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A convergent uptake route for peptide- and polymer-based nucleotide delivery systems

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ABSTRACT

Cell-penetrating peptides (CPPs) have been used as vehicles to deliver various cargos into cells and are promising as tools to deliver therapeutic biomolecules such as oligonucleotides both *in vitro* and *in vivo*. CPPs are positively charged and it is believed that CPPs deliver their cargo in a receptor-independent manner by interacting with the negatively charged plasma membrane and thereby inducing endocytosis. In this study we examine the mechanism of uptake of several different, well known, CPPs that form complexes with oligonucleotides. We show that these CPP:oligonucleotide complexes are negatively charged in transfection-media and their uptake is mediated by class A scavenger receptors (SCARA). These receptors are known to promiscuously bind to, and mediate uptake of poly-anionic macromolecules. Uptake of CPP:oligonucleotide complexes was abolished using pharmacological SCARA inhibitors as well as siRNA-mediated knockdown of SCARA. Additionally, uptake of CPP:oligonucleotide was significantly increased by transiently overexpressing SCARA. Furthermore, SCARA inhibitors also blocked internalization of cationic polymer:oligonucleotide complexes. Our results demonstrate that the previous held belief that CPPs act receptor independently does not hold true for CPP:oligonucleotide complexes, as scavenger receptor class A (SCARA) mediates the uptake of all the examined CPP:oligonucleotide complexes in this study.

1. Introduction

Biomolecules such as plasmid DNA and siRNA can potentially be used as drugs to treat a wide variety of diseases [1]. If they are to be used to this end, these molecules need access to the intracellular environment to exert their function, however, due to their charge and/or size they are unable to bypass the cellular plasma membrane. Therefore, several distinct classes of delivery systems have been developed, one of which is comprised of a group of peptides collectively termed CPPs. CPPs are usually 5–30 amino acids in length, cationic and/or amphipathic and are defined by their ability to pass the cellular membrane with attached cargo [2]. The uptake of CPPs is considered to occur in a receptor-independent manner [3]. CPPs have been used to deliver a wide range of cargos (plasmid DNA, siRNA, proteins, liposomes, quantum dots, small molecules etc.) into cells both *in vitro* and *in vivo*, either covalently coupled to or non-covalently complexed with cargo. This unusual ability has made CPPs an active field of research since the discovery of the first CPP over 20 years ago. Despite huge efforts to identify the exact mechanism of uptake of CPPs, no such general mechanism has been found [4]. These efforts are hampered by the fact that experimental conditions such as CPP concentration, cargo properties and cell lines differ between studies and may therefore influence the mechanism of uptake. Additionally, the primary and secondary structure of different CPPs may affect the uptake route. Furthermore, there may be differences in the uptake mechanism if the CPP is in monomeric form or has formed complexes. Nevertheless, consensus in the field is that cellular uptake is mostly mediated by various endocytosis pathways, although direct penetration may occur under certain conditions such as when high CPP concentrations are used and small molecules (dyes) are covalently attached [5]. It has been proposed that interactions between positively charged CPPs and negatively charged cell surface proteoglycans are important for accumulation of CPPs at the cell membrane, which in turn leads to actin re-organization and subsequent uptake into endosomes [6–10]. However, other studies have shown that proteoglycans are not important for cellular uptake of CPPs [11]. In a recent publication Ezzat et. al. showed for the first time that a cell surface receptor was responsible for the uptake of a CPP, the stearylated TP10 analog PepFect14 (PF14), in complex with splice correcting oligonucleotides (SCO) [12]. Later, structurally related peptides PepFect15 and NickFects in complex with oligonucleotides (ONs) (SCO and plasmid respectively) were also shown to be taken up by the same receptors, namely class A scavenger

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receptors (SCARA) [13,14]. These findings came from the observation that the ζ-potential of PF14:SCO complexes is not positive but negative in transfection-relevant media, making direct membrane interaction or engagement of negatively charged proteoglycans unlikely to be the initial contact due to charge repulsion.

SCARA belongs to the group of pattern recognition receptors called scavenger receptors (SRs), originally defined by their ability to bind and endocytose acetylated low-density lipoprotein. SCARA displays promiscuous binding of many poly-anionic molecules such as polyribonucleotides, sulfated polysaccharides, dsRNA and spherical nucleotide particles [15–17]. SCARA were first isolated from macrophages but have since been found to be expressed in many types of cells such as fibroblasts, endothelial-, epithelial-, and dendritic cells and has a broad expression profile in tissues [18,19].

Recently, several different CPP:ON complexes were shown to, have a negative ζ-potential in transfection media [20,21]. Therefore, we speculated that SCARA might not only be involved in uptake of PepFect:ON and Nickfect:ON, but that this is a more general uptake mechanism for several types of CPP:ON complexes. To test this hypothesis we selected well-studied CPPs that have been shown to efficiently deliver either non-covalently complexed siRNA or plasmid DNA (pDNA) into cells. The CPPs investigated were members of the PepFect family, stearylated RxR_4 and CADY [20,22–24] (Table 1). While PepFects have been reported as vectors for various nucleotide-based cargoes, CADY and s-RxR_4 have only been reported as vectors for delivery of siRNA and pDNA respectively. CPPs were only used in experiments to deliver cargoes for which they had previously been reported capable of delivering. Pharmacological inhibitors, RNAi and overexpression were used to elucidate the role of SCARA in the uptake of these CPP:ON complexes. We show that SCARA is responsible for uptake of all CPP:ON complexes included in this study. Furthermore, we show that SCARA inhibitors abolish intracellular delivery of pDNA by cationic polymers poly-L-ornithine (PLO) and polyethyleneimine (PEI). Elucidating the exact mechanism of CPP uptake is essential for improving CPP design, therefore these findings should be of great value to the CPP field and potentially for the drug delivery field as a whole.

