Biochimica et Biophysica Acta 1858 (2016) 1860–1867

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Glycosaminoglycans are required for translocation of amphipathic cell-penetrating peptides across membranes

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ARTICLE INFO

Article history:
Received 14 January 2016
Received in revised form 21 March 2016
Accepted 21 April 2016
Available online 23 April 2016

Keywords:
Cell-penetrating peptide
Tat peptide
Transportan
Nona-arginine
Direct translocation
Glycosaminoglycan

ABSTRACT

Cell-penetrating peptides (CPPs) are considered as one of the most promising tools to mediate the cellular delivery of various biologically active compounds that are otherwise cell impermeable. CPPs can internalize into cells via two different pathways — endocytosis and direct translocation across the plasma membrane. In both cases, the initial step of internalization requires interactions between CPPs and different plasma membrane components. Despite the extensive research, it is not yet fully understood, which of these cell surface molecules mediate the direct translocation of CPPs across the plasma- and endosomal membrane. In the present study we used giant plasma membrane vesicles (GPMVs) as a model membrane system to elucidate the specific molecular mechanisms behind the internalization and the role of cell surface glycosaminoglycans (GAGs) in the translocation of four well-known CPPs, classified as cationic (nona-arginine, Tat peptide) and amphipathic (transportan and TP10). We demonstrate here that GAGs facilitate the translocation of amphipathic CPPs, but not the internalization of cationic CPPs; and that the uptake is not mediated by a specific GAG class, but rather the overall amount of these polysaccharides is crucial for the internalization of amphipathic peptides.

1. Introduction

The majority of molecules possessing a high biomedical or biotechnological potential are unable to cross the barrier of the plasma membrane, which limits their biological activity and impedes usage. Cell-penetrating peptides (CPPs) are short amino acid sequences that can efficiently surpass the plasma membrane and internalize without affecting cell viability [1–3]. Furthermore, CPPs can transport a variety of biologically active molecules into cells, making them highly potent cellular delivery vectors [4–7].

The mechanism underlying the internalization of CPPs and its payloads is the focuses of interest within the field of CPPs. Although it is now well established that different endocytosis pathways are involved in the uptake of CPPs [8–10], as well as direct translocation across the plasma membrane [11–13], it is still not clear which cell surface molecules are the main binding partners that lead to efficient internalization. However, a full understanding of CPPs cellular uptake mechanisms is vitally important for the design and development of peptide vectors.

The abundance of glycosaminoglycans (GAGs) on the cell surface and their high negative charge suggest that these are the first molecules interacting with positively charged CPPs before the peptides are taken up by cells. Several studies have confirmed the main role of GAGs in endocytosis [14–17], among which heparan sulfates are known to be involved in endocytosis most often [18]. Cell surface proteoglycans have been shown to mediate the macropinocytic uptake of Maurocalcine [17] and arginine-rich CPPs; in fact, cellular internalization of Tat peptide was highly dependent on heparan sulfate proteoglycans (PG), but the uptake of octa-arginine was not specifically mediated by these PGs [16]. Similarly, it was demonstrated that heparan sulfates were needed for efficient cellular internalization of Tat and Antennapedia peptides; and in contrast with the previous study, also for octa-arginine [14,15,19]. These results are consistent with the findings from other studies, in which heparan sulfates increased the uptake of human lactoferrin-derived CPP [20,21]. However, another major internalization mechanism of CPPs, direct translocation across the membrane, is not understood sufficiently, and it is not clear whether binding to different GAG types also contributes to this cellular uptake route of CPPs.

To elucidate specific molecular mechanisms behind the internalization, we examined the role of cell surface GAGs in the direct membrane translocation process of CPPs. The continuous flow of cellular processes complicates the elucidation of specific interactions between CPPs and

Abbreviations: Arg_9, nona-arginine; CHO, Chinese Hamster Ovary; CPP, cell-penetrating peptide; GAG, glycosaminoglycan; GAG^{neg}, glycosaminoglycan deficient CHO cells; GPMV, giant plasma membrane vesicle; HS^{neg}, heparan sulfate deficient CHO cells; pTat, Tat peptide; TP, transportan; WGA, wheat germ agglutinin; WFA, *Wisteria floribunda* agglutinin.

