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Penetration without cells: Membrane translocation of cell-penetrating peptides in the model giant plasma membrane vesicles

Pille Säälik, Aira Niinep, Janely Pae, Mats Hansen, Dmitri Lubenets, Ülo Langel, Margus Pooga

Graphical abstract

Supplementary Fig. 1 Characterization of giant plasma membrane vesicles (GPMVs) from RBL cells (A–C). A—GPMVs were incubated with Alexa Fluor 594-labeled cholera toxin B subunit (CtxB) and Alexa Fluor 647-labeled Annexin V (AnV) and visualized by confocal microscopy at low temperature to reveal the L_o and L_d lipid phases, respectively (see Materials and methods). B—GPMVs were incubated with DAPI (1 μg/ml) or PI (5 μg/ml) and visualized 30 min after addition of the stains at low temperature. C—GPMVs were incubated either with 1 μM fluorescein-labeled β1 peptide or FITC-dextran for 1 h. D—HeLa or RBL cells were incubated with 3 μM β1 peptide at 37 °C, and visualized after 30 min at the same temperature. In all images one confocal plane is presented. The corresponding DIC images of vesicles/cells are represented at the right of the respective image. Scale bar — 10 μm.

Supplementary Fig. 2 The preference of amphipathic CPPs for the L_d phase is not dependent on the presence of phase markers. GPMVs were incubated with 1 μM fluorescein- (MAP, pAntp, TP10) or Oregon Green 488-labeled (TP) CPPs (green) either in the absence (two left panels) or presence (two right panels) of 8 μM Laurdan membrane dye (blue). All images were recorded in 40–60 min after the start of the incubation. L_d phase in lower right image was visualized by incubating GPMVs with annexin V-647 (AnV, pseudo-colored as white) to corroborate the selectivity of Laurdan for L_o phase. Scale bar 10 μm.

Supplementary Fig. 2 CPPs accumulate in giant plasma membrane vesicles obtained with N-ethylmaleimide treatment. 1 μM respective CPP/control peptide together with lipid phase markers (red — CtxB-AF594, liquid-ordered phase; pseudo-white — AnV-AF647, liquid-disordered phase) was added to the N-ethylmaleimide-induced giant plasma membrane vesicles and visualized at low temperature between 40 and 70 min after the addition of the CPPs.

Supplementary Fig. 4 Internalization of fluorescently labeled CPPs into giant plasma membrane vesicles prepared from HeLa cells. GMPVs were incubated with fluorescein- (R9, pTat, pAntp, MAP, and TP10) or Oregon Green 488-labeled (TP) CPPs (1 μM, green) at low temperature for 45 min. L_d phase is labeled with annexin V-647 (pseudo-colored as white). Scale bar 10 μm.

Supplementary Fig. 5 Characterization of GPMVs’ properties by flow cytometry. A—Association of DAPI and propidium iodide (both 1 μg/ml) with GPMVs. B—Association of TP-Oregon Green to GPMVs at 4 °C (left panel) or room temperature (right panel). Different concentrations of TP are indicated as: filled squares — 0.01 μM, empty squares — 0.1 μM, filled triangles — 0.5 μM, empty triangles — 1 μM. C–E, histogram plots of GPMVs incubated with 1 μM fluorescein-labeled β1 peptide (A), R9 (B) or 1 μg/ml DAPI (C) for 0 (left column) or 60 min (right column) at low temperature. Note the single population of GPMVs marked with fluorescent compounds.
Penetration without cells: Membrane translocation of cell-penetrating peptides in the model giant plasma membrane vesicles

