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Peptide-assisted traffic engineering for nonviral gene therapy

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Many of the challenges facing nonviral gene therapy, to make it as effective as the viral-based version, have yet to be overcome. The technology possesses sufficient biosafety advantages to make the construction of 'artificial viruses' a worthwhile undertaking. The impact of vehicle architecture on traffic regulation, and the convergence of several intracellular pathways in late endosomes, indicates that the particular intracellular route might be less relevant than formerly believed. Proper functional tuning of artificial viruses by the use of full proteins or protein stretches, and especially, the incorporation of membrane-active peptides, would improve transgene expression levels and convert artificial viruses into powerful tools for gene medicine.

Introduction

Gene therapy offers an unusual biomedical approach for the treatment of many severe human diseases. Although theoretically highly promising, it needs complex adjustment to be implemented into general practice. Viruses, being strict intracellular parasites, are natural vehicles for cell-targeted delivery of nucleic acids; following appropriate engineering, members of several families (including Poxviridae, Adenoviridae, Herpesviridae, Parvoviridae and Retroviridae) have been adapted to deliver therapeutic doses of nonviral nucleic acids [1]. Many viral vehicles have entered clinical trials, but only one of them, till date, has been licensed for use [2]. Crucial obstacles for the complete development of virus-based gene therapy are the adverse effects associated with the administration of viral agents, including inflammation, cellular transformation and death of the patients [3–5]. For these reasons, nonviral constructs are under continuous development, and despite being less efficient than their natural counterparts, with respect to transgene expression levels, their administration is biologically safe and represents an appealing alternative for therapeutic gene delivery. Nonviral vehicles, however, present major drawbacks, such as low and transient gene expression levels [6,7], that are essentially because of their inability to promote integration of transported DNA and, therefore, stable gene expression. Because biosafety is not such a major concern for nonviral approaches, efforts have been focused on enhancing biological efficiency through fine-tuning of their functional components. Those nonviral constructs designed to mimic viral properties are referred as 'artificial viruses' [8].

Artificial viruses can be constructed from lipids, cationic polymers, peptides, or combinations thereof, associated with the cargo nucleic acid [8]. Their functional components should enable the transgene to complete an intricate intracellular tour from the cell surface to the nucleus, overcoming a sequential series of biological obstacles. The main steps of gene delivery include binding to the cell surface; endocytosis; escaping from endocytic vesicles; evading cytoplasmic nucleases; reaching the nucleus by circumventing a cytoskeletal meshwork and entering through the nuclear pore complex. As a quantitative indication of this complexity, around 10^6 naked plasmid molecules are needed to transform efficiently a single cell, from which only 10^2 to 10^4 reach the nucleus [9]. The transfer process needs to be fast, because the half-life of plasmid DNA in the cytoplasm is 50–90 min and it is highly sensitive to calcium-dependent cytosolic nucleases [10].

DNA viruses and retroviruses have evolved to overcome such cellular barriers in their passage to the nuclear compartment. In adenoviruses and other DNA viruses, such a complex transfer is extremely efficient and it can be completed in about an hour [11].

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Although specific components relevant to particle uptake are well known in several virus species, including cell ligands, endosomal escape peptides, dinein-binding sequences and nuclear localization signals (NLSs) [12], precise trafficking routes for most of these pathogens, including those from families commonly used for viral gene transfer, are still to be solved [12,13]. Single-particle tracking (SPT) has given, in recent years, insights with respect to intracellular virus particle dynamics [14], and particularly, information about host factors modulating cytoplasmic motility and nuclear transport [12]. Most of these new data, however, cannot yet contribute to the construction of nonviral gene therapy vehicles, because they imply a fine structural disposition of viral components that cannot be fully reproduced in artificial constructs. Individual proteins or protein segments determining particular viral life cycle steps and intracellular pathways could, however, serve as functional elements to develop better manmade gene delivery constructs [7]. In this context, the performance of artificial viruses can be improved by adding (i) cell-targeting ligands to enhance cellular uptake through receptor-mediated endocytosis; (ii) fusogenic peptides to avoid endosomal degradation and (iii) nuclear localization signals to improve nuclear entry [15]. Such rational vehicle design would gain from a progressive understanding of viral biology that could prove essential in improving the internalization and trafficking properties of nonviral vectors. We will discuss, in the next sections, crucial steps in the internalization and intracellular trafficking mechanics that are of special relevance for the design of artificial viruses, and how functional peptides can modulate the traffic pattern and delivery efficiency of such constructs.