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http://dx.doi.org/10.1016/j.bbamem.2016.04.010
0005-2736/

membrane constituents in living cells. Therefore, in the present study we used giant plasma membrane vesicles (GPMVs) as a model membrane system. GPMVS are derived from cells and obtained by chemical induction, which maintains the complex nature of the plasma membrane [22,23]. Vesicles released by cells contain cytoplasm, but lack organelles and therefore also cellular energy-driven processes, including endocytosis [22]. The similar composition to the plasma membrane and lack of endocytosis makes GPMVs a highly relevant model system to study the interactions between cell membrane constituents and CPPs, and translocation of peptides across biological membranes [24–26]. In order to examine specific interactions between CPPs and GAGs, we prepared GPMVs from three different Chinese Hamster Ovary (CHO) cell lines and two of these were deficient in specific GAGs. We further degraded particular GAGs by enzymatic digestion on the surface of GPMVs and examined the translocation of four well-known representatives of two different classes of CPPs: arginine-rich nona-arginine and Tat peptide, and amphipathic transportan and TP10. The first two are considered as cationic CPPs based on their high arginine content. TP and TP10 contain hydrophobic amino acids in their sequence in addition to cationic residues and after acquiring alpha-helical structure form two distinguishable hydrophobic and hydrophilic domains, which are responsible for the amphipathic nature of these peptides. By using different GAG-deficient CHO cells and targeted degradation of GAGs we demonstrate here that the internalization of amphipathic peptides is dependent on the presence of GAGs, but the translocation of arginine-rich CPPs is not influenced by these polysaccharides. We also show here that transportan can reorganize the membrane of the vesicles and cluster GAGs into liquid disordered membrane lipid phase.

2. Materials and methods

2.1. Cell culture

Chinese Hamster Ovary CHO-K1 cells (WT), GAG-deficient CHO pgsA-745 cells (GAG^{neg}) and heparan sulfate deficient CHO pgsD-677 cells (HS^{neg}) were cultured in HAM's F12 medium supplemented with 10% FBS (Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin (Corning). Cell cultures were maintained at 37 °C in a humidified atmosphere (5% CO_2) and were passaged every second or third day.

2.2. Peptides

Transportan (TP; GWTLNSAGYLLGKINLKALAALAKKIL-NH_2), transportan 10 (TP10; AGYLLGKINLKALAALAKKIL-NH_2), Tat peptide (pTat; GRKKRRQRRRPPQ-NH_2), nona-arginine (Arg_9; RRRRRRRRR-NH_2) and a peptide from the C-terminus of β1 adrenergic receptor (β1A; CSSLDEPGRGGFSSESKV-NH_2) were synthesized in a stepwise manner in 0.1 mmol scale on an automated peptide synthesizer (Applied Biosystems) using the *tert*-butyloxycarbonyl (*t*-Boc) or for TP10 fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide sythesis strategy. To obtain C-terminally amidated peptides, p-methylbenzylhydrylamine (Neosystem) or Rink-amide methylbenzylhydrylamine resin (Orpegen Peptide Chemicals GmbH) was used in *t*-Boc strategy and Fmoc strategy, respectively. Fluorescent label (fluorescein; FAM) was coupled manually to the side chain of Lys13 or Lys7 in transportan and TP10, respectively, or to the N-terminus in case of pTat, Arg_9 and β1A. In *t*-Boc strategy peptides were finally cleaved from the resin using liquid HF at 0 °C for 1 h in the presence of p-cresol (1:1) and in Fmoc strategy using trifluoroacetic acid (TFA) with 2.5% triisopropylsilane and 2.5% water. Peptides were purified by reverse-phase HPLC on a C18 column, using 20–100% acetonitrile gradient. The molecular mass was determined by MALDI–TOF mass spectrometry (The Voyeger DE TM PRO Biospectrometry TM System) and calculated molecular weights were obtained each time.

