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The role of endocytosis on the uptake kinetics of luciferin-conjugated cell-penetrating peptides

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ABSTRACT

Cell-penetrating peptides (CPPs) are short cationic/amphipathic peptides that can be used to deliver a variety of cargos into cells. However, it is still debated which routes CPPs employ to gain access to intracellular compartments. To assess this, most previously conducted studies have relied on information which is gained by using fluorescently labeled CPPs. More relevant information whether the internalized conjugates are biologically available has been gathered using end-point assays with biological readouts. Uptake kinetic studies have shed even more light on the matter because the arbitrary choice of end-point might have profound effect how the results could be interpreted. To elucidate uptake mechanisms of CPPs, here we have used a bioluminescence based assay to measure cytosolic delivery kinetics of luciferin–CPP conjugates in the presence of endocytosis inhibitors. The results suggest that these conjugates are delivered into cytosol mainly via macropinocytosis; clathrin-mediated endocytosis and caveolae/lipid raft dependent endocytosis are involved in a smaller extent. Furthermore, we demonstrate how the involved endocytic routes and internalization kinetic profiles can depend on conjugate concentration in case of certain peptides, but not in case of others. The employed internalization route, however, likely dictates the intracellular fate and subsequent trafficking of internalized ligands, therefore emphasizing the importance of our novel findings for delivery vector development.

1. Introduction

Cell-penetrating peptides (CPPs) are relatively short cationic and/or amphipathic peptides capable of delivering many types of cargos into mammalian cells. The first CPPs, penetratin [1] and Tat [2], were discovered in 1994 and 1997, respectively, and numerous other CPPs have been reported ever since. The range of CPP-compatible cargo molecules is wide and it includes many types of therapeutic proteins and peptides, nucleic acids, cytotoxic agents and imaging contrast agents [3]. In order to mediate its corresponding biological effect, each cargo type needs to reach the specific intracellular compartment, such as the cytoplasm or the nucleus [4–6]. However, it is still actively debated which mechanisms are involved in CPP-mediated cargo delivery and whether the mechanisms might differ for reaching separate intracellular targets.

While early reports suggested the involvement of direct (energy independent) pathways in the internalization process, the conclusion was based largely on cell fixation artifacts [3]. Thereafter endocytosis has been considered as the predominant uptake route, especially when CPPs are carrying cargo molecules. However, some more carefully controlled recent studies still suggest that direct translocation mechanism cannot be overruled in case of some naked or fluorescently labeled CPPs at certain conditions [7–9].

For both the endocytosis and direct translocation the CPPs interact with the cell membrane prior to being taken up by cells [8]. The cell membrane, however, contains many components that are required for different types of endocytosis [10–14] and different cell lines might express these constituents in various levels. Composite CPP properties could therefore lead to multifaceted membrane interactions, and it is thus not surprising why the results of CPP uptake mechanism studies are not always converging.

It seems that, similarly to uptake of many ligands and receptors [13], different endocytosis sub-types can be involved simultaneously in the CPP uptake process [3,15]. Their relative importance and the subsequent intracellular fate of the internalized material may depend on numerous factors. For example, at low concentration epidermal growth factor (EGF) triggers its receptor (EGFR) internalization via

Abbreviations: CPP, Cell-penetrating peptide; siRNA, Short interfering ribonucleic acid; EGF, Epidermal growth factor; EGFR, Epidermal growth factor receptor; CME, Clathrin-mediated endocytosis; SPPS, Solid phase peptide synthesis; *t*-Boc, *tert*-Butyloxycarbonyl; HOBt, Hydroxyl-benzotriazole; DCC, *N,N′*-Dicyclohexylcarbodiimide; DIEA, *N,N′*-Diisopropylethylamine; DMF, *N,N′*-Dimethylformamide; Cpz, Chlorpromazine; CyD, Cytochalasin D; MP, Macropinocytosis; Nys, Nystatin; C/LR, Caveolae/lipid raft dependent endocytosis; CQ, Chloroquine; TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate; LDH, Lactate dehydrogenase; AUC, Area-under-curve; RLU, Relative luminescence unit

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clathrin-mediated endocytosis (CME) after which the receptor is recycled back to the membrane. However when the EGF concentration is increased the uptake of EGFR is routed to a non-CME pathway during which the receptor will be degraded [13]. Since many CPPs are derived from proteins it could be hypothesized that similar processes to the previously described example could also occur when delivering cargos with CPPs, thus making it important to study their internalization pathways [13,16].

Most studies in the field regarding CPP uptake mechanisms are end-point studies. While biological readout systems are exploited in some investigations (e.g. the delivery of enzymes or oligonucleotides), mostly fluorophore-labeled peptides have been used. Furthermore, CPP uptake kinetic measurements should be preferred to end-point studies because in an arbitrarily chosen end-point certain effects of endocytosis inhibitors and thus the involvement of certain pathways could be left unregistered. Further, differences between peptides that might display similar overall internalization degree may have completely different kinetic profiles which could not be noticed using end-point assays [17].

In this paper we have used luciferin-conjugated CPPs (luciferin–CPPs) to evaluate CPP uptake mechanisms. Previously we have used a semi-biological real-time uptake kinetics assay [18,19] to characterize CPPs based on their uptake kinetics profiles [17]. In the latter study we showed that CPPs can be divided into two distinct groups —the fast internalization group (TP10, MAP and Tat) whose uptake kinetics profile resembled the behavior of membrane permeable free luciferin, and the slow uptake group (TP10(Cys), *p*Vec, M918, penetratin and EB1) whose uptake profile was more consistent with the uptake rates conventionally observed in case of endocytosis.