Uptake pathways

General considerations

Vesicular uptake can be broadly classified into clathrin-mediated endocytosis (CME), caveolae/lipid-raft, macropinocytosis, clathrin/caveolae-independent endocytosis and nonendocytic entry, whose different properties are outlined in Table 1. All these different endocytic pathways have been previously described [15–18] and extensively reviewed elsewhere [15–20]. Several considerations with respect to the uptake mechanisms and the interpretation of internalization data are, however, crucial in the

TABLE 1

Markers and properties of each main endocytic pathway regarding artificial viruses

	Clathrin-mediated endocytosis	Caveolae/lipid rafts	Macropinocytosis	Endocytosis-independent
INHIBITORS				
Chlorpromazine	Inhibits	No effect	No effect	Inhibits
Filipin (cholesterol ligand)		**Inhibits**		
Other cholesterol ligands (MβCD, nystatin)	Inhibits	Inhibits	Inhibits	**No effect**
Cytosol acidification	**Inhibits**			
Inhibitor PI3K (wortmannin)			Inhibits	
Heparan sulphate inhibitors				Inhibits
GTPase Dynamin inhibitors	Inhibits	Inhibits		Inhibits
GTPase Rab protein inhibitors	Inhibits		Inhibits (Rho)	
Actin inhibitor (cytochalasin)	**Hardly Inhibits**	Inhibits	Inhibits	Inhibits
Microtubule inhibitor (nocodazole)		Inhibits		
PKC inhibitors			Inhibits	
Na+/H+ inhibitors (Amiloride)			**Inhibits**	
TK inhibitor	Inhibits	Inhibits	Inhibits	
Ammonium chloride				**Inhibits**
Potassium depletion	**Inhibits**			
Hypertonicity	**Inhibits**			
Low temperature	Inhibits	Inhibits	Inhibits	
Chloroquine				No effect
Rottlerin	No effect			**Inhibits**
STIMULATOR				
PKC activators (Phorbol ester)			**Stimulates**	
MARKERS				
Transferrin	Labels	No labelling	No labelling	
Dextran	No labelling	No labelling	Labels	
CTB	No labelling	Labels	No labelling	
RELEVANT PROPERTIES				
ADVANTAGES	Universal, fast	Not degraded in lysosomes	Leaky, not degraded in lysosomes, uptake of macromolecules	Fast, massive
DISADVANTAGES	Lisosomal degradation	Not universal, low capacity, slow	Not for pH-dependent fusogenic peptides (eg. histidylated peptides)	High exposure to cytoplasmic nucleases
EXAMPLES	Transferrin	Tat	Tat, 8R	Tat, antennapedia, vp22, R9

The fields filled in gray indicate that these inhibitors are specific for one internalization pathway. CTB, cholera toxin beta subunit; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; MβCD, methyl-beta-cyclodextrin; PI3K, phosphoinositide-3-kinase; PKC, protein kinase C; TK, tyrosine kinase; 8R, octaarginine; vp22, herpes virus vp22 structural protein.

