Effective siRNA delivery and target mRNA degradation using an amphipathic peptide to facilitate pH-dependent endosomal escape

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Effective delivery of siRNA (small interfering RNA) into the cells requires the translocation of siRNA into the cytosol. One potential delivery strategy uses cell-delivery peptides that facilitate this step. In the present paper, we describe the characterization of an amphipathic peptide that mediates the uptake of non-covalently bound siRNA into cells and its subsequent release into the cytosol. Biophysical characterization of peptide and peptide/siRNA mixtures at neutral and lysosomal (acidic) pH suggested the formation of α-helical structure only in endosomes and lysosomes. Surprisingly, even though the peptide enhanced the uptake of siRNA into cells, no direct interaction between siRNA and peptide was observed at neutral pH by isothermal titration calorimetry. Importantly, we show that peptide-mediated siRNA uptake occurred through endocytosis and, by applying novel endosomal-escape assays and cell-fractionation techniques, we demonstrated a pH-dependent alteration in endosome and lysosome integrity and subsequent release of siRNA and other cargo into the cytosol. These results indicate a peptide-mediated siRNA delivery through a pH-dependent and conformation-specific interaction with cellular membranes and not with the cargo.

Key words: amphipathic peptide, endosomal escape, small interfering RNA (siRNA) delivery, small interfering RNA (siRNA) trafficking.

INTRODUCTION

Specific gene silencing by RNAi (RNA interference) requires formation of the RISC (RNA-induced silencing complex). Endogenously processed miRNA (microRNA) or exogenously added siRNA (small interfering RNA) is assembled into the RISC loading protein complex containing Ago2 (Argonaute 2), TRBP (TAR RNA-binding protein) and Dicer proteins [1]. Specific base pairing between the miRNA or siRNA and the corresponding mRNA leads to precise degradation or, in the case of miRNAs, translational repression of target mRNAs. The decrease in target mRNA levels reduces the steady-state levels of the encoded proteins over time, depending on their respective half-lives. This specific reduction in target-gene mRNA levels allows studying individual gene function in cells, target validation for small-molecule drug discovery and the opportunity to develop siRNA or miRNA as therapeutic agents.

Successful delivery of siRNAs into cells, however, remains a major obstacle in the development of siRNA therapeutics. Since the RISC proteins and the target mRNAs are located in the cytosol, it is necessary for the siRNAs to be delivered into the cytosol where they can enter the RISC complex. Because the siRNA is negatively charged and has a relatively high molecular mass, it cannot cross directly through the plasma membrane. To address these issues, researchers have implemented a number of strategies to improve siRNA uptake into cells. These include packaging or complexing siRNA into LNPs (lipid nanoparticles) [2–4], conjugation of siRNA with lipids such as cholesterol [5] or conjugating siRNA with polymers [6]. The exact molecular mechanisms of endocytosis and endosomal escape for all of these delivery platforms remains unknown.

Another approach to siRNA delivery is to use specific peptides known as CPPs (cell-penetrating/cell-delivery peptides) [7–10], which have also been described as PTDs (protein transduction domains). The first peptide that was discovered to be able to cross membranes was the TAT (transactivator of transcription) peptide [11], which subsequently led to the development of arginine-rich peptides [9,12,13]. Other CPPs like Antennapedia, which is derived from the Drosophila antennapedia homeobox domain [14–16], have also been investigated. For most of the CPPs, it is still under debate as to whether their uptake mechanism involves endocytosis [17–22]. We hypothesize that most siRNA-delivery technologies use endocytosis for cell entry: the siRNA is taken up into transport vesicles and subsequently into intracellular organelles such as endosomes. Ultimately, the internalized siRNA has to escape from the endosome. This could involve a non-specific disruption of the endosome or the formation of specific pores through which siRNA can pass.

Particularly interesting for nucleic acid delivery are amphipathic peptides, which may contain high numbers of histidine...
residues. These peptides have previously been shown to interact with DNA and efficiently promote nucleic acid uptake into cells [23,24]. Most recently, this class of cell-delivery peptides has been shown not only to deliver DNA but also siRNA into human cells; importantly, these peptides showed very favourable toxicity profiles [25].

In the present paper, we describe the characterization of the histidine- and leucine-containing amphipathic peptide Endoporter [26] that has been demonstrated to deliver morpholino–RNA into cells [26]. We show that Endoporter effectively delivers siRNA into cells. By applying a set of assays to characterize its siRNA delivery properties, we show that the peptide not only promotes the uptake of siRNA into cells, but also facilitates pH-dependent endosomal escape to the cytosol where the siRNA is loaded into RISC and target mRNA is degraded. We also obtained CD spectra and applied ITC (isothermal titration calorimetry) to investigate the biophysical properties of the peptide and siRNA and their interactions at different pH values. Importantly, we evaluated protein reporter molecules in endosomal-escape assays and demonstrated a pH-dependent Endoporter-mediated release of these molecules into the cytosol, similar to that observed with siRNA.