2. Results and discussion

2.1. Pharmacological inhibition of SCARA

Since the mechanism of uptake of a given CPP may be influenced by the cargo it delivers we investigated the role of SCARA in the uptake of PF6 and PF14 when complexed with different cargos. For plasmid as a cargo, PF14 and PF6 in complex with the luciferase expressing plasmid pGL3 was used together with specific SCARA inhibitors and their respective controls [18,25]. HeLa cells were incubated with inhibitor (Dextran sulfate (Dex), polyinosinic acid (Poly I) or fucoidin (Fuc)) or their respective control (Chondroitin sulfate (Chon), polycytidylic acid (Poly C) or galactose (Gal)) for 1 h before addition of CPP:pGL3 complexes. After 24 h luciferase activity was measured. Plasmid delivery by PF14 and PF6 was totally inhibited by all inhibitors while their respective control inhibitors did not significantly inhibit the plasmid delivery as measured by luciferase activity (Fig. 1). A de-complexation assay was performed to verify that the presence of inhibitors did not disrupt the integrity of the CPP:pDNA complexes even at higher concentrations of inhibitors (Supplementary fig. 1).

Table 1
CPPs included in the study.

PepFect 6 (PF6)	Stearyl-AGYLLG(K^a)INLKALAALAKKIL-NH_2
PepFect 14 (PF14)	Stearyl-AGYLLGKLLOOLAAAALOOLL-NH_2
dPF14 [b]	Stearyl-agyllgkllоolaaaalooll-NH_2
PF14-Fl	Stearyl-AGYLLG(K^c)LLOOLAAAALOOLL-NH_2
s-RxR_4 [d]	Stearyl-RxRRxRRxRRxR-NH_2
CADY	Ac-GLWRALWRLLRSLWRLLWRA-Cya

[a] Four chloroquine-analogs coupled via a lysine tree. [b] Small letters indicate D-amino acids. [c] 5,6-carboxyfluorescein. [d] x = 6-aminohexanoic acid.

2.2. ζ-Potential measurements

A number of CPPs that have been shown to be effective in delivering non-covalently complexed ONs were chosen for investigating if they are also recognized and taken up by SCARA. We measured these CPP:ON complexes ζ-potential in transfection-relevant media. To measure the physiochemical properties in transfection-relevant media is important as the complexes are affected by media components such as salts and proteins. We have previously shown that CPP:ON complexes can have positive ζ-potential in water but negative ζ-potential in transfection-relevant media [12]. CADY and s-RxR_4 were complexed with ON and ζ-potential was measured (Table 2). CADY:siRNA complexes were negatively charged with a ζ-potential close to −20 mV. s-RxR_4:pDNA complexes showed near neutral charge. As PF6 and PF14 has already been shown to form negatively charged complexes with ONs they were not analyzed here.

2.3. RNAi of SCARA and subsequent plasmid delivery

To further investigate the role of SCARA in the uptake of CPP:pGL3 complexes we performed RNAi of SCARA and subsequent cellular delivery. CPPs used for this experiment were PF14, the all D analog of PF14 (dPF14) and structurally very different s-RxR_4. HeLa cells have previously been shown to express SCARA 3 and SCARA 5 [12], therefore, the cells were treated with either siRNA targeting SCARA3 and SCARA5 or scrambled siRNA as control and incubated for 48 h. Cells were then transfected with CPP:pGL3 complexes for 24 h, luciferase activity was measured and normalized to protein content. Cells pre-treated with SCARA 3 and SCARA 5 siRNA showed significantly lower luciferase activity than did cells pre-treated with control siRNA or cells with no pre-treatment for all CPPs examined (Fig. 2). This demonstrates that PF14 and its D analog dPF14, as well as the structurally different sRxR_4, are all taken up by SCARA. The fact that D-analogs of CPPs are taken up into cells has been considered as proof for receptor-independent uptake [3], but since SCARAs interact with polyanionic particulate ligands with low specificity, the L- or D-configuration should not be important as a factor for receptor recognition as long as they both produce negatively charged complexes. An experiment using LF2000:pGL3 complexes as negative control was performed in order to show that SCARA down-regulation does not indiscriminately inhibit transfection. Results show no effect on cellular uptake of LF2000:pGL3 complexes by SCARA down-regulation (Supplementary fig. 2).

2.4. Up-regulation of SCARA and subsequent plasmid delivery

If SCARA are responsible for the uptake of CPP:pGL3 complexes it should be possible to increase CPP:pGL3 uptake by over-expressing SCARA. Therefore, HeLa-cells were treated with plasmids expressing SCARA3 and SCARA5 two days prior treatment with PF14, dPF14 or s-RxR_4 in complex with pGL3. Indeed, by overexpressing SCARA the uptake was increased 4–6 fold compared to control as measured by luciferase expression, further indicating that SCARA mediates the uptake of these complexes (Fig. 3). An experiment using LF2000:pGL3 complexes as negative control was performed in order to investigate whether SCARA up-regulation affects transfection in general. Results show no statistically significant effect on cellular uptake of LF2000:pGL3 complexes by SCARA up-regulation (Supplementary fig. 2).