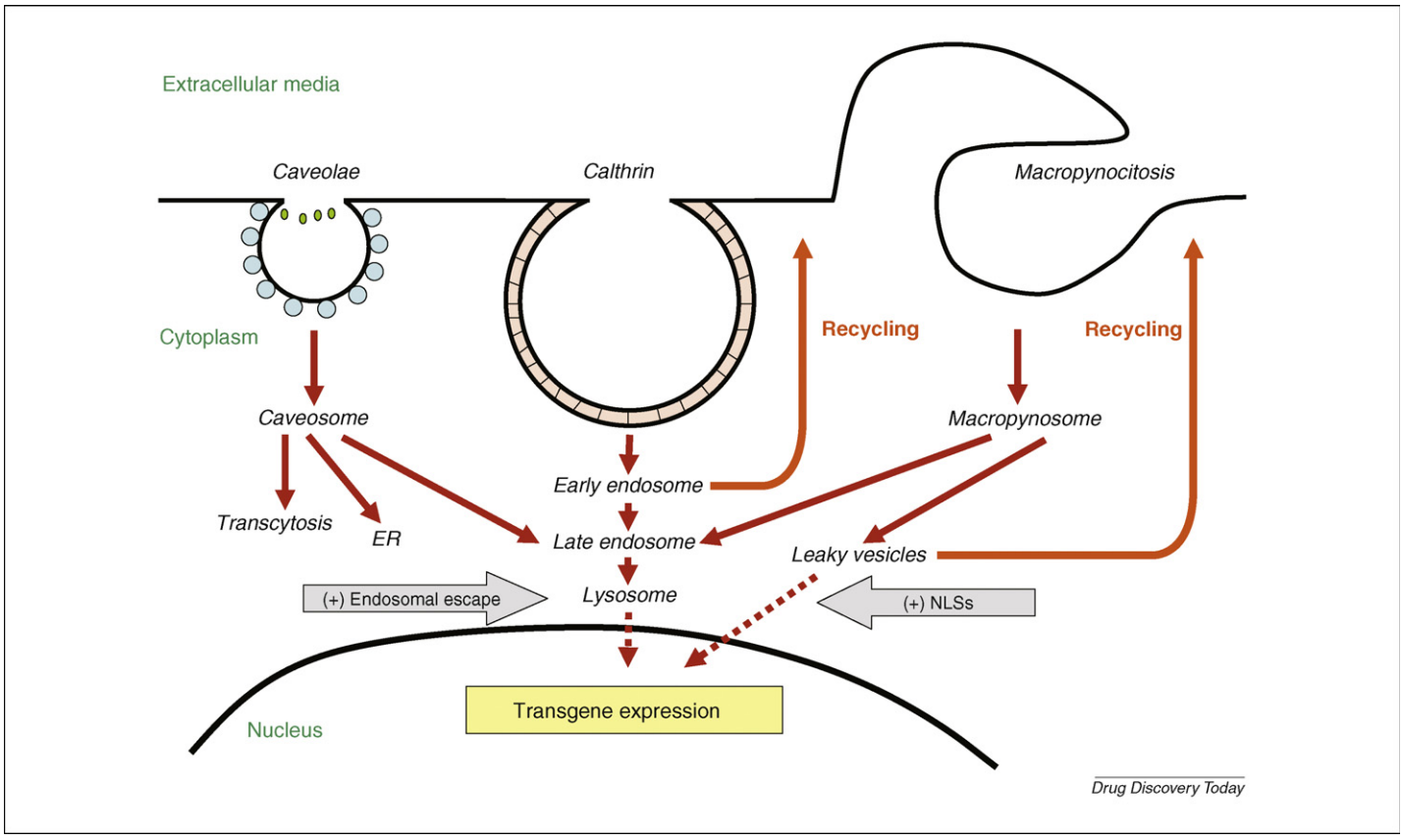

FIGURE 1

Classical endocytic pathways toward gene expression. Dynamic features of particular traffic pathways taken by artificial viruses (i.e. caveosomes may suffer transcytosis or arrive to the ER; macropynosomes may become leaky vesicles) are indicated by red arrows. Most nonviral vectors for gene therapy end up in cytoplasmic vesicles, independently of the uptake pathway, as inferred by the convergence of late endosomes and endocytic vesicles. This fact stresses the importance of incorporating peptides for endosomal escape and NLSs that dramatically favor the release of the vector into the nucleus and the levels of transgene expression. Brown arrows indicate membrane recycling during cytoplasmic traffic.

context of artificial virus engineering and deserve further attention. First, the endocytic pathway, undertaken by an internalized particle, determines probable cellular fate, namely delivery into lysosomes in clathrin-mediated endocytosis, caveosomes or lysosomes from caveolae [17] and macropinosomes in macropinocytosis [15]. Most of these resulting vesicles, however, converge to later stage endosomes that eventually fuse with lysosomes for the degradation of their content [18] (summarized in Figure 1). Second, only very few inhibitors used in internalization studies are specific for a single pathway [15,21], which makes the results hard to interpret. For instance, cholera toxin beta (CTB), the most used marker for caveolae, can also be internalized by clathrin- and CDC42-dependent pathways [18].

Finally, avoiding clathrin endocytosis might not be the best strategy to escape degradation in the lysosomes, because other endocytic routes could also be ineffective regarding reached gene expression levels. Macropinocytosis, for instance, can drive the vector to a dead-end within macropinosomes, where the pH is not sufficiently acidic to allow rescue by histidilated endosomal-escape peptides [22]. Therefore, the embedded vehicle is neither degraded nor released to the cytoplasm [23]. On the contrary, caveolae-dependent endocytosis represents, in general, a minority gate to cell entry, given that most cellular types (with the exception of endothelial cells) have a very low proportion of caveolae in their membranes. Indeed, endosomes move to the nucleus along the microtubule highway, safe from cytoplasmic nucleases (in common with many viruses). Therefore, endosomes offer an excellent trafficking route, provided the transported vehicles can escape before their degradation in the lysosome. In all cases, and regardless of the cell entry pathway, efficient endosomal escape is required to achieve high transgene expression levels [24] (Figure 2).

