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Cell surface clustering of heparan sulfate proteoglycans by amphipathic cell-penetrating peptides does not contribute to uptake

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ABSTRACT

For arginine-rich cell-penetrating peptides (CPPs), an association with heparan sulfate (HS) chains is considered the first step in the stimulation of uptake for many cells. Much less is known about the role of HS chains in the cell-association and internalization of arginine-free amphipathic CPP such as transportan-10 (TP10). Here, we report that various TP10 analogs differ in their capacity to accumulate on HS-rich plasma membranes in an HS-dependent manner. No accumulation was observed on HS-poor plasma membranes or when HS was removed by enzymatic cleavage. The TP10 analog that strongly clustered on the cell surface, also showed a pronounced capacity to form clusters with HS chains in solution. However, aggregation occurred in a thermodynamically different way compared to the interaction of arginine-rich CPP with HS. To monitor the impact of the peptide on the aggregation of the glycocalyx by time-lapse microscopy, sialic acids were visualized by metabolic labeling using copper-free click chemistry to attach fluorophores to metabolically incorporated azido sugars. Strikingly, a highly enhanced HS-mediated accumulation on the plasma membrane of a particular TP10 analog did not correlate with a better uptake. These findings illustrate that the mode of interaction between cell-penetrating peptides and HS chains has important functional consequences regarding peptide internalization and that there is no direct coupling of interaction, accumulation and uptake.

1. Introduction

Cell-penetrating peptides (CPPs) are a promising class of molecules that are being applied to shuttle membrane-impermeable cargo into cells. To date, most applications relate to biomedical research. Ultimately, the goal is to apply these molecules for therapeutic purposes. After almost twenty years of investigation, there is now a considerable understanding about the routes CPP may exploit to enter into cells [1,2] and the kind of effects they have on the physiology of the cell [3]. There is a widespread agreement that the route of uptake depends on multiple factors, including the type and concentration of the CPP, the cell-type and the cargo, and that multiple import pathways may be exploited simultaneously [1,4,5]. The most prominent types of CPP that can be distinguished are the arginine-rich CPP and the cationic amphipathic CPP. The TAT-peptide and nonaarginines are important representatives of the first class [6,7]. Transportan and its shorter analog TP10 are members of the second one [8,9]. There is ample evidence that in spite of the physicochemical differences, transportans and the arginine-rich CPP are internalized by endocytosis, even though uptake routes may differ in details [10,11].

In the analysis of molecular interactions leading to peptide uptake, studies in particular have addressed the electrostatic binding to lipids and glycosaminoglycans (GAGs), which are negatively charged sugars with a high abundance on the surface of most cell types [12–14]. One of the most prominent findings that has been obtained from GAG-binding studies is that arginine-rich peptides have submicromolar affinities for heparan sulfate (HS) chains, whereas affinities for lipid membranes are much lower [13]. Indeed, abundant evidence exists for the notion that cationic CPPs have the ability to interact with GAGs in biological systems and that this interaction contributes to uptake [10,15–21].

According to a recent model, the initial interaction with arginine-rich CPP leads to GAG clustering and activation of intracellular signaling cascades, and both processes have been proposed to facilitate CPP uptake [20–22]. Specifically, syndecan-4, was shown to be closely involved in uptake [15]. This protein is a ubiquitously expressed member of the syndecan protein family, which next to glypicans is one of the two main families of membrane-bound HS-carrying proteins [23,24]. But other membrane-associated proteoglycans may be involved as well [20]. The Rho GTPase Rac-1 has been suggested as a critical factor in

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peptide internalization [20]. Interestingly, highly similar mechanisms have been described for, amongst others, growth factors [25], nonviral delivery systems [26–28], viruses [29,30], and bacteria [31]. These examples suggest that the exploitation of membrane-associated proteoglycans, and in particular syndecans, is a mechanism widely associated with cellular internalization. Additionally, HS chains have been reported to contribute to the uptake of guanidinium-rich CPP mimics [32,33]. For $S4_{13}$-PV peptides HS on the cell surface was required for particle formation [34]. For complexes between DNA and the more hydrophobic MPG-β, HS was required for the induction of lamellipodia formation and for a Rac-dependent cytoskeletal reorganization, associated with uptake [35].

In the analysis of HS clustering, the introduction of fluorescent reporter groups is a challenge. So far, studies have either employed fluorescent fusion proteins of syndecans [15] or immunofluorescent labeling [36]. However, C-terminal fusions of syndecans abolish their PDZ motif and may therefore impair interactions of the cytoplasmic domain while immunofluorescent labeling of the extracellular domain or sugars may affect HS dynamics through cross-linking. Recently, metabolic labeling has been introduced as a powerful approach in chemical biology to introduce sugars for bioorthogonal labeling [37,38]. However, in CPP research, this is a novelty. Cells are cultured in the presence of cell-permeable sugar esters carrying azido-functionalities. Inside the cells, the ester groups are cleaved off through the action of esterases and the sugars with the azido functionalities are incorporated into glycans analogous to the non azido-functionalized endogenous counterparts. After trafficking to the cell surface, labels can be introduced through copper-free click chemistry [39].

Here, we show for transportan-10 (TP10) and a set of TP10 analogs that these CPPs also interact with heparan sulfates on the cell surface but that this interaction is thermodynamically and functionally different from the one described for arginine-rich CPP. Metabolic labeling of sialic acids was employed for the introduction of fluorescent reporter groups in the glycocalyx and for monitoring the colocalization of peptide and sugar clustering on the cell surface. Significantly, the results indicate that HS chains have the capacity to sequester amphipathic CPP in a manner that is unproductive for uptake.

