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Translocation of cell-penetrating peptides across the plasma membrane is controlled by cholesterol and microenvironment created by membranous proteins

Janely Pae [a], Pille Säälik [b], Laura Liivamägi [a], Dmitri Lubenets [a], Piret Arukuusk [c], Ülo Langel [c,d], Margus Pooga [a,*]

[a] Institute of Molecular and Cell Biology, University of Tartu, Tartu, Estonia
[b] Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu, Estonia
[c] Institute of Technology, University of Tartu, Tartu, Estonia
[d] Department of Neurochemistry, Stockholm University, Stockholm, Sweden

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ABSTRACT

Despite the extensive research in the field of CPPs' cell entry the exact mechanisms underlying their cellular uptake and the role of involved cell surface molecules in the internalization process have remained controversial. The present study focused on the interactions between CPPs and plasma membrane compounds using giant plasma membrane vesicles (GPMVs). GPMVs have shown to be a suitable model to study the translocation of CPPs across the plasma membrane in conditions lacking endocytosis. Our results show that higher cholesterol content and tighter packing of membrane predominantly reduce the accumulation of transportan, TP10 and model amphipathic peptide (MAP) in vesicles, indicating that the internalization of CPPs takes place preferentially via the more dynamic membrane regions. The partial digestion of membrane proteins from GPMVs' surface, on the other hand, drastically reduced the accumulation of nona-arginine and Tat peptide into vesicles, suggesting that proteins play a crucial role in the uptake of arginine-rich CPPs.

1. Introduction

Cell-penetrating peptides (CPPs) are a class of delivery vehicles that mediate the cellular uptake of various macromolecules with high biomedical or technological potential. Multiple factors participate in dictating the cellular internalization routes of CPPs, and targeting to two major uptake modes — endocytosis and direct crossing the plasma membrane named translocation. Basically the factors that determine the uptake route of CPPs can be divided into two groups: on one hand, the physico-chemical properties, the concentration of peptide and its cargo molecule, and on the other, the properties of plasma membrane — its lipid composition and protein content. For example, it is now well-established that when the peptide is applied at a high concentration, direct translocation across the plasma membrane can take place also in physiological conditions [1–3]. However, the used uptake route is highly influenced by the size of the cargo linked to CPP [4] and by the properties of the cargo. Even the fluorochromes conjugated for detection have been shown to modify the properties of CPPs and influence their localization in cells [5–7].

When looking at the CPP uptake process from the aspect of cellular determinants, endocytosis as the main cellular internalization mode prevails at physiological conditions and at low peptide concentrations [2,8,9], even if accompanied by penetration in small extent in parallel [10]. A clear deviation from this rule is penetratin, which at low concentration preferentially directly translocates across plasma membrane into cells and switches to endocytosis at higher concentrations [11]. The balance between endocytosis and penetration, however, seems to shift towards a direct translocation when cellular energy-dependent processes are inhibited, which control the mechanisms governing the dynamics and organization of membrane contents and regulating the subcortical cytoskeleton attached to it [12,13]. Therefore, in order to analyze the peptide–lipid interactions in an environment lacking the cellular energy-driven processes, artificial lipid vesicles have been exploited in parallel to research in cell culture models [14]. However, the exactly defined composition of artificial vesicles is their biggest drawback at the same time — it is almost impossible to recapitulate the vast cellular lipid and protein diversity of biological membranes, and most often the synthetic vesicles contain only two or three different lipids without the supplement of proteins or cholesterol. Cholesterol, on the other hand, is known to govern the formation of more densely packed domains called membrane rafts in artificial as well as in natural lipid membranes, but is lacking in vesicles formed from phospholipids only. We and others have recently introduced giant plasma membrane vesicles (GPMVs) as a novel model system to assess the CPP-membrane interactions in a biologically complex and highly relevant environment that lacks cellular energy-driven processes [15,16]. As GPMVs are released from cells by

* Corresponding author at: Institute of Molecular and Cell Biology, 23 Riia Str, Tartu 51010, Estonia. Tel.: +372 7375049; fax: +372 7420286.
E-mail address: mpooga@ut.ee (M. Pooga).

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chemical induction [17,18], their lipid and protein constitution is similar to the composition of the plasma membrane of living cells [19,20]. This quality enables to consider GPMVs organelle-free models of cell that is depleted of cellular energy and lacks cytoskeleton-controlled membrane dynamics — the processes that in cellular context fuel active endocytosis and membrane reorganization by actin rearrangements. In addition, at low temperature the membrane of GPMVs is prone to segregate into two multi-micrometer coexisting fluid phases that differ in packing density and lipid/protein composition [18]. These phases are designated as liquid-ordered (L_o) and liquid-disordered (L_d) membrane microdomains, and the former are thought to resemble the plasma membrane rafts by their properties.

We have shown earlier that a number of CPPs accumulate into GPMVs' lumen both at room as well as low temperature, and that more hydrophobic/amphipathic CPPs preferentially concentrate at liquid-disordered membrane phase [15]. These results impelled us to examine in more detail how different plasma membrane constituents and their organization affect CPPs' internalization. In this article we focused on the membrane components which govern the stability and formation of lipid rafts — cholesterol and ceramide as well as on the role of proteins on the plasma membrane.