On the contrary, the role of endocytosis in coordinating cell signaling [25] is a main issue when attempting to design a particular trafficking route. The caveolar internalization of SV40, for example, involves 80 different kinases [26] that connect endocytosis with other cellular activities such as cell cycle, adhesion and metabolism [25]. Therefore, the uptake of the nonviral vectors might interfere with other intracellular trafficking pathways, as observed for adenovirus 5 (Ad5) penton base protein and the epithelial secretion of the lachrymal gland [27]. Contrarily, cellular secretion can also modulate endocytosis [28].

Cell binding

The uptake of artificial viruses, in common with the natural versions, involves their attachment to the cell surface, either by nonspecific binding, such as electrostatic interaction with negatively charged cell surface proteoglycans, or by specific interactions, which

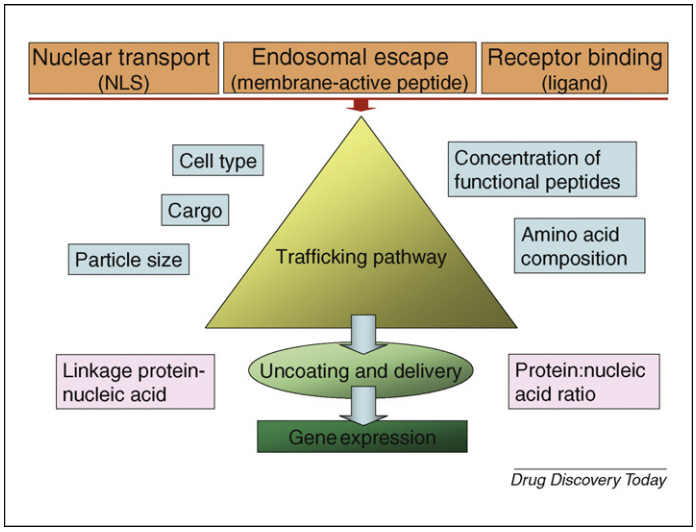

FIGURE 2

Positive modulators of artificial virus-mediated nuclear delivery of DNA. Functional peptides addressing cell binding, endosomal escape and nuclear delivery issues are main and general contributors to the biological efficacy of artificial viruses and indirectly determine gene expression levels. In addition, other factors such as particle size and organization, concentration and amino acid sequence of functional peptides, type of cargo and cell type significantly influence the trafficking route and efficiency. Finally, the linkage type and ratio between cargo DNA and carrier proteins specifically modulate the uncoating and delivery efficiency once in the nucleus.

are mediated by receptors. To a great extent, the therapeutic potential of artificial viruses depends on tissue targeting; the presence of a specific ligand-binding molecule promotes the internalization process into a target cell type and it is a main functional requirement of such constructs. Appropriate cell targeting has, so far, been achieved with a short list of ligands including transforming growth factor (TGF)-α, insulin, folate, bovine fibroblast growth factor (bFGF), Arg-Gly-Asp (RGD)-containing peptides, heregulin, transferrin, epithelial growth factor (EGF), avian erythroblastosis oncogene B 2 (ErbB2) and asialoglycoprotein [16]. In this respect, phage display and directed evolution approaches are expected to provide new tissue-specific peptidic ligands with which the solvent-exposed surface of nonviral vectors can be functionalized [29,30]. The incorporation of new natural or artificially obtained ligands would be desirable and would allow the use of a wider catalog of cell types and tissues than is currently available.

Most cell-surface receptors, together with bound ligands, are internalized by clathrin-coated pits. Nonviral constructs attached to cell receptors through epithelial growth factor (EGF) or RGD-containing domains, both internalized by clathrin-mediated endocytosis, might be delivered to lysosomes for degradation. Albumin, a paradigm of caveolae-mediated endocytosis, is, however, transported together with its receptor to a different plasma membrane domain [31]. Transferrin has often been incorporated into gene delivery constructs to mediate receptor-driven endocytosis for anticancer therapy, because tumor cells overexpress transferrin receptors [31]. Transferrin receptor-mediated endocytosis occurs via clathrin vesicles that evolve to endosomes and lysosomes. In this, and similar cases, an endosomal escape peptide is then needed to prevent degradation of the macromolecular complex [24]. On the contrary, transferrin linked to lipoplexes enters via nonreceptor endocytosis, apparently as a consequence of the large size of the complex [32].