EXPERIMENTAL

Materials

Anti-biotin antibody (mouse, monoclonal) was purchased from Sigma (cat. no. B7653). Anti-GAPDH (gyceraldehyde-3-phosphate dehydrogenase) antibody (mouse, monoclonal) was obtained from Applied Biosystems/Ambion (cat. no. AM4300); HRP (horseradish peroxidase)-conjugated secondary antibodies were from Bio-Rad Laboratories. HRP (Type II), dinaose and SLO (streptolysin O) were from Sigma, and biotin–BSA was from Thermo Fisher Scientific. Endoporter (aqueous, in 300 mM mannitol or in DMSO) was obtained from Gene Tools, and other reagents were purchased from AnaSpec and reconstituted in water or DMSO. Bafilomycin A, concanamycin A and cytochalasin D were purchased from EMD Biosciences; sucrose was from USB. Heparin was obtained from Hospira. Acetic acid, sodium acetate, or DMSO. Bafilomycin A, concanamycin A and cytochalasin D peptides were purchased from AnaSpec and reconstituted in water (1.5 μM) or in DMSO) was obtained from Gene Tools, and other reagents were usually washed with 15 IU/ml heparin in medium B at the end of siRNA/peptide incubation. Cell viability was determined using MTS reagent (Promega) according to the manufacturer’s protocol.

CD

CD measurements were performed with a Jasco J-810 spectrometer. Samples of Endoporter peptide and Ssb siRNA (25 μM each) were prepared at pH 5.0 (20 mM acetate and 140 mM NaCl), pH 6.0 (20 mM Mes and 140 mM NaCl) and pH 7.4 (20 mM Hepes and 140 mM NaCl). CD signals were collected in the range 200–300 nm at 20°C using a 0.1 cm path length cuvette. A resolution of 1 nm and a scanning speed of 20 nm/min were used. CD spectra are presented as an average of three consecutive measurements. Buffer spectra were subtracted from peptide/RNA spectra and raw CD signals were converted to molar ellipticity using Jasco spectral analysis software.

ITC

ITC was conducted on a VP-ITC Microcalorimeter (MicroCal) with a cell volume of 1.43 ml. Endoporter (aq. 50 μM) and siRNA (1.5 μM) were prepared in buffers at pH 6.0 (20 mM Mes and 140 mM NaCl) and pH 7.4 (20 mM Hepes and 140 mM NaCl).

All of the buffers also contained 15 mM mannitol to match the concentration in the Endoporter stock solution. All samples were generally stored at 4°C for >2 days prior to usage and they were all degassed in a ThermoVac (MicroCal) prior to experiments. Water was used in the ITC reference cell. For each titration, 10 μl of Endoporter peptide in a computer-controlled pipette stirring at 307 rev./min was automatically added to siRNA in the sample cell of the calorimeter equilibrated at 37°C. The addition was programmed to occur within 20 s and at an interval of 300 s. A total of 250 μl of peptide was added to the siRNA. The titration of peptide to a buffer solution without siRNA was also conducted to serve as a control.

Cell culture

HeLa cells were purchased from A.T.C.C. HeLa, Huh-7 and HEK-293T (human embryonic kidney-293 cells expressing the large T-antigen of simian virus 40) cells were cultured at 37°C under a 5% CO2 atmosphere in DMEM (Dulbecco’s modified Eagle’s medium) (containing 4.5 g/l glucose) supplemented with 10% (v/v) FBS (fetal bovine serum) (Thermo Fisher Scientific), 100 IU/ml penicillin G and 100 μg/ml streptomycin sulfate (medium A). Medium B was medium A without FBS and supplements.

On day 0, cells were set up in medium A at a density of 1 × 104 cells per well of a 96-well plate (flat bottomed), 5 × 105 cells per well of a 12-well plate or 2 × 105 cells per well of a 6-well plate. For 150-mm-diameter dishes, cells were seeded at a density of 5 × 105 cells per dish. Cell treatment was performed on day 1; if cells were incubated with inhibitors, treatment occurred for 30 min at 37°C prior to incubation with siRNA/Endoporter. Cells were usually washed with 15 IU/ml heparin in medium B at the end of siRNA/peptide incubation. Cell viability was determined using MTS reagent (Promega) according to the manufacturer’s protocol.

siRNA incubation and stem-loop PCR

Peptides were mixed with chemically modified siRNA [2,27] targeted against Sjögren’s syndrome antigen B (Ssb; GenBank accession number NM_003142) using the following sequences: sense, 5′-ACACACGACUUAAGUAAATT-3′; antisense, 5′-UUACAUUAAAGUCUGUUGUTT-3′; or a siRNA targeted against Lamin A/C [28], which was obtained from Applied Biosystems. The control (non-target) sequence used was 5′-AAUAUCGACUGUCCAGCUAG-3′. Peptides and siRNA were combined in medium B and incubated for 15 min at 20°C prior to adding the mixture to cells. Ssb siRNA stem–loop PCR was conducted as described elsewhere [2,29] using the following primers: RT (reverse transcription) primer, 5′-GTCATATCCAGTGGTTCCAGGTATCTCGCAGTACGACAAAACACAGA-3′; forward primer, 5′-GGCGGTATTCAATAAGTC-3′; reverse primer, 5′-GTCAGGAGGATCCGAGGTTGTTCAG-3′; and stem–loop primer, 5′-GTCATATCCAGTGGTTCCAGGTATCTCGCAGTACGACAAAACACAGCUUAAGUAAAGCCCA-3′. Quantitative PCR reaction was performed on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems) using standard cycling conditions [2]. The derived C values from each experiment were normalized to that of the endogenous miRNA mir-16 (Applied Biosystems) and transformed into copy numbers using a linear equation derived from the standard curve, which was run in parallel. For the determination of the total associated siRNA in cells, a whole-cell lysate was prepared and processed with a TaqMan
MicroRNA Cells-to-CT Kit (Applied Biosystems) according to the manufacturers’ protocol.

**Liposome-based transfection**

The lipid-based transfection reagents DOTAP [N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate, also called dioleoyltrimethylammonium propane] (Roche) and ESCORT, a 1:1 lipid formulation of DOTAP/DOPE (dioleoyl phosphatidylethanolamine) (Sigma) were mixed with siRNA according to the manufacturers’ protocols, and complexes were incubated on cells for up to 6 h followed by cell lysis.