Still, besides multiple evident advantages of GPMVs as a plasma membrane model, these have also revealed some drawbacks. The group of Simons recently demonstrated the protein palmitoylation defects [21] and protein relocation from the ordered to disordered lipid membrane phase in the membrane of GPMVs that were induced by formaldehyde/dithiothreitol, and suggested an alternative method for the preparation of vesicles [22]. Therefore, in order to avoid the cross-linking-derived artifacts, in the current study we used N-ethylmaleimide (NEM)-induced GPMVs for a more detailed analysis of interactions of fluorescently labeled CPPs with biological membranes and translocation across it. Our results demonstrate that amphipathic CPPs cross more efficiently the membranes that are partially depleted from cholesterol or are less ordered. Arginine-rich CPPs translocation, on the other hand, is dependent on membrane proteinaceous components.

2. Materials and methods

2.1. Cells

Rat basophilic leukemia RBL-2H3 cells (ATTC CRL-2256) were cultured in MEM, enriched with Earle's salts and stable L-glutamine with 100 IU/ml penicillin, 100 μg/ml streptomycin and 15% fetal bovine serum (FBS). Chinese-Hamster-Ovary cells (CHO-K1) were cultured in Ham's F12 medium containing 10% FBS, penicillin and streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were passaged every second or third day and cell culture media, FBS and enzymes were from PAA (Austria).

2.2. Peptides

All peptides were synthesized in a stepwise manner in 0.1 mmol scale on an automated peptide synthesizer (Applied Biosystems, USA). For transportan (GWTLNSAGYLLGKINLKALAALAKKIL-NH_2), MAP (KLALKLALKALKAALKLA-NH_2), pTat (GRKKRRQRRRPPQ-NH_2), pAntp (RQIKIWFQNRRMKWKK-NH_2), Arg_9 (RRRRRRRRR-NH_2), and a peptide from C-terminus of β1 adrenergic receptor (β1A; CSSLDEPGRGGFSSESKV-NH_2) *tert*-butyloxycarbonyl (t-Boc) and for TP10 (AGYLLGKINLKALAALAKKIL-NH_2) fluorenylmethyloxycarbonyl (Fmoc) the solid-phase peptide synthesis strategy was used. In t-Boc strategy p-methylbenzylhydrylamine (Neosystem, Strasbourg, France) and in Fmoc strategy Rink-amide methylbenzylhydrylamine resin (Orpegen Peptide Chemicals GmbH, Germany) were used to obtain C-terminally amidated peptides. Fluorescent labels (fluorescein for TP10, MAP, Arg_9, pAntp, pTat, β1 adrenergic receptor peptide, and Oregon Green 488 for TP) were coupled manually to the side chain of Lys13 or Lys7 in transportan and TP10, respectively, or to the N-terminus of MAP, pTat, pAntp, 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). The concentration of the peptides was determined based on dilutions of accurately weighted substances.

2.3. GPMV preparation

2×10^5 cells were plated per well in a six-well plate (Greiner Bio-One, Germany) 48 h before induction of GPMVs. Vesicles were generated as described earlier [23] with minor modifications. Briefly, adherent cells were washed twice with buffer containing 2 mM $CaCl_2$; 150 mM NaCl; 10 mM HEPES (pH 7.4) (GPMV buffer, GPMVb). Vesicles were induced in the same buffer supplemented either with 2 mM N-ethylmaleimide (Fluka) or with 25 mM formaldehyde (Fluka) and 2 mM DTT (AppliChem GmbH, Darmstadt; Germany) by mild shaking (300 r/min) (Thermomixer comfort, Eppendorf AG, Germany) at 37 °C for 1,5 h or 2,5 h in the case of RBL and CHO cells, respectively. Vesicles were stored in ice and used in experiments on the same day.

2.4. Confocal laser scanning microscopy

GPMVs were applied to microscopy analysis as described before [15]. CPPs, control peptide (β1) or FITC-labeled dextran (Mw = 3000; Pharmacia, Sweden) were added at 1 μM concentration. Cholera toxin B subunit labeled with Alexa Fluor 594 (CtxB-AF594) and annexin V labeled with Alexa Fluor 647 (AnV-AF647) (Invitrogen) were used for the visualization of liquid-ordered and liquid-disordered phases of GPMVs, respectively. CtxB-AF594 was added to the GPMV suspension at a concentration of 10 μg/ml, and AnV-AF647 at 1:100 dilution. 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 and Oregon Green 488), 559 nm (AF 594) and 635 nm (AF 647). The lasers were run in a sequential scanning mode to avoid the spectral overlap. Obtained images were processed with Adobe Photoshop CS4.

2.5. Quantification of CPPs in GPMVs by flow cytometry analysis

The GPMVs were applied to flow cytometry (FACS) analysis in GPMVb and fluorescent CPP was added directly before the analysis. The voltage settings for forward and side scatter were kept constant through all experiments being identical to experiments performed on FA/DTT GPMVs [15]. 1 μM peptide was used per $1.5–2 \times 10^6$ of GPMVs and 10^4 events were counted per sample. Filipin complex (Sigma-Aldrich) was added to vesicle solution to a final concentration of 30 μg/ml. 405 nm and 488 nm lasers were used for the quantification of filipin and CPP fluorescence, respectively. The results were analyzed by FACS Diva and MS Excel software. Values in graphs represent the mean value of the histogram plot from at least three separate experiments. Error bars represent standard deviation.

2.6. Depletion and enrichment of GPMV membrane cholesterol

For depletion of cholesterol from GPMV membranes, methyl-β-cyclodextrin (MCD) (Sigma-Aldrich) was added to GPMV solution to 5 mM final concentration and incubated at 37 °C for 1 h. The reagent was present in the GPMV solution during the experiment. Enrichment of GPMVs with cholesterol was performed as described previously [24] with some modifications. Briefly, 0.8 mg of cholesterol (Sigma-Aldrich) was dissolved in 35 μL of 1:1 chloroform/methanol and dried