Interestingly, heparan sulfate proteoglycans (HSPGs) constitute a major source of macromolecular polyanions that surround almost every cell type. They are involved in the activity of growth and coagulation factors, microbe–host interactions and lipoprotein metabolism, upon the docking with natural cationic ligands, such as extracellular matrix proteins and growth factors. HSPG also regulates nonviral gene transfer mediated by peptides rich in basic amino acids, like the HIV Tat transduction domain, polylysines and polyarginines, among others. The participation of HSPG in classical endocytosis occurs through its role as a coreceptor for the initial attachment site [33]. HSPG, however, is also involved in direct membrane penetration in endocytosis-like processes that are insensitive to endocytic inhibitors [34].

Cell entrance of peptide-driven artificial viruses

It has been shown that endocytosis is the main mechanism for the import of peptide-based vectors [35] and nonviral vectors [16]. Conflicting evidence, however, has been presented with respect to the role of the individual endocytic pathways. This is probably a consequence of the different experimental conditions regarding cell line, incubation time and peptide concentration [20], under which trafficking studies have been performed. Furthermore, the properties of the gene transfer vehicles such as DNA cargo size, type of linkage to the vehicle and whole construct architecture, might determine the cellular uptake route, independently of the properties of the driver peptide. Some examples of entry pathways for Tat, one of the cationic peptides most studied in the artificial virus context are presented in Table 2. Unfortunately, many of the gene delivery studies do not discriminate between the different endocytic pathways, but only between endocytic (vehicles observed into vesicles) and nonendocytic (vehicles diffusely distributed in the cytoplasm) entry.

Furthermore, conclusions from many studies on peptide-mediated endocytosis need to be carefully re-evaluated, because enlightening experiments performed in different laboratories [35,36] have shown that cell fixation, even under mild conditions, alters the cellular distribution of at least several membrane-active peptides and their cargoes, including vp22, Tat, 8K and 8R. In living unfixed cells, such peptides do not translocate membranes as suggested in previous studies, but they internalize passively during the endocytic turnover of the plasma membrane to which they are attached by electrostatic interaction. In the context of artefacts relevant to trafficking studies, flow cytometry analysis has been shown to be poorly robust with respect to the investigation of cellular uptake, unless a step of trypsin digestion of the cell membrane adsorbed peptide is added to the protocol [36], and this has not often been the case.

Caveolae are commonly responsible for the uptake of conjugates containing pathogen-derived peptides, such as those from simian virus 40 (SV40) and cholera toxin [37]. In cells lacking caveolae, however, endocytosis of these markers still occurs through alternative routes [38]. Alternatively, vesicular stomatitis virus (VSV) and human immunodeficiency virus (HIV) viral protein R (Vpr) enter through CME [39,40]. Macropinocytosis has been observed for polyoma virus vp1 and for Ad5 penton proteins through CAR receptor, and proteoglycans acting as coreceptors

TABLE 2

Examples of different intracellular distribution patterns of Tat under diverse experimental conditions, examined in nonfixed living cells

Vector	Type of linkage	Cargo	Peptide concentration	Cells	Uptake	Ref
Tat	AEEA linker	PNA	1 and 10 μM	HeLa, CHO	Endocytic vesicles	[36]
Tat	Disulfide bond	PON	0.05–1 μM	HeLa	Endocytic vesicles and nucleus	[64]
Tat-biotin	Avidin–biotin	Avidin	0.5 μM	HeLa	Endocytic clathrin-dependent and -independent and diffuse in cytoplasm	[45]
Tat	Disulfide bond or maleimide coupling	PNA	2 μM	HeLa, SK-BR-3, U937	Endocytic	[72]
Tat	Disulfide bond or maleimide coupling	PNA	2 μM	IMR-90	Endocytic and weak diffuse staining	[72]
Tat	Disulfide bond or maleimide coupling	PNA	>10 μM	HeLa, SK-BR-3, U937, IMR-90	Whole cell staining (cytotoxic explanation)	[72]
Tat	–	–	>10 μM	HeLa	Whole cell staining (specifically inhibited; also at 5 μM if endocytic entry is inhibited)	[20]
Tat	–	–	<10 μM	HeLa	Macropinocytosis and caveolae/lipid raft	[20]
GST-Tat-EGFP	Fusion protein	–	2 μg/ml	HeLa, Cos-1, Jurkat	Caveolae/lipid raft	[47]
Tat-Cre	Fusion protein	–	0.25–2 μM	Mouse reporter T cells	Macropinocytosis	[23]
Full-length Tat	–	–	0.05	Jurkat	Clathrin-mediated endocytosis	[46]