**bDNA (branched DNA) assay**

HeLa cells grown as monolayers in 96-well plates were incubated as indicated and lysed. $Ssb$ mRNA levels were determined using a bDNA assay according to the manufacturer’s protocol (Panomics). Values were normalized to that of endogenous levels of cyclophilin B [also called PPIB (peptidylprolyl isomerase B); Panomics] or to total protein levels as determined by bicinchoninic acid assay (Bio-Rad).

**Cell fractionation**

HeLa cells were seeded on to one 150 mm dish per experimental condition the day before the experiment. On the day of the experiment, cells were incubated as indicated and then washed twice with PBS on ice. The cells were then scraped into 5 ml of ice-cold PBS and pelleted (500 g for 5 min at 4°C) and finally resuspended in 1 ml of buffer A (250 mM sucrose and 3 mM imidazole, pH 7.4, containing the manufacturer’s recommended concentration of Protease Inhibitor Cocktail Set III (EMD Biosciences)). After incubation for 20 min on ice, cells were homogenized with a ball-bearing homogenizer (6 strokes, 10 μm clearance; Isobiotec). Lysates were centrifuged at 1000 g for 10 min at 4°C, and the resulting PNS (post-nuclear supernatant) (approx. 1 ml) was carefully loaded on to 10 μl of a sucrose cushion (2.5 M) and centrifuged at 100000 g (45 000 rev./min) for 30 min at 4°C in a Beckman Optima TL table-top ultracentrifuge using a TLA 45 rotor. The supernatant (cytosol fraction) was recovered and the pellet (total membrane fraction) was washed once with PBS, centrifuged (45 000 rev./min for 10 min at 4°C) and resuspended in buffer A.

**HRP endosomal-escape assay**

HeLa cells were cultivated on 12- or 6-well plates and washed twice with medium B prior to loading with 5 mg/ml HRP in medium B containing 1 mg/ml BSA for 3 h at 37°C. After incubation, cells were washed repeatedly with serum-free medium and PBS. Permeabilization and cytosol recovery was then conducted as described in [30,31] using the pore-forming toxin SLO. Briefly, cells were washed at 4°C with cold ICT (intracellular transport buffer) composed of 50 mM Hepes, 78 mM KCl, 4 mM MgCl₂, 8.37 mM CaCl₂ and 10 mM EGTA (adjusted to pH 7.2 with KOH) and freshly added DTT (dithiothreitol) (1 mM final concentration). SLO was activated by incubation for 10 min at 37°C in the presence of 10 mM DTT and diluted in ice-cold ICT buffer, followed by treatment of cells at 4°C (10 min). The low temperature allowed specific binding to the plasma membrane only while preventing internalization of SLO and subsequent pore formation on internal membranes. Unbound SLO was then removed by washing the cells twice with ice-cold ICT. Cytosol was released by SLO-mediated pore formation (∼30–35 nm) in the plasma membrane as previously described [32] by incubation for 10 min at 37°C in pre-warmed ICT followed by an incubation for an additional 10 min at 4°C. Released cytosol was centrifuged for 10 min at 100000 g and 4°C; HRP activity was measured in triplicates using a peroxidase assay kit from Anaspec.

**BSA proteolysis assay**

HeLa cells were grown on 12- or 6-well plates and washed twice with medium B and then loaded with 1 mg/ml b-BSA (biotinylated BSA; Thermo Scientific) in serum-free medium for 1 h at 37°C in the absence or presence of Endoporter. Cells were then washed repeatedly (4–5 times) with serum-free medium containing 1 mg/ml BSA (non-biotinylated) and incubated for the indicated times in the absence or presence of Endoporter. The assay was stopped by washing cells with serum-free medium (1 mg/ml BSA) and PBS before adding reducing SDS sample buffer [2% (v/v) 2-mercaptoethanol] to the wells. Lysates were transferred into tubes, sonicated and heated for 5 min at 95°C. Samples were separated by SDS/PAGE and transferred on to a PVDF membrane as described previously [33]. Proteins were detected by probing the membranes with antibodies against biotin (Sigma) and GAPDH (Ambion) using the VersaDoc™ Imaging System (Bio-Rad Laboratories).

**RESULTS**

Endoporter facilitates siRNA-mediated reductions in $Ssb$ mRNA

A set of cell-penetrating peptides was tested for their ability to deliver siRNA into cells and facilitate the reduction of target mRNA levels (Table 1). Because conjugation of reporter molecules (e.g. fluorophores) to the siRNA or peptides could change their biophysical properties and affect their uptake and delivery characteristics, we did not measure their internalization directly. Instead, we quantified siRNA-mediated mRNA reduction using a bDNA assay [34] as a surrogate for cytosolic siRNA delivery. siRNA was mixed with peptide (50 μg/ml final concentration) prior to adding them to cells. Figure 1 shows an experiment in which we incubated HeLa cells for 6h with a mixture of the indicated peptides (Table 1) and 20 nM siRNA targeted against $Ssb$ mRNA, which expresses the La antigen protein [35]. $Ssb$ is a good model target because of its robust expression in most tissue-culture cell lines and animal tissues. Furthermore, a reduction in $Ssb$ levels by as much as 80% in numerous cell types is non-toxic (results not shown). Of all the peptides tested, only Endoporter, either dissolved in DMSO (D) or in aq. 0.3 M mannitol (A), showed significant and specific mRNA degradation compared with control-treated cells (Figure 1A). None of the peptides had a significant effect on the viability of cells as measured by the MTS cell-proliferation assay (Figure 1B).

