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The role of endocytosis in the uptake and intracellular trafficking of PepFect14–nucleic acid nanocomplexes via class A scavenger receptors

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ABSTRACT

Cell penetrating peptides are efficient tools to deliver various bioactive cargos into cells, but their exact functioning mechanism is still debated. Recently, we showed that a delivery peptide PepFect14 condenses oligonucleotides (ON) into negatively charged nanocomplexes that are taken up by cells via class A scavenger receptors (SR-As). Here we unraveled the uptake mechanism and intracellular trafficking of PF14–ON nanocomplexes in HeLa cells. Macropinocytosis and caveolae-mediated endocytosis are responsible for the intracellular functionality of nucleic acids packed into nanocomplexes. However, only a negligible fraction of the complexes were trafficked to endoplasmic reticulum or Golgi apparatus — the common destinations of caveolar endocytosis. Neither were the PF14–SCO nanocomplexes routed to endo-lysosomal pathway, and they stayed in vesicles with slightly acidic pH, which were not marked with LysoSensor. "Naked" ON, in contrary, was rapidly targeted to acidic vesicles and lysosomes. The transmission electron microscopy analysis of interactions between SR-As and PF14–ON nanocomplexes on ultrastructural level revealed that nanocomplexes localized on the plasma membrane in close proximity to SR-As and their colocalization is retained in cells, suggesting that PF14–ON complexes associate with targeted receptors.

1. Introduction

Cell penetrating peptides (CPPs) are gaining popularity as non-viral delivery vectors for transfecting nucleic acids into cells, including oligonucleotide- based therapeutics [1–3]. To achieve sufficient transfection efficiency and functionality, several biological hurdles have to be overcome. The plasma membrane is the first barrier, regulating the trafficking between the interior and exterior of the cell. Uptake of CPPs is considered to start upon the interaction with negatively charged plasma membrane components, followed by internalization via different endocytosis mechanisms. The internalization mostly takes place by clathrin-dependent pathway [4,5], caveolin-dependent endocytosis [6,7] and via macropinocytosis [8]. However, the exact mechanism may depend on CPP, cargo, used cell line or particular experimental conditions and frequently different pathways may be utilized in parallel [4,9,10]. The above mentioned features significantly complicate the design and development of new CPP-based pharmaceuticals. Although, some CPPs can penetrate into cells independently from endocytosis and this pathway has been suggested as the main cell entry mechanism for example for CADY peptide [11] and for some arginine-rich peptides [9,12]; still if CPPs are coupled to big macromolecules, endocytosis is considered as the main uptake mechanism. Once inside the cells, the intracellular fate and trafficking of CPPs or CPP-cargo complexes is even less understood. Entrapment of CPPs in endosomes is the next obstacle that limits their access to intracellular targets.

Recently it was demonstrated that, in contrary to expected, the second generation CPP, PepFect14 (PF14) forms negatively charged nanocomplexes with splice correcting oligonucleotides (SCOs) [13], which cannot associate with negatively charged proteoglycans for induction of endocytosis. This finding implies that the cellular uptake of PF14–SCO nanocomplexes has to be mediated by other types of receptors. The plausible candidates, scavenger receptors, form a large superfamily, whose first members were initially characterized by their ability to bind and internalize modified low density lipoproteins. However, they also associate with various other polyanionic ligands

Abbreviations: CPP, cell penetrating peptide; TP10, transportan 10; PF, PepFect; ON, oligonucleotide; SCO, splice-correcting oligonucleotide; pDNA, plasmid DNA; SR-A, class A scavenger receptor.

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[14], which made them good candidates for PF14–SCO nanocomplexes. Moreover, scavenger receptors are known to facilitate the cellular uptake of nucleic acids, like polyribonucleotides [15,16], dsRNA [17–19] and polyvalent oligonucleotide-functionalized gold nanoparticles [20]. Further experiments with SR-A specific inhibitory ligands revealed that the uptake of PF14–SCO complexes was indeed mediated by these receptors, whose two subtypes SR-A3 and SR-A5 were present in HeLa cells. The presence of inhibitory ligands abolished both the binding of nanocomplexes at the plasma membrane and internalization into cells [21]. Involvement of SR-As was further confirmed by fluorescence microscopy, which demonstrated the colocalization of nanocomplexes with both receptors on the plasma membrane as well as after internalization inside the cells. Type A scavenger receptors were recently shown to mediate the cellular uptake of PF14–siRNA nanocomplexes [22], PepFect15 nanoparticles with SCO [23], PF14–pDNA and NickFect nanoparticles with pDNA [24–26]. Therefore it is reasonable to assume that other gene delivery vectors of PepFect and NickFect family also utilize SR-As to gain access into cells.

Internalization of scavenger receptors may also occur via different endocytosis pathways, such as clathrin-mediated endocytosis [14], caveolin-dependent endocytosis [27,28] and macropinocytosis [29]. In order to gain more insight into mechanism used by PF14–SCO nanocomplexes, we first aimed to pinpoint the particular endocytic pathways responsible for the uptake and functionality of the nanocomplexes as well as their intracellular trafficking in reporter HeLa pLuc705 cell line. Secondly, we aimed to obtain more detailed information about the interactions between the nanocomplexes and SR-As by using transmission electron microscopy (TEM) to examine how nanocomplexes interact with receptors and whether they can bind directly to receptors. We demonstrate that PF14–SCO nanocomplexes localize in very close proximity to both receptors, indicating that their binding to receptors might be direct. Surprisingly, in cells that were not treated with PF14–SCO nanocomplexes, SR-A3 and SR-A5 were not exposed on the plasma membrane, whereas their intracellular concentration was high.