PNA, peptide nucleic acids; PON, phosphorotioate antisense oligonucleotides; AEEA, 2-aminoethoxy-2-ethoxyacetic acid; GST, glutathione *S*-transferase; aas, amino acids. A vesicular pattern is usually assumed as resulting from an endocytic pathway, while a cytosolic diffuse pattern as derived from a nonendocytic entrance.

have been identified [41]. For polyoma vp1, clathrin microtubule-dependent endocytosis has also been described [42,43].

Regarding Tat, probably the most studied membrane-active peptide for artificial nanoconjugates, different, contradictory, uptake mechanisms have been described in the past decade, including clathrin-mediated endocytosis [44–46], caveolae/lipid-rafts [20,23,47], macropinocytosis [20,23] and nonendocytic processes [20,48]. It is now becoming clear that Tat (and probably some of the arginine-rich peptides closely related to Tat), can use any available internalization pathway (including the plasma cell membrane turnover, that can be completed in less than one hour) in the target cell type. In the same line, its uptake can occur upon specific binding to a cell membrane integrin [49] or upon non-specific binding to cell surface proteoglycans [50].

Endosomal escape

There is a long list of natural membrane-crossing peptides from different origins that enhance the nuclear delivery of nonviral vectors, irrespective of their precise endocytic pathway, by favoring the release of complexes from endosomal entrapment (see Table 1 in [24]). Some synthetic versions of natural peptides, rationally designed to improve the efficacy of the natural counterparts, have also been successfully used as components of artificial viruses [24]. The mechanisms of endosomal escape are often not well characterized, but they could be classified as pH-dependent (such as in diptheria toxin, GALA, influenza virus HA2 peptide and histidine-rich peptides), or pH-independent (in KALA and gramicidin S). Peptides displaying pH-dependent endosomal escape can be, in turn, classified as fusogenic (like HA2 and ppTG20 peptides that form amphipathic structures at low pH), or histidine-rich peptides (like H5WYG and LAH, in which the imidazole group acts as a proton sponge). Interestingly, different peptides seem to be especially appropriate for specific molecular sizes of the cargo DNA. So, rhinovirus VP1 and influenza virus HA2 proteins only allow the endosomal escape of small molecules (i.e. 10 kDa), while Ad5 penton protein enables the release of molecules larger than 70 kDa [51].

Cytosol trafficking

In eukaryotic cells, the route from the cell membrane to the nucleus can be as long as up to 100 μm, and it occurs through a large and extremely crowded cytoplasm crossed by a cytoskeletal meshwork that impedes the free diffusion of macromolecules such as DNA. Twenty-one-bp oligonucleotides and DNA fragments smaller than 250 bp freely diffuse into the nucleus, 500 bp-DNA molecules diffuse throughout the cytoplasm, but do not enter the nucleus, and DNA segments larger than 2 kb do not move through the cytoplasm [52].

On the contrary, the cytoskeleton facilitates the intracellular transport of endosomes and lysosomes to the nuclear periphery (endosomes require intact microtubules to translocate from the plasma membrane to lysosomes) and also directly transport macromolecules associated with the cytoskeletal motor.

This dual role of the cytoskeleton on cytoplasmic trafficking might explain why, in some cases, the cytoskeleton helps in the appropriate delivery, while in other cases, and depending on the particular vehicle, might impair its mobility.

In any case, early release of DNA from the carrier into the cytoplasm could have a detrimental effect, because naked DNA will be degraded by cytosolic nucleases in one to two hours [53]. In this regard, a prolonged association with cationic components would keep it in a condensed, nuclease-resistant status during its passage to the cell nucleus.

