic molecules *in vivo*.

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