Here we used the same assay to assess the effects of selected endocytosis inhibitors on the CPP cytosolic uptake kinetics profiles of the both CPP groups. We will show that endocytosis is extensively involved in the cytosolic delivery of all the tested luciferin–CPP conjugates, even in case of the fast uptake group peptides despite their behavior resembles the membrane permeable free luciferin. We will also discuss how different luciferin–CPP conjugates can utilize preferred endocytosis sub-type depending on the conjugate concentration.

2. Materials and methods

2.1. Peptide synthesis

The peptides used in this study (Table 1) were synthesized using a solid phase peptide synthesis (SPPS) method on an automated peptide synthesizer (ABI 433A, Applied Biosystems, USA). In the synthesis *tert*-butyloxycarbonyl (*t*-Boc) chemistry was used. Shortly, *t*-Boc amino acids (Neosystem, France; Iris Biotech, Germany; Bachem AG, Switzerland) were coupled as hydroxyl-benzotriazole (HOBt) esters (Iris Biotech, Germany) in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC; Iris Biotech, Germany) and *N*,*N*′-diisopropylethylamine (DIEA; Iris Biotech, Germany) to a 4-methylbenzhydryl-amine resin (Iris Biotech, Germany). This yielded in C-terminally amidated peptides.

TP10 peptide was modified by manual coupling of the Cys residue to the ε-amino group on its Lys^7 to obtain TP10(Cys) peptide. The Cys residue was activated by HOBt, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyl-aminium tetrafluoroborate (TBTU) and DIEA.

Before being cleaved from the resin, peptides containing His(Dnp) or Trp(For) were deprotected with 20% thiophenol in *N*,*N*′-dimethylformamide (DMF) for 1 h or 20% piperidine in DMF for 1 h, respectively. All peptides were cleaved from the resin using anhydrous hydrogen fluoride/*p*-cresol/*p*-thiocresol (90/5/5) solution for 1 h at 4 °C. After cleavage the peptides were ether-precipitated and finally purified by semi-preparative reverse-phase HPLC column (Discovery®BIO Wide Pore C-18, Supelco®, Sigma-Aldrich, Sweden).

Table 1
Cell-penetrating peptides used in this paper.

CPP	Cys-CPP sequence	Origin	Type	Ref.
MAP	C-KLALKLALKALKAALKLA-amide	Synthetic	Secondary amphipathic	[52]
TP10	C-AGYLLGKINLKALAALAKKIL-amide	Chimeric	Primary amphipathic	[53]
TP10(Cys)	AGYLLGK(C)[a]INLKALAALAKKIL-amide	Chimeric	Primary amphipathic	[53]
EB1	C-LIRLWSHLIHIWFQNRRLKWKKK-amide	Chimeric	Secondary amphipathic	[54]
Penetratin	C-RQIKIWFQNRRMKWKK-amide	Protein derived	Secondary amphipathic	[1]
*p*Vec	C-LLIILRRRIRKQAHAHSK-amide	Protein derived	Secondary amphipathic	[55]
Tat	C-GRKKRRQRRRPPQ-amide	Protein derived	Non-amphipathic/cationic	[2]
M918	C-MVTVLFRRLRIRRASGPPRVRV-amide	Protein derived	Non-amphipathic/cationic	[56]

[a] Luciferin was coupled to the cysteine at the ε-amino group on Lys^7.

Their purity was determined by RP-HPLC analytical column (Discovery® C-18, Supelco®) and correct molecular weight was verified by MALDI-TOF (Perkin Elmer prOTOF™ 2000, Perkin Elmer, Sweden) mass spectrometry.

2.2. Synthesis of luciferin–CPP conjugates

Luciferin-linker was synthesized as previously reported (see Scheme 1 in [20]). Luciferin-linker and Cys-CPPs were mixed in 1:1 molar ratio at 0.88 mM concentration in DMF/acetic acid buffer (pH 5, 50 mM) for 1 h under nitrogen. Luciferin–CPP conjugates were purified by semi-preparative RP-HPLC column, and their purity (>99%) was analyzed by RP-HPLC analytical column. The correct molecular weight was verified by MALDI-TOF mass spectrometry.

2.3. Cell culture

HeLa pLuc 705 cells, kindly provided by Ryszard Kole [21], were grown in DMEM (Dulbecco's modified Eagle's medium) with glutamax and supplemented with 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin (hereafter referred to as complete medium). For uptake kinetics experiments, complete medium without phenol red was used. Cells were grown in a humidified 5% CO_2 environment at 37 °C. Cell culture media and supplements were purchased from Invitrogen, Sweden.

2.4. Uptake kinetics

2×10^5 cells were seeded onto a 6-well cell culture plate (day 1). 24 h later (on day 2) 4 μg luciferase encoding pGL3 plasmid (Promega, Sweden) was complexed with 10 μl Lipofectamine™ 2000 reagent (Invitrogen, Sweden) and the cells were transfected with the plasmid according to the manufacturer's instructions in 2.5 ml complete medium for 4 h. After that the transfection medium was replaced with fresh complete medium and the cells grown for further 20 h. After that, on day 3, 9×10^3 cells were seeded in a white 96-well clear-bottom plate (Greiner Bio-One, Germany) and 24 h after seeding, on day 4, the cells were treated with luciferin–CPP conjugates.

On the day of the experiment the cells were washed once with 100 μl complete cell culture media, after which 30 min pre-incubation with endocytosis inhibitors was conducted. The inhibitors