Based on the low toxicity profile and effectiveness of siRNA delivery and target mRNA reduction, we selected the Endoporter peptide for further mechanistic studies. To determine whether the internalized siRNA is able to facilitate mRNA degradation in a concentration-dependent manner, cells were incubated with Endoporter peptide mixed with siRNA ($Ssb$) in two different titration experiments: first, while leaving the concentration of Endoporter constant at 5 μM, we increased the concentrations of siRNA from 0.39 nM to 5 μM (1:1 siRNA/peptide). Reductions in $Ssb$ mRNA levels were quantified after 6 h of incubation using a bDNA assay. $Ssb$ mRNA levels
Figure 1  Endoporter facilitates siRNA-mediated target mRNA reduction

(A) Evaluation of peptide toxicity and ability to effectively deliver Ssb siRNA. The indicated peptides at a concentration of 50 μg/ml in combination with 20 nM Ssb siRNA in medium B were incubated for 20 min and then added to HeLa cells. After 6 h, target-specific reductions in mRNA levels were determined using the bDNA assay. Cell viability was determined in parallel using the MTS cell-proliferation assay (B). (C) siRNA concentration response. siRNA concentration response is the equivalent experiment to ‘Endoporter concentration response’ (D). Endoporter was kept constant and the siRNA concentration was changed. HeLa cells (10^4) were seeded into each well of a 96-well plate at day 0. On day 1, 5 μM (∼18.7 μg/ml) Endoporter was mixed with the indicated concentration of siRNA in medium B and added to cells for 6 h at 37 °C. After 6 h, cells were lysed and mRNA levels were determined using the bDNA assay. Values were normalized to values from cells that did not receive any treatment. (D) Endoporter concentration response. HeLa cells were grown and incubated as in (C). The siRNA concentration was kept constant (25 nM) and siRNA was mixed with the indicated Endoporter concentrations in medium B. After 6 h, cells were lysed and mRNA levels were determined as described above. (E) Determination of Endoporter toxicity. HeLa cells were seeded on to 96-well plates as described above and on the next day were incubated with the indicated concentrations of Endoporter in medium B. All treatment groups had the same vehicle concentration of mannitol. After 18 h, cell viability was determined using the MTS assay as described in the Experimental section and values were normalized to the group that did not receive any treatment. Ant, Antennapedia leader peptide; Endoporter A, Endoporter dissolved in aq. 0.3 M mannitol; Endoporter D, Endoporter dissolved in DMSO; LMTP, lipid-membrane-translocating peptide; MPS, membrane-permeable sequence; NGR, aminopeptidase N ligand; Pep-1, Pep-1-cysteamine; R9, arginine 9; TAT, HIV transactivator of transcription; 105Y, [Cys58]105Y, cell-penetrating peptide, α1-antitrypsin.

were reduced by >50% at a concentration of 1.56 nM siRNA; the maximum Ssb mRNA reduction (approx. 75%) was observed with Ssb siRNA concentrations above 50 nM. In a second set of experiments, the siRNA concentration was kept constant (25 nM) and mixed with increasing concentrations of Endoporter peptide. At a ratio of 40:1 (1 μM peptide/25 nM siRNA), Ssb mRNA levels were reduced to approx. 50%. The maximum reduction in mRNA levels (∼80%) was achieved at a peptide/siRNA (Ssb) ratio of 7 μM peptide/25 nM siRNA (Figure 1D). No reductions in mRNA levels were observed in control experiments with Ssb...
siRNA in the presence of equivalent volumes of the mannitol vehicle. Importantly, Endoporter was able to effectively deliver siRNA against another target, *Lamin A/C* (Supplementary Figure 1A at http://www.BiochemJ.org/bj/435/bj4350475add.htm), and into the additional cell lines Huh-7 and HEK-293T (Supplementary Figure 1B). To verify that the Endoporter concentrations used in the experiments were non-toxic, cells were treated with up to 32 μM Endoporter without any impact on cell viability as determined with a MTS cytotoxicity assay (Figure 1E).

### Transfection efficiency of Endoporter in comparison with lipid-based transfection reagents

First, we compared the transfection efficiencies and impact on cellular viability of Endoporter to that of lipid-based transfection reagents. We tested the cationic lipid formulations DOTAP and ESCORT by incubating cells with a constant amount of Ssb siRNA (50 nM) with increasing volumes of lipids (Figure 2). Since the concentration of total lipid in ESCORT was 2 mg/ml and in DOTAP it was 1 mg/ml, equal volumes of each lipid formulation were used for complex formation in order to incubate cells with identical concentrations of DOTAP. The ESCORT lipid formulation was not toxic at any of the concentrations tested as shown by cell viability (Figure 2A). In contrast, DOTAP alone affected cell viability at concentrations > 12 μM/ml lipid as measured by a MTS assay. Both DOTAP and ESCORT effectively delivered siRNA and mediated reductions in Ssb mRNA levels at a concentration of 12.5 μM/ml lipid, the highest non-toxic concentration for DOTAP (Figures 2B and 2C). Importantly, similar mRNA reductions were observed when cells were incubated with siRNA in the presence of Endoporter. Furthermore, lipid-based and Endoporter-mediated delivery showed similar kinetics (compare 3 h with 6 h).