2. Material and methods

2.1. Cell culture

HeLa and Jurkat E6.1 cells were maintained in DMEM and RPMI 1640 (Gibco, Invitrogen, Eugene, U.S.A.), respectively, supplemented with 10% fetal calf serum (FCS; PAN Biotech). All cells were incubated at 37 °C in a 5% CO_2-containing, humidified incubator. Cells were passaged every 2 to 3 days.

2.2. Solid-phase peptide synthesis

All peptides were synthesized on an automated Syro II multiple peptide synthesizer (MultiSynTech, Witten, Germany) with standard Fmoc protocols and HOBt/HBTU as coupling reagents. The unnatural amino acid CF_3-Bpg was coupled manually for 2 h using diisopropylcarbodiimide (DIC) and HOBt. Peptides were N-terminally labeled with 5(6)-carboxyfluorescein before cleavage off the resin and side chain deprotection. For fluorescein labeling, a 5-fold molar excess of 5(6)-carboxyfluorescein:DIC:HOBt (1:1:1) in dimethylformamide (DMF) was added to the peptide on the resin and was allowed to react for 12 h at room temperature. After washing with DMF, dichloromethane (DCM), methanol (MeOH) and diethyl ether, piperidine (20% v/v in DMF) was added for 30 min. Afterwards the resin was washed with DMF, DCM and MeOH, dried under reduced pressure and the peptide cleaved off the resin using a mixture of trifluoroacetic acid (TFA) (93.5%), triisopropylsilane (TIS, 4%) and H_2O (2.5%). The filtrate was evaporated under a gentle stream of N_2 and the crude peptide was precipitated using diethyl ether and lyophilized. The crude peptides were purified by high-performance liquid chromatography (HPLC) on a preparative C18 column (22 × 250 mm) (Vydac, Hesperia, CA, USA) using water-acetonitrile gradients supplemented with 5 mM HCl. The identity of all peptides was confirmed by mass spectrometry. The purity of the peptides was found to be over 95%. The concentration of fluorescein-labeled peptides was determined by measuring A_{492} in Tris–HCl buffer (pH 8.8), assuming a molar extinction coefficient of 75,000 M^{-1} cm^{-1}.

2.3. Bioorthogonal labeling of cells

HeLa cells (40,000 cells/well) were seeded one day before the experiment in 8-well microscopy chambers (Nunc, Wiesbaden, Germany) and incubated in the absence or presence of Ac4ManNAz (50 μM) [38,39] over night. For live-cell labeling, adhered cells were washed three times with HBS and incubated with BCN-biotin (60 μM, SynAffix, Nijmegen, The Netherlands) [40] or buffer (1 h, 20 °C), washed three times with HBS and incubated with HBS containing Alexa Fluor 647-conjugated streptavidin (5 μg/ml, Invitrogen). After incubation (30 min, 4 °C), cells were washed three times and kept in pre-warmed culture-medium for 1 h at 37 °C, 5% CO_2 before treatment with CPPs and confocal microscopy studies.

2.4. Imaging of peptide uptake by confocal microscopy

HeLa cells were seeded one (40,000 cells/well), two (20,000 cells/well) or three (10,000 cells/well) days before the experiment in 8-well microscopy chambers and grown to 75% confluence. Cells were incubated with the indicated concentrations of peptides for 30 or 60 min at 37 °C, as indicated per experiment. Cells were washed twice after incubation and living cells were analyzed immediately by confocal microscopy using a TCS SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with an HCX PL APO 63× N.A. 1.2 water immersion lens. Cells were maintained at 37 °C on a temperature-controlled microscope stage. Annexin V-Alexa Fluor 647 was used at a dilution of 1:50 (Invitrogen). For heparan sulfate removal HeLa cells were pre-incubated with 3 mIU of heparinase III (Ibex, Quebec, Canada) in DMEM containing 1% FCS for 1 h at 37 °C. For time-lapse confocal microscopy, cell culture medium was replaced by pre-warmed culture medium including 10% FCS, containing TP10 or TP10 Gly2 → *L*-CF_3-Bpg in a final concentration of 5 μM, followed by life cell confocal imaging at 37 °C using excitation at 488 nm and detection of fluorescence over 500–550 nm (fluorescein) and additional excitation at 633 nm and emission of detection over 650–750 nm (BCN-biotin/Streptavidin Alexa Fluor 647 labeled sialic acids) over a timespan of 30 min.

2.5. Flow cytometry

HeLa cells were seeded in 24-well plates (Sarstedt, Numbrecht, Germany) at a density of 80,000 cells/well one day prior to the experiment. Heparinase pre-treatments were performed as described above. Cells were incubated with 5 μM of the indicated peptide for 30 min or 1 h at 37 °C, as indicated. When incubating the peptides at 4 °C, cells were pre-incubated at 4 °C for 10 min to ensure temperature adjustment. Subsequently, cells were washed, detached by trypsinization for 5 min or using a commercial cell-dissociation solution (Merck Millipore, Billerica, USA), spun down and re-suspended in 200 μl RPMI + 10% fetal calf serum in the presence or absence of 0.4% (w/v) trypan blue. When cells were analyzed with trypan-blue solutions *via* flow cytometry, the high concentration of 0.4% (w/v) ensured that quenching was maintained. Propidium iodide was included at a concentration of 2.5 μg/ml, where indicated. Cellular fluorescence was measured using a FACS-Calibur flow cytometer using the 488 nm line of an argon ion