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Secondary conformational conversion is involved in glycosaminoglycans-mediated cellular uptake of the cationic cell-penetrating peptide PACAP

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ABSTRACT

Glycosaminoglycans (GAGs) contribute to the cellular uptake of cationic cell-penetrating peptides (CPPs). However, molecular details about the contributions of GAGs in CPP internalization remain unclear. In this study, we examined the cellular uptake mechanism of the arginine-rich CPP pituitary adenylate-cyclase-activating polypeptide (PACAP). We observed that the uptake efficacy of PACAP is dependent on the expression of cell surface GAGs. As the binding of PACAP to sulfated GAGs induced a random coil-to-α-helix conformational conversion, we investigated the role of the helical formation in PACAP internalization. Whereas this secondary structure was not crucial for efficient internalization in GAGs-deficient cells, PACAP α-helix was essential for GAGs-dependent uptake.

1. Introduction

Over the last two decades, cell-penetrating peptides (CPPs) have gained increase of interest as chemical tools for the intracellular delivery of macromolecular cargoes intended for biological and medical applications [1]. CPPs are a class of diverse peptides, usually ranging from 5 to 30 residues, which can cross the plasma membrane through a variety of mechanisms that remain partially elusive. According to their physicochemical properties, CPPs can be classified into three major classes: (i) cationic, (ii) amphipathic and (iii) hydrophobic [2]. Cationic CPPs are short peptides that are rich in arginine and lysine residues [3,4]. The vast majority of CPPs derived from natural protein motifs and were identified in DNA/RNA-binding proteins, viral proteins, signal peptides or heparin-binding proteins [2]. Interestingly, we recently reported that an endogenous peptide neurohormone, pituitary adenylate cyclase-activating polypeptide (PACAP), can cross efficiently the plasma membrane in a specific receptor-independent manner, mainly by active endocytosis involving clathrin-dependent pathway and micropinocytosis [5]. This peptide was highly effective to mediate the uptake of a variety of cargoes, including protein and DNA plasmid [6]. The cellular uptake efficacy of PACAP was 3 times as high as that observed for the TAT peptide [5], underlining the potent ability of this peptide to cross plasma membrane. These studies have identified PACAP as a new member of the CPP family and suggest that PACAP derivatives represent excellent vectors for the intracellular delivery of non-permeable (bio)molecules. Nonetheless, as for other CPPs, the elucidation of the molecular requirements of PACAP internalization is necessary to improve its delivery efficiency.

PACAP is a 38-amino acid C-terminally-α-amidated peptide that encompasses 11 basic residues, *i.e.* 4 arginines and 7 lysines, conferring a polycationic nature to this peptide (Fig. 1A) [7]. PACAP exhibits a random coil conformation in aqueous solutions whereas the central and C-terminal domains of the polypeptide chain readily adopt a helical structure in membrane mimicking milieu, such as dodecylphosphocholine (DPC) micelles (Fig. 1B) [8–10]. Helical wheel representation of this putative helical segment shows that

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cationic residues are dispersed on both sides of the α-helix (Fig. 1C), conferring a highly positive charge distribution on the overall surface of the peptide. For cationic CPPs, it has been reported that membrane-associated glycosaminoglycans (GAGs) contribute significantly to their cellular uptake [11]. For instance, the removal of cell surface sulfated GAGs was shown to decrease substantially the cellular uptake of the TAT peptide [12], penetratin [13,14] and the R8 poly-arginine peptide [15].

GAGs, including heparan sulfate (HS) and chondroitin sulfate (CS), are long and linear polysaccharides composed of repeating disaccharide units [16]. They are abundant on the outer leaflet of the plasma membrane of every cell type of metazoan organisms where they are O-linked to proteoglycans [17]. Owing to their high content in carboxylate and sulfate groups, GAGs are highly negatively charged biopolymers that surround cells. Despite the well-recognized importance of GAGs in the uptake of cationic CPPs, the molecular details regarding the roles of proteoglycans are still a matter of controversy and several mechanisms have been inferred from biophysical and biochemical investigations. It has been recently proposed by Favretto et al. [11] that the roles of GAGs in the endocytosis of CPPs could be ascribed to either (i) GAGs clustering upon peptide binding, (ii) co-clustering of a receptor and GAGs upon CPP binding and/or (iii) GAGs mediating peptide adsorption to the plasma membrane. Particularly, in contrast to amphipathic CPPs, the importance of the secondary structure of cationic CPPs has been poorly investigated so far.

In this study, we first investigated the roles of cell surface GAGs in the adsorption of PACAP to the outer leaflet of plasma membrane and its subsequent cellular uptake. Considering that the binding of PACAP to sulfated GAGs induced a random coil-to-α-helix conformational conversion, we studied the contribution of this secondary structure in cellular uptake. This study highlights the mechanistic elements of PACAP endocytosis and exposes a new molecular basis of GAGs-mediated uptake of cationic CPPs.