### Endoporter-mediated reductions in Ssb mRNA levels requires endosomal acidification

To elucidate the peptide-mediated siRNA-delivery mechanisms, we determined whether Endoporter increased the total amount of cell-associated siRNA (bound to the plasma membrane and internalized) by direct quantification of the siRNA using stem–loop PCR [2]. HeLa cells were incubated with a mixture of Endoporter ranging from 20 μM to 10 nM and 50 nM siRNA (*Ssb*) for 6 h. Whole-cell lysates were prepared and the ratio of *Ssb* siRNA to *miR-16* copy numbers was determined by stem–loop PCR. siRNA alone did not associate with cells; however, increasing concentrations of Endoporter at non-toxic levels (Figure 1E) enhanced the amount of associated siRNA (Figure 3A).

Since Endoporter stimulates the association of siRNA with cells, we designed experiments that tested whether the siRNA was internalized by endocytosis. Since cells usually use different pathways of endocytosis (e.g. clathrin- and non-clathrin-mediated endocytosis), one can selectively manipulate these with pharmacological inhibitors. However, it is known that inhibitors of endocytic trafficking often display non-specific side effects depending on cell type, concentration and/or exposure time [36]. To minimize exposure times, we determined the minimum treatment time that was required to observe Endoporter-mediated siRNA delivery and reductions in target mRNA levels. Endoporter/siRNA mixtures (5 μM Endoporter/50 nM siRNA) were incubated with HeLa cells for 0.5, 1, 3 and 18 h. Cells were then washed and incubated in the absence of Endoporter and siRNA for a total of 18 h (Figure 3B). Incubation of siRNA and Endoporter with cells for as little as 30 min resulted in a reduction in Ssb mRNA levels of more than 50 %. The maximum reduction in mRNA levels (appro. 75 %) was observed after 3 h of treatment, and no further reduction was detected after 18 h. Since the incubation of cells with Endoporter and siRNA for 3 h followed by a treatment in the absence of nucleotide and peptide for 15 h was sufficient for efficient mRNA knockdown, the 3 h incubation time was chosen for the next experiments.

Low temperatures are known to reduce endocytic processes [37–39], therefore we tested the effect of temperature reduction on the ability of Endoporter to deliver siRNA and reduce target mRNA levels. Endoporter/siRNA mixtures were incubated with cells for 3 h at 4°C followed by the removal of unbound material through several washes. The temperature was then raised to 37°C and cells were incubated in the absence of Endoporter/siRNA for an additional 15 h. The *Ssb* mRNA levels were quantified and found to be comparable with those of control-treated cells (Figure 3C), indicating that Endoporter/siRNA-mediated knockdown was abolished at low temperatures.

We also examined the effects of different endocytic pathway inhibitors for their ability to block peptide-mediated siRNA delivery. Cells were pre-treated for 30 min with either cytochalasin D [40–42], an inhibitor of F-actin polymerization, or dynasore, an inhibitor of dynamin-dependent endocytosis [43,44]. Cells were then incubated for 3 h with Endoporter/siRNA mixtures in the presence of the drugs. After incubation, cells were washed and incubated for an additional 15 h in the absence of siRNA, peptide or drugs. In contrast with the 4°C block, none of the treatments reduced delivery, and mRNA levels remained
A may affect the endocytosis of certain cargo into cells [38], we tested whether the drug altered the uptake of siRNA by analysing the total cell-associated siRNA using stem–loop PCR. The siRNA present in lysates from control- and bafilomycin A-treated cells was quantified and similar levels were found in both (Figure 3E). We conclude that Endoporter facilitates the release of siRNA out of endosomes and that acidification of endo-lysosomal compartments is required for this release.

Endoporter facilitates pH-dependent endosomal escape of siRNA

In the previous experiments, we characterized the siRNA-delivery properties of Endoporter by quantification of the siRNA associated with cells and by measuring siRNA-mediated reductions in target mRNA levels. To expand on these results, we evaluated the effect of bafilomycin A treatment on siRNA intracellular localization by the most direct means possible. The cells were fractionated by standard techniques and the siRNA from specific cellular compartments quantified by stem–loop PCR following Endoporter-mediated delivery of siRNA. Control cells or cells growing in the presence of bafilomycin A were incubated with an Endoporter/siRNA mixture for 6 h and harvested. After gentle homogenization [47], lysates were centrifuged (1 000 g) to remove the nuclei and unbroken cells. The integrity of endosomes after cell breakage had been evaluated in independent experiments [31] (results not shown). The resulting PNS was centrifuged further and separated into a TM (total membrane) fraction and a cytosolic fraction. Bafilomycin A-treated cells showed a similar uptake of siRNA when compared with control cells. Bafilomycin A treatment did not reduce the amount of siRNA in the PNS (Figure 4A), but caused an accumulation of siRNA in the membrane fraction (Figure 4B) and a significant decrease in the cytosolic fraction (Figure 4C). Samples from the same experiment were used to verify bafilomycin A-dependent mRNA reductions (Figure 4D).