2.3. GPMV preparation

For GPMV preparation, 2×10^5 cells per well were plated into a six-well plate (Falcon, Corning) and allowed to grow for 48 h. GPMVs were formed using a method described by Holowka and Baird [23] with minor modifications [24]. First, cells were washed twice with GPMV buffer (2 mM $CaCl_2$, 150 mM NaCl, 10 mM HEPES, pH 7.4) and then induced with GPMV buffer, supplemented with 25 mM formaldehyde (Fluka Chemie GmbH) and 2 mM DTT (AppliChem GmbH). Cells were incubated for 2.5 h at 37 °C with gentle shaking (300 r/min). After incubation, vesicles were kept in ice and centrifuged for 2 min at 300 *g* prior experiments to separate GPMVs from cells that had detached from plastic. Vesicles were used in experiments on the same day.

2.4. Removal of glycosaminoglycans from GPMVs membrane

For enzymatic treatment of GPMVs, CHO wild type vesicles were incubated with hyaluronidase VIII (hyalVIII) or heparinase III (hepIII) or chondroitinase ABC (chABC) at a final concentration of 2 mg/ml, 1 U/ml and 2 U/ml, respectively. All used enzymes were from Sigma-Aldrich. To remove the enzyme and detached GAGs from the suspension of GPMVs, all enzymatic treatments were performed on membrane filters (0,45 μm, Ultrafree®-MC-HV, Merck KGaA). First, vesicles were concentrated by adding 600 μl of GPMV suspension to a filter tube and then certifuged at 100 *g* for 2 min at room temperature. In individual experiments, the enzymes were applied to GPMVs suspension at respective concentrations in a filter tube and incubated at 37 °C for 30 min in case of hyaluronidase and 1 h for heparinase III and chondroitinase ABC treatment. After that, GPMV-enzyme suspension was centrifuged at 100 *g* for 1 min at room temperature. Next, GPMVs were washed twice by adding GPMV buffer and centrifuged as described above. GPMVs were then resuspended in 100 μl GPMV buffer for confocal microscopy analysis.

2.5. Confocal laser scanning microscopy

GPMVs were analyzed by confocal scanning microscopy as described earlier [24]. All microscopic observations were performed in chambered glass slides (8-well, 0.7 cm^2 of area per well, Nalge Nunc International) containing 50 μL of freshly prepared GPMV solution per well. CPPs and the control peptide (β1A) were added at 1 μM concentration, and TRITC or Alexa Fluor 647 labeled wheat germ agglutinin (WGA-TRITC, WGA-AF647; Life Technologies) or fluorescein labeled *Wisteria floribunda* agglutinin (WFA-FITC Vector Laboratories) was added at 1 μg/ml and 10 μg/ml concentration, respectively. Annexin V labeled with Alexa Fluor 647 (AnV-AF647) (Invitrogen) at 1:100 dilution was used for the visualization of liquid-disordered (L_d) lipid phases of the vesicles. GPMVs were imaged with an Olympus IX81 inverted microscope equipped with the FluoView1000 confocal system using a 60× water-immersion objective and excitation at 488 nm (fluorescein) or 559 nm (TRITC) or 635 nm (AF647). The lasers were run in a sequential scanning mode to avoid the spectral overlap. The images were recorded from the center of the vesicle, representing the internalized peptide; and images were processed with Adobe Photoshop CS4. *AutoQuant X*3 was used to quantify the intensity of fluorescence signal in confocal images. The fluorescence intensity of each vesicle was measured and divided by its ROI area to obtain the fluorescence intensity per unit area. The mean fluorescence intensity was calculated by analyzing 80 vesicles from at least three independent experiments.

2.6. Flow cytometry

1.5–2 × 10^6 GPMVs in 300 μl were subjected to flow cytometry (FACS) analysis in GPMV buffer and fluorescent CPP was added to reach 1 μM concentration directly before the analysis. For every sample 10^4 events were counted using constant voltage settings of forward and side scatter. 488 nm laser was used for the quantification of fluorescein-