2. Materials and methods

2.1. Peptide synthesis, purification and characterization

Peptides were synthesized on solid phase, purified by reverse-phase high performance liquid chromatography and characterized by mass spectrometry as described in the Supplementary Materials.

2.2. Peptide uptake

CHO-K1 and CHO-pgs-A-745 were seeded in 12-well plates at a density of 30000 cells/well for 48 h. Cells were incubated in presence of fluorescein-labeled peptides for 1 h at 37 °C and 5% CO_2. Time of incubation was defined according to our previous study [5]. After incubation, cells were washed twice with HBSS buffer, treated for 5 min with 100 μg/ml heparin to remove the excess of peptide bound to the cell surface [18,19], washed once again and detached by trypsinization. Trypsin action was stopped and cells were centrifuged. Cells were resuspended in 500 μl sorting buffer and kept on ice until flow cytometry analysis. For heparinase treatment, CHO-K1 cells were seeded as above-described. After 24 h incubation, cells were treated overnight with 8 UI/ml of heparinase. One hour before cell treatment with peptide, heparinase was added and experiments were performed as above-described. These methods are described in details in Supplementary Materials.

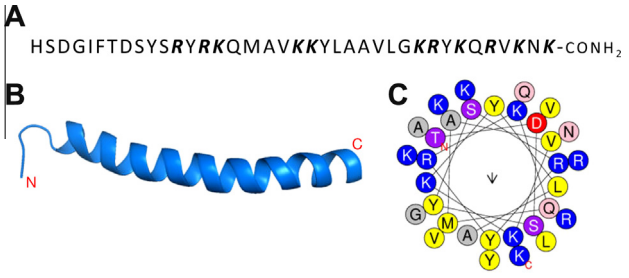

Fig. 1. Sequence and structure of PACAP. (A) Primary structure of PACAP with basic residues indicated in italic bold letters. (B) Schematic ribbon representation of micelle-bound PACAP secondary structure (PDB code: 2D2P). (C) Helical wheel representation of the putative α-helix segment of PACAP (Thr^{7}-Lys^{38}) with basic residues indicated in blue.

2.3. Membrane binding

Cell surface absorption was assessed using a protocol adapted from Gump et al. [20]. Cells were seeded as above-described and after 48 h, cells were incubated on ice at 4 °C for 10 min. Cells were then incubated in presence of fluorescein-labeled peptide for 15 min at 4 °C. Cells were then washed 3 times and detached manually. Cells were centrifuged at 4 °C before being resuspended in sorting buffer. Flow cytometry analyses were performed using a FACS Calibur instrument (BD Biosciences) and a minimum of 10000 gated cells per sample were analyzed. These methods are described in details in Supplementary Materials.

2.4. Characterization of PACAP-sulfated GAGs interactions

Affinity chromatography, circular dichroism (CD) spectroscopy and static light scattering were used to probe PACAP interaction with heparin as described in the Supplementary Materials.

3. Results

3.1. Cell surface glycosaminoglycans promote cellular uptake and membrane binding of PACAP

According to the high positive net charge of PACAP, we initially investigated the potential role of cell surface GAGs in the uptake and membrane binding. We used the CHO-pgs-A-745 cells, which are deficient in xylosyltransferase, an enzyme that catalyzes the transfer of a D-xylosyl group to the side chain of a serine, a key step in the synthesis of proteoglycans. As a consequence, these cells do not express any GAGs on their plasma membrane [21]. As observed by flow cytometry, pgs-A-745 cells were significantly less effective than their wild type counterpart to internalize fluorescein-labeled PACAP (Fig. 2A and B). To confirm that these results were not a consequence of a defect in the endocytosis machinery of CHO-pgs-A-745, we exposed the CHO-K1 cells to an enzymatic treatment to remove cell surface GAGs. To digest cell surface HS, two successive treatments with 8 UI/ml heparinase I/III were performed. Such treatment was previously shown to be effective for decreasing the amount of cell surface GAGs, while not affecting the integrity of the plasma membrane [22,23]. It is worth mentioning that HSPGs constitute the major proteoglycans of CHO-K1 cells [24]. The uptake of PACAP was reduced by approximately 50% upon HS enzymatic removal with heparinase (Fig. 2C and D). Particularly, CHO-K1 heparinase-treated cells showed a comparable uptake of PACAP to pgs-A-745 cells. Secondly, we addressed the contributions of proteoglycans in extracellular membrane association by incubating the cells at 4 °C for 15 min before detaching them with a non-enzymatic treatment. Such conditions are known to inhibit active endocytic pathways [14,20]. As observed