Biophysical characterization of Endoporter and siRNA

Since the endosomal release of siRNA by Endoporter depends on the acidification of the organelles, we next examined the biophysical properties of the peptide at different pH values. First, we applied CD to analyse the conformation of Endoporter and siRNA at acidic (pH 5.0 and 6.0) and near-neutral pH (pH 7.4). At low pH, the CD spectra showed a double minimum near 208 and 220 nm, suggesting that the peptide contains a significant amount of α-helical structure (Figure 5A). In contrast, no such structural features were observed at near-neutral pH (Figure 5A, pH 7.4). Then, we examined potential pH-dependent conformational changes of the peptide in the context of RNA molecules. Natural RNA duplexes usually adopt to an A-form conformation, showing a large positive CD band above 260 nm and a large negative band near 210 nm [48]. CD spectra of the Ssb RNA duplex alone, however, only exhibited a broad positive peak above 260 nm, while several much less intense bands are observed near 240, 225 and 210 nm at pH 6.0 (Figure 5B). This might be due to the various chemical modifications on the Ssb siRNA [2] that could affect the arrangement of its higher-order structure. As expected, no changes in the CD spectra of Ssb siRNA were observed as the pH shifted from the neutral to the acidic range (Figures 5B and 5C). Next, we analysed potential interactions of siRNA and Endoporter at different pH values by obtaining CD spectra of 1:1 mixtures (molar ratio) of peptide/siRNA. At pH 7.4, the CD spectrum of the mixture is not significantly different from combined spectra of the peptide and RNA when acquired
Figure 3  Endoporter mediates siRNA uptake and target mRNA reduction

(A) Increasing concentrations of Endoporter enhance siRNA uptake into cells. HeLa cells (10^4) were seeded on to 96-well plates and, on the next day, 50 nM siRNA was mixed with the indicated concentrations of Endoporter in medium B and added to cells for 6 h at 37°C. After incubation, cells were washed with PBS and lysed. Ssb copy numbers were quantitatively determined as described in the Experimental section and normalized to copy numbers of the endogenous miR-16. As a control, cells were either left untreated or incubated in the presence of 50 nM siRNA alone. All treatment...
HeLa cells were incubated with 100 nM bafilomycin A or vehicle (DMSO) for 30 min at 37°C and then incubated with a mixture of Ssb siRNA (50 nM) and Endoporter (5 μM) in serum-free medium in the presence or absence of drugs for 6 h at 37°C. After incubation, cells were harvested, resuspended in buffer A and lysed gently using a ball-bearing homogenizer. PNS (A), the membrane fraction (B) and cytosol (C) were prepared as described in the Experimental section, and aliquots of all three fractions were analysed by stem–loop PCR to determine the Ssb siRNA copy numbers as described previously. An aliquot of PNS from the same experiment was used to determine Ssb mRNA knockdown using a bDNA assay (D).

Similarly, the effects of temperature and endocytosis inhibitors on Endoporter-mediated siRNA delivery and reductions in target mRNA levels. Cells were pre-incubated with either 2.5 μM cytochalasin D, 100 μM dynasore or vehicle (DMSO) for 30 min at 37°C prior to incubation with Endoporter/siRNA mixture in the presence of drugs or vehicle control. After incubation for 3 h at 37°C, cells were washed and incubated in medium A for an additional 15 h. For the temperature-block experiment, cells were chilled to 4°C before adding the Endoporter/siRNA mixture. After 3 h, cells were washed and incubated in medium A at 37°C for 15 h. The incubation was stopped by adding cell-lysis buffer, and Ssb mRNA levels were determined by a bDNA assay as described in the Experimental section. (D) Bafilomycin A treatment blocks the effective delivery of siRNA. Cells were pre-incubated with 100 nM bafilomycin A or vehicle (DMSO) for 30 min at 37°C prior to addition of the Endoporter/siRNA mixture for 6 h in the presence of bafilomycin A. Cells were lysed and mRNA levels were determined by the bDNA assay. (E) siRNA uptake in the presence of bafilomycin A. Cells were treated as in (D) except cells were lysed after 3 h and siRNA copy numbers were determined as described in the Experimental section. Baf. A, Bafilomycin A; si, siRNA.
After subtracting the results of titrating Endoporter to buffer from these signals, little to no heat could be observed, suggesting that the peptide does not interact with siRNA at pH 7.4 (Figure 6A, lower panel). Similar experiments were conducted at acidic pH 6.0 to evaluate a potential interaction between Endoporter and siRNA in the acidic environment of endosomes and lysosomes. Figure 6(B) shows that small exothermic signals could be observed for the first several injections, followed by a gradual decrease in heat after that. The excess heat generated due to the titration of peptide to siRNA suggests an interaction between Endoporter and RNA at pH 6.0. The raw ITC data were processed and fitted to obtain the thermodynamic parameters of the interaction (Table 2). The results generated suggest that the peptide–siRNA interaction has a molar stoichiometry of \( \sim 3 \), and the process appears to have very low enthalpy and is mostly driven by entropy. This kind of thermodynamic feature is consistent with an ionic interaction process, i.e. charge neutralization [51]. Taken together, these results suggest that the binding of Endoporter and siRNA at low pH might be due to the ionization of the peptide and could be at least partially attributed to the electrostatic interaction between positive charges of peptide and negative charges of siRNA.

### Table 2 Thermodynamic parameters of the interaction between Endoporter and siRNA at pH 6.0

<table>
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<th>Stoichiometry (peptide/RNA)</th>
<th>( \Delta H ) (cal/mol)</th>
<th>( K ) (10^9 M(^{-1}))</th>
<th>( \Delta S ) (cal/(mol \times K))</th>
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Endoporter facilitates endosomal escape of reporter molecules