2. Materials and methods

2.1. Cells and materials

HeLa pLuc705 cells [30], kindly provided by Prof. R. Kole, were grown in humidified atmosphere containing 5% CO_2 at 37 °C in Iscove's Modified Dulbecco's Medium (IMDM). Culture medium was supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin. PF14 was synthesized and purified as described before [13]. Phosphorothioate 2′OMe splice correction oligonucleotides with Cy5 labeling at the 5′ end were purchased from Microsynth AG, Switzerland.

2.2. Formation of PF14–SCO complexes

To form complexes, Cy5 labeled SCO was mixed with PF14 (molar ratio MR 1:5) in MQ-water in 1/10 of the final treatment volume to reach 0.2 μM and 1 μM concentration, respectively. Complexes were formed for 30 min at room temperature.

2.3. Cellular uptake experiments

5×10^4 HeLa pLuc705 cells were seeded 2 days before the experiment into 24-well plate. Cells were washed and pre-treated for 30 min at 37 °C in serum containing IMDM with 10 μM chlorpromazine, 50 μM nystatin or 1 mM amiloride (all from Sigma-Aldrich). Lipofectamine 2000 (Invitrogen) was used as a control according to the manufacturer's protocol. After that PF14–SCO complexes were added and incubated for additional 4 h in serum containing media. Transfection medium was replaced with fresh media and incubated for 20 h. Before the measurement, cells were washed twice with PBS and lysed with 0.1% Triton X-100 in PBS buffer for 30 min at 4 °C. Luciferase activity was measured using Promega's luciferase assay system on GLOMAX 96 microplate luminometer (Promega, Sweden). For control experiments, AF-594-conjugated transferrin, AF-555-labeled cholera toxin B subunit (CtxB) and 70 kDa TAMRA-dextran (all from Molecular Probes) were applied to cells at 20 μg/ml, 3 μg/ml, 0.25 mg/ml concentration to test efficacy of the inhibitors to block the uptake of respective endocytic markers.

2.4. RNAi experiments

4×10^4 HeLa pLuc705 cells were seeded onto 12-well culture plate 24 h before siRNA transfection. siRNAs against SR-A3 and SR-A5 (Ambion, USA) were used as a mixture at final concentration of 25 nM of each and cav-1 siRNA (Santa Cruz Biotechnology, Heidelberg, Germany) was used at final concentration of 100 nM. Negative control siRNA (Ambion, USA) was used at 50 nM or 100 nM concentration and siRNAs were transfected into HeLa cells using Lipofectamine RNAiMax (Life Technologies) according to the manufacturer's protocol. 48 h after the transfection cells were treated with pre-formed PF14–SCO nanocomplexes for 1 h, cells were then washed, lysed and analyzed with Tecan Infinite 200 M plate reader.

2.5. Localization of PF14–SCO complexes in relation to Golgi apparatus, caveolin-1 and LAMP2

5×10^4 HeLa pLuc705 cells were seeded onto round glass coverslips in 24-well plate 2 days before the experiment. Cells were washed and incubated with complexes of 0.2 μM SCO and 1 μM PF14 for 30 min or 1 h at 37 °C in serum-free IMDM. Treated cells were then fixed with 4% paraformaldehyde in PBS for 30 min, washed, permeabilized and blocked with 5% non-fat dry milk solution in PBS for 30 min. Cells were then treated with anti-TGN46 rabbit polyclonal antibody (1:100, Abcam, UK) for 1 h at room temperature followed by incubation with Alexa Fluor 488-conjugated anti-rabbit secondary antibody (1:500, Invitrogen) for 30 min at RT. Colocalization of nanocomplexes with caveolin-1 was analyzed by using anti-caveolin-1 rabbit polyclonal antibody (1:100, BD Transduction Laboratories, Belgium) and Alexa Fluor 488-conjugated goat-anti-rabbit secondary antibody (1:500, Invitrogen) and LAMP2 was visualized with mouse polyclonal antibody LAMP2 (1:100, H4B4, DSHB) and with Alexa Fluor 488-conjugated goat-anti-mouse secondary antibody (1:500, Invitrogen). After washing, the coverslips were mounted with Fluoromount G (Electron Microscopy Sciences, PA). Images were captured by Olympus FluoView FV1000 confocal laser scanning microscope using excitation at 488 nm (for TGN46, cav-1 and LAMP2) and 633 nm (for SCO-Cy5) and processed with Adobe Photoshop CS6.

2.6. Co-localization of PF14–SCO complexes with Golgi apparatus, endoplasmic reticulum, dextran and CellMask in live cells

7×10^4 HeLa pLuc705 cells were seeded onto 8-well chambered coverglasses (Lab-Tek, Nalge Nunc International, NY) 2 days prior to experiment. At 80% of confluence, cells were washed and incubated in serum-free media for 2 h. After that cells were pre-incubated with Golgi marker 0.5 μM BODIPY-TR-C_5 ceramide (Molecular Probes, UK) in serum-free media for 30 min on ice. Cells were washed again and incubated with PF14–SCO complexes in serum-free media for an additional 30 min or 1 h at 37 °C. To visualize the localization of complexes in relation to endoplasmic reticulum, cells were incubated with PF14–SCO complexes and 0.2 μM Blue–White DPX (Molecular Probes, UK) in serum-free media for 30 min or 1 h at 37 °C. Images were captured by Olympus FluoView FV1000 confocal laser scanning microscope using excitation at 590 nm (for Golgi marker), 380 nm