Nuclear entry

Particles over 30 nm in diameter or 40 kDa molecular weight (that of a standard plasmid DNA molecule is 2–10 MDa) require active transport through the nuclear pore complex (NPC) to reach the

nucleus, with associated energetic expense. Such nuclear transport can be accomplished by incorporating appropriate NLSs in the shuttle vehicle. They are usually contained in short peptide stretches present in diverse proteins, which, upon binding to cytoplasmic importins, dock to the NPC. This nuclear import style, which depends on the NLS protein-machinery seems to apply only to plasmid-containing protein complexes, while nuclear transport of linear DNA under 1 kb might proceed by an NLS-independent route [54].

A very commonly used NLS is the PKKKRKV amino acid sequence from the SV40 large T antigen, whose positive charges not only help in DNA condensation, but also allow nuclear transport [55]. Other nuclear-targeting proteins of viral origin are Tat; Ad hexon protein; Ad2 protein VII; HIV Vpr, Epstein–Barr virus nuclear antigen (EBNA)-1 and Rep 68/71 proteins from adeno-associated viruses [16]. Peptide stretches from cellular transcription factors, such as the yeast GAL4 amino terminal domain, can also be exploited as NLSs in nonviral vectors. Furthermore, nuclear proteins like ribonucleoprotein A1, H1 histones, protamines and high motility group (HMG) proteins contain highly basic sequences, rich in arginines, which not only show activity in DNA binding, but also guide it to the nucleus. Intriguingly, the combination of several of these peptides has an additive effect [16], which could be highly convenient with respect to nuclear entry efficiency and final transgene expression levels. NLS peptides, however, because of their basic nature, may be incapable of releasing their cargo into the nucleus for further transcription, a fact that might limit their molar abundance in peptide–DNA complexes.

Of course, strategies to promote transcription of delivered DNA in the cytoplasm are under investigation, because their application would allow skipping the nuclear transport step. In this regard, the cotransfection of a phage T7 RNA polymerase gene and a transgene controlled by the T7 promoter has offered some promising results [56]. The natural nuclear transgene expression, however, can offer a further tool to engineer the appropriate tropism in the systemic delivery of an artificial virus, complementary to the incorporation of a receptor-binding peptide. Cell-targeted gene expression can be gained using cell-specific promoters to control the transgene expression. In this regard, smooth muscle gamma actin (SMGA) promoter [57], as well as several endothelial promoters [58] has proven to confer tissue-specificity in the transgene expression.

Regulatable features of artificial viruses that influence the internalization pathway

In all possible internalization pathways, the amino acid composition of the guiding peptide is crucial for efficient vector internalization. In this regard, the number of basic residues (from 5 to 11) is crucial for the efficiency of polyarginine peptides, with no significant improvement of internalization upon further chain elongation [59]. On the contrary, Thoren *et al.* have shown that the cytoplasmic distribution pattern of penetratin can be modified by arginine substitutions from endocytic to diffuse, and that penetratin entrance can be fully inhibited through lysine substitutions [60]. Also, the actual number of active peptides incorporated in a nanocomplex is crucial for efficient cell internalization, especially in very large vehicles [61]. From the architectural point of view, and concerning the rational design of artificial viruses, the type of linkage to the cargo is extremely important, as it can strongly affect the solvent exposure of the guiding peptide and the biological activity of the cargo once inside the cell.

The size of the complexes is also crucial for the internalization pathway. Particles below 200 nm in diameter enter through clathrin-coated pits and spheres of 500 nm in size enter via macropinocytosis/caveolae, in an energy-dependent endocytosis [62]. Instead, individual peptides, or those conjugated to small molecules, penetrate target cells via electrostatic interactions in an energy-independent manner [63]. The nature of the cargo itself might influence the final distribution of the conjugate, as reported in independent studies; peptidic nucleic acid (PNA)–Tat (or R9) conjugates were found predominantly segregated in endocytic vesicles [36], whereas phosphorothioate antisense oligonucleotides conjugated to such peptides were, at least partly, found in nuclei [64].

Recently, very interesting studies by Duchardt and colleagues [20] have shown that in HeLa cells, each of the studied peptides (tat, R9 and antennapedia) internalizes through different endocytic, as well as nonendocytic, pathways simultaneously. One route predominated over the others, however, depending on the peptide concentration and the chemical or biological inhibition of alternative endocytic routes. These results are in accordance with the fact that some plasma-membrane receptors (e.g. TGF-β, EGFR) are internalized through different endocytic routes depending on the ligand concentration [25], by a route that is believed to be regulated by ubiquitylation [65]. Therefore, the concentration of functional uptake peptides on the artificial virus seems to be crucial for further intracellular trafficking.