Since our biophysical characterization did not suggest a direct interaction between siRNA and peptide at neutral pH, we wanted to determine whether Endoporter was capable of mediating the release of molecules other than siRNA from endosomes when co-administered with peptide. To explore this, we developed a novel set of assays using two different protein reporter molecules for endosomal escape. First, we designed an assay (Figure 7A) that measures the degradation of b-BSA. BSA is known to be internalized into cells by endocytosis and rapidly degraded in lysosomal compartments [52]. We reasoned that if the peptide released BSA from lysosomes into the cytosol, then less b-BSA would be degraded. Cells were incubated with b-BSA for 1 h followed by the removal of non-internalized and plasma-membrane-bound b-BSA by several washes. The cells were then incubated for up to 10 h in the absence of b-BSA. Western blot analysis of total cell lysates with antibodies against biotin showed that b-BSA was internalized and detectable immediately after b-BSA had been removed by several washes (Figure 7B, upper panel), but was not detected after more than 3 h. This is presumably due to lysosomal proteolysis [52]. To test if this degradation can be prevented by blocking acidification, cells were incubated with the V-ATPase inhibitors bafilomycin A or concanamycin A [46,53]. As shown in Figure 7B (lower panel), the amount of internalized b-BSA was similar to the control samples (Figure 7B, upper panel) zero chase time. When b-BSA was incubated in the presence of Endoporter, an overall increase in internalization was observed. Importantly, the amount of cell-associated b-BSA was not reduced, suggesting that Endoporter-mediated escape of b-BSA into the cytosol protected b-BSA from lysosomal degradation.

Measuring lysosomal proteolysis of a reporter molecule is an indirect approach to studying endosomal escape. To address this issue, we developed a second assay in which cytosolic translocation of HRP as a reporter molecule for Endoporter-mediated endosomal escape was measured more directly. HRP is a well-established enzyme marker that is internalized into cells by fluid-phase endocytosis [54]. This assay is based on the ability to detect HRP enzymatic activity in the cytosol following release from endosomes. The amount of HRP that was internalized and subsequently translocated into the cytosol was quantified by measuring peroxidase activity in the cytosol following selective pore formation at the plasma membrane using SLO while leaving internal membranes intact [55,56]. Cytosolic levels of HRP were low in the absence of Endoporter (DMSO vehicle alone); however, with increasing concentrations of the peptide, the amount of HRP...
in the cytosol increased. To verify that the endosomal release of HRP was dependent on the acidification of endosomes, the experiment was also conducted in the presence of bafilomycin A. Bafilomycin A treatment reduced the amount of HRP in the cytosol to the levels observed in the control experiment (Figure 7C).

**DISCUSSION**

Even though the mechanism by which CPPs/PTDs translocate and deliver charged molecules into cells remains poorly understood, the ability of these peptides to translocate molecules into cells makes them a potentially useful component of a delivery vehicle for therapeutic applications. In order to gain understanding of the mechanism of CPPs, we developed a novel set of assays that were used to investigate the ability of peptides to deliver siRNA into cells, facilitate escape of molecules out of endosomes into the cytosol and to subsequently reduce mRNA levels in a specific manner.

After incubating different CPPs with siRNA to test for their ability to reduce mRNA target levels, we focused on the Endoporter peptide because it effectively delivered siRNA and mediated reductions in mRNA levels in a relatively short period of time. This was somewhat surprising, as it had been reported that Endoporter was effective at delivering morpholino antisense oligonucleotides, but not polyanions such as siRNA [26]. The ability of Endoporter to effectively deliver siRNA was comparable with known lipid-based transfection reagents (DOTAP or DOTAP/DOPE mixture), with a reduced toxicity compared with DOTAP.

Endoporter is a relatively short (approx. 30 amino acids) amphiphilic peptide [26] that is composed of leucine and histidine residues. The amino acid sequence was designed to form an α-helical structure with a proposed lipophilic face and a weak-base face [26]. Endoporter belongs to the class of histidine-rich amphipathic peptides that have been previously described for the delivery of nucleic acids [23–25]. It was shown that these peptides form complexes with plasmid DNA followed by internalization, pH-dependent dissociation of nucleic acid in the endosome and subsequent endosomal release [24]. The proposed mechanism of action for Endoporter may not involve binding to the cargo, but is suggested to consist of three steps: (i) binding to the plasma membrane; (ii) internalization and transport into endosomes; and (iii) pH-dependent pore formation in the endosomal membrane. On the basis of this cellular-uptake model, cargo that has been co-internalized with Endoporter will be ultimately released into the cytosol.

We showed that Endoporter is able to effectively deliver siRNA into cells in a concentration-dependent manner. In addition, the siRNA ultimately enters the cytosol and is incorporated into the RISC complex, where it mediates significant reductions in mRNA levels after just 30 min of incubation (Figure 3). The precise mechanism of peptide/siRNA uptake remains to be
Figure 7  Endoporter facilitates endosomal escape of reporter molecules

(A) Experimental rationale. (B) BSA proteolysis protection by Endoporter. Cells grown on 12-well plates were incubated with 100 nM bafilomycin A (upper panel, lanes 5–8), 200 nM concanamycin A (lower panel, lanes 1–4) or the equivalent concentration of DMSO (upper panel, lanes 1–4, and lower panel, lanes 5–8) in serum-free medium for 30 min at 37°C before adding 1 mg/ml b-BSA in serum-free medium in the presence of drugs or 4 μM Endoporter (lower panel, lanes 5–8). After incubation for 1 h, the reaction was either stopped (chase time 0 h) or cells were washed with medium B containing 1 mg/ml non-biotinylated BSA for the indicated time (chase times 3, 6 and 10 h) in the presence of Endoporter or drugs. After incubation, cells were washed and the reaction was stopped by adding 500 μl of SDS sample buffer. Aliquots of each sample (30 μl) were subjected to SDS/PAGE (10% gel) and BSA immunoblot analysis using monoclonal anti-biotin antibody. Membranes were re-probed with a monoclonal anti-GAPDH antibody to verify equal protein loading. (C) Endosomal Escape of HRP is mediated by Endoporter. HeLa cells were grown on 12-well plates and incubated with 5 mg/ml HRP in serum-free medium containing 1 mg/ml BSA in the presence of the indicated concentrations of Endoporter (1–3 μM), Endoporter and 100 nM bafilomycin A or bafilomycin A alone for 3 h at 37°C. After incubation, cells were chilled on ice and cytosol was released using SLO as described in the Experimental section. Enzymatic activity of HRP was determined in the supernatant of centrifuged cytosol. AU, arbitrary units.