Finally, it is also well known that the endocytic pathway of the peptide-based vehicles depends also on the cell type (e.g. Tat uses caveolae in COS-1 [66] and HeLa cells [47] and clathrin in T cells [46]), a fact related to the endocytic pathways predominating in each cell type. Therefore, fine-tuning of artificial viruses should carefully consider, if known, the particular trafficking scheme preferred by the target cells.

Peptide-mediated traffic engineering

How functional peptides can modulate the traffic of artificial viruses and/or improve the achieved gene expression levels can be exemplified by several representative studies. In an elegant analysis [67], Kichler manipulated conformational preferences and concentration of the cationic LAH4 peptide, modulating the delivery of expressible DNA-mediated by peptide–DNA nanoconjugates. Similarly, the manipulation of amino acid sequence and type of linkage to the cargo, among others, resulted in enhanced metabolic stability of arginine-rich CPPs [68] that favors endosomal escape and mediates further trafficking of the vehicle. The hemolytic peptide melittin has similarly been engineered as a powerful sulfhydryl polymerized peptide, acting as a DNA-binding agent that shows interesting properties in promoting endosomal escape and nuclear transport of peptide–DNA complexes [69].

In other studies, the fine amino acid engineering of CCPs in artificial viruses enhanced endosomal escape and nuclear or cytoplasmic delivery of expressible DNA or small interfering (siRNA), respectively, stressing the importance of endosomal escape over the precise uptake way. In this context, Wadia *et al.* [23] described that nanoconjugates internalized via macropinocytosis and, therefore,

supposedly avoiding the lysosomal pathway, do not promote a high level of transgene expression, unless the endosomal escaping peptide HA2 is added to the complex. The membrane disruption activity of penetatin can be stimulated by the addition of His residues that promote the formation of an active α-helix upon protonation and enhances cytosolic delivery of model siRNAs [70]. Interestingly, sequence engineering of CCPs affects not only endosomal escape, but also endocytosis efficiency itself, a fact that had not been observed previously. In this regard, Amand *et al.* have just shown that complexes containing penetratin derivatives enriched with arginine residues are internalized more efficiently than those based on the natural sequence, probably through enhanced macropinocytosis [71].

Conclusions and future prospects

Intracellular trafficking for nuclear delivery of gene therapy vehicles is a major issue in emerging biomedical fields. The routing of internalized model vehicles has been the object of detailed analyses to gain a rationale for traffic engineering and delivery into the appropriate cell compartment, usually the nucleus. In this context, it has been largely believed that avoiding the lysosomal fate would necessarily render enhanced expression levels of the cargo transgene. However, confusing and even contradictory results obtained in the past can be accounted for by the diversity of cell lines used in *in vivo* experiments and it is now becoming clear that in different cell types, specific trafficking routes are naturally favored. Also, the diversity of protocols used to test the performance of vehicles, involving different drug concentration and incubation times, have generated inconsistent data, through what is now considered to be as a consequence of environmental conditions of the internalization routes. Furthermore, the architectural and chemical nature of nonviral vehicles regarding size, linkage DNA–protein and orientation of functional peptides with respect to the cargo, modulates the nuclear trafficking route. Therefore, the intracellular fate of different vehicles may be different even when internalized by the same mechanism.

From a different point of view, the classical clathrin/caveolae/macropinocytosis/nonclathrin noncaveolae uptake route classification is now observed as oversimplified. The clathrin-independent route is, in fact, branching into several endocytic, cross-regulated pathways, all of which merge at the early or late endosome stage. Furthermore, a particular vehicle can simultaneously use different endocytic pathways not only in different cell types but also in a single cell line. Under this novel concept, the particular endocytic pathway to be taken by an artificial virus might be less relevant than previously believed. In this regard, the nature and combined abilities of the functional peptides incorporated into a vehicle promoting nuclear delivery (those regulating cell binding, DNA stability, endosomal escape and nuclear transport), are probably much more relevant in terms of transgene expression levels than the precise route taken by the vehicle. Therefore, functional peptides, and especially CPPs, are progressively observed as very crucial components of artificial viruses, guiding the cargo, whatever is the used trafficking route, to the nuclear compartment for the successful delivery of therapeutic genes in an expressible form.

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