elucidated, since reversible inhibition of pinocytosis with cytochalasin D [41,42] or dynamin-dependent endocytosis with dynasore [43,44] did not block Endoporter-mediated knockdown, whereas lower temperatures did abolish mRNA degradation. The simplest hypotheses are that Endoporter/siRNA molecules are rapidly taken up by multiple temperature-sensitive endocytic pathways or that reduction of particular pathways (e.g. dynamin-dependent endocytosis) may be compensated by the up-regulation of alternative pathways (dynamin-independent uptake). Even though Endoporter stimulates the uptake of siRNA (Figure 3) and
specific mRNA reduction is concentration-dependent (Figure 1), there appears to be no direct interaction between Endoporter and siRNA at neutral pH as suggested by CD and ITC data (Figures 5 and 6). This is in contrast with previous reports, where a direct interaction between siRNA or DNA and amphipathic peptides (presumably similar to Endoporter) was observed [23,25]. We speculate that in the case of Endoporter, such interaction between siRNA and peptide might not be necessary, since binding of the peptide to the plasma membrane may stimulate endocytic events with a subsequent increase in fluid-phase uptake of siRNA; this may apply also for other fluid-phase cargo, since Endoporter stimulated the uptake of b-BSA (Figure 7B).

As previously mentioned, there continues to be some debate regarding the ability of peptides and bound cargo to cross the plasma membrane directly. We cannot rule out the possibility that Endoporter facilitates the translocation of a small amount of siRNA into the cytosol directly across the plasma membrane by processes that do not involve endocytosis. However, when we tested whether effective delivery is dependent on the reduction of pH in intracellular organelles, we observed that Endoporter requires the acidification of endosomes, since activity can be blocked by bafilomycin A, even though treatment with the inhibitor did not block the uptake of siRNA into cells. Furthermore, we demonstrated that other cargos besides siRNA (BSA or HRP) require acidification of endosomes prior to translocation into the cytosol when co-administered with Endoporter.

The importance of acidification is also supported by our biophysical characterization showing that the peptide changes conformation in response to pH. At physiological pH (pH 7.4) the peptide does not appear to form any secondary structures, but does form an α-helical structure at endosomal/lysosomal pH (pH 5.0–6.0; Figure 5). Based on charge, it seems likely that Endoporter and siRNA interact at lower pH. We do not know how the interaction affects endosomal release of siRNA; however, it is possible that the complexes contain significant amounts of Endoporter that is not tightly associated and that unpacking may not be a significant barrier to delivery. We speculate that this α-helical structure might be able to interact with endosomal membranes and may subsequently disrupt these membranes, releasing endosomal content into the cytosol. Our results suggest either the formation of a large pore in the endosomal membrane or a less organized disruption of the organelle, since both HRP and BSA (44 kDa and 66 kDa) were able to translocate into the cytosol (see Figure 7).

Molecules, which are internalized into cells by means of endocytosis, are transported into early endosomes and then, through late endosomes or MVBs (multivesicular bodies), into lysosomes, where the bulk of lysosomal hydrolases are located. This presents a problem for siRNA delivery, since oligonucleotides may undergo degradation in these compartments. Since transport of cargo (like HRP) into lysosomes occurs within 2 h [54], the window for endosomal escape is narrow. When the acidification was inhibited by bafilomycin A, more siRNA could be detected in membrane fraction (Figure 4), suggesting that siRNA might not only be trapped in endosomal and lysosomal compartments, but that the elevated pH may reduce the degradation of the siRNA.

The successful delivery of siRNA into cells requires both sufficient uptake and a rapid and efficient means of endosomal escape to avoid lysosomal degradation. The assays described in the present paper can be used to evaluate other molecules in addition to peptides for their endosomal-escape properties, since endosomal escape is likely to be a property of most nucleic-acid-delivery technologies (liposomes, lipid nanoparticles, endolytic polymers and cholesterol conjugates). Using the approach described in the present paper, it may be possible to design therapeutics that have the appropriate siRNA potency, stability, pharmacodynamics, biodistribution and toxicity profiles.

**AUTHOR CONTRIBUTIONS**


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SUPPLEMENTARY ONLINE DATA

Effective siRNA delivery and target mRNA degradation using an amphipathic peptide to facilitate pH-dependent endosomal escape

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Figure S1 Test of Endoporter with different siRNAs and in different cell lines

(A) siRNA concentration response. HeLa cells (10⁴) were seeded into each well of a 96-well plate at day 0. On day 1, Endoporter (4 or 6 μM) was mixed with the indicated concentration of Lamin A/C siRNA in medium B and added to cells for 6 h at 37 °C. After 6 h, cells were lysed and mRNA levels were determined using quantitative PCR as described in the Experimental section in the main paper. Values were normalized to values from cells that had been treated with mannitol (control). (B) siRNA delivery into HEK-293T (Hek293T) and Huh-7 cells. Indicated cells were seeded and incubated as described above except that Ssb siRNA was used in combination with 6 μM Endoporter. After 6 h, the experiment was stopped by adding cell-lysis buffer and Ssb mRNA levels were determined by a bDNA assay as described in the Experimental section in the main paper.

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