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The cellular internalization of cell-penetrating peptides (CPPs) is proposed to take place by both endocytic processes and by a direct translocation across the plasma membrane. So far only scarce data is available about what determines the choice between the two uptake routes, or the proportion of used pathways when both are active simultaneously. Furthermore, the mechanism(s) of membrane penetration by peptides is itself still a matter of debate. We have introduced the giant plasma membrane vesicles (GPMVs) to study the interaction of six well-described CPPs (fluorescently labeled nona-arginine, Tat peptide, Penetratin, MAP, Transportan and TP10) in a model system of native plasma membrane without the interference of endocytic processes. The membranes of GPMVs are shown to segregate into liquid-ordered and liquid-disordered phases at low temperatures and we demonstrated by confocal microscopy that amphipathic CPPs preferentially associate with liquid-disordered membrane areas. Moreover, all tested CPPs accumulated in the lumen of GPMVs both at ambient and low temperature. The uncharged control peptide and dextran, in contrary, did not translocate from the medium into the lumen of vesicles. The absence of energy-dependent cellular processes and the impermeability to hydrophilic macromolecules makes the GPMVs a useful model to study the translocation of CPPs across the plasma membrane in conditions lacking endocytosis.

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1. Introduction

Cell-penetrating peptides (CPPs) are defined as sequences of up to 40 amino acids and with a characteristic ability to translocate through cell membranes [1], a property that is retained also after conjugation of various small cargoes. Initially, the membrane-permeable properties of these peptides were thought to be dependent solely on the cationic nature of the molecule, because most CPPs have a high content of basic amino acids that makes them positively charged at physiological pH. However, in addition to the high number of cationic amino acids in the primary structure the current view also emphasizes the significance of the secondary structure and hydrophobicity of CPPs. Moreover, different cellular factors are suggested to participate in the uptake process.

By now it is appreciated that multiple cellular uptake routes exist for CPPs [2–4]. Both, different types of endocytosis, as well as direct translocation through the membrane have been shown to mediate the cellular uptake of CPPs or CPP-cargo complexes, whereas the mode of internalization can be dependent on the peptide concentration [2,3], the size of the cargo molecule [5], temperature [4] and the specificity towards lipids irrespective of their charge, giving reason to classify these as primary amphipathic CPPs [14]. However, a few data is available on the extent to which the cellular plasma membrane and its lipids can be reached by CPPs because the plasma membrane is heavily populated with proteins. Furthermore, the extracellular protein domains often carry anionic glycosylations and various sulfated polysaccharides that create a sort of shield over the lipid
bilayer. The high content of sulfates confers on polysaccharides in heparan sulfate proteoglycans (HSPG) a negative charge. Hence, positively charged CPPs, like Tat(48–60) [15] and oligoarginines [16,17], classified as non-amphipathic CPPs [14], possess high affinity towards HSPGs, which has also been convincingly demonstrated [18,19]. The absence of HSPGs, in turn, leads to the inhibition of the cellular uptake of the cationic CPPs either in naked [20] or in cargo-conjugated form [21,22] revealing the importance of negatively charged entities in forming portals for cellular entry of the cationic CPPs. However, it is still hotly debated by which way exactly the hydrophilic peptides cross the hydrophobic core of the plasma membrane, because the heparan and chondroitin sulfate-independent uptake of Tat-fusion protein has been also reported recently [23]. Based on the computer simulation, Herve and colleagues suggest the formation of a short-lived pore in the membrane by Tat peptide and Arg9, where the peptide translocation across the bilayer is mainly driven by the interactions and dynamics between the positively charged arginines and phosphate groups of the phospholipids [24,25]. Up to now, this was the most well-defined system for studying the CPP translocation mechanisms. Intriguingly, very recently Verdunnen and colleagues reported acidic sphingomyelase and subsequent ceramide formation in the plasma membrane to be responsible for the rapid cellular uptake of R8 via nucleation zones [26]. Despite the first demonstration of CPPs as the inducers of enzymatic activity, it is still unclear whether the sphingomyelase activity is induced via extra- or intracellularly located CPP.

Since the lipid composition of a eukaryotic cell membrane is very complex and dynamic, different lipid bilayer models have been introduced in order to simplify the studied system and to facilitate the visualization of individual components. In addition, the lipid bilayer model systems enable to monitor the initial steps of cell entry — binding to membrane and translocation through it as well as to simultaneously assess the organization and dynamics of the membrane [27]. Large and less frequently giant unilamellar vesicles (LUVs and GUVs, respectively) have been the most relevant model system to study the properties of CPPs necessary for the interaction with membranes of various lipid compositions [14]. In addition, the experiments with artificial vesicles have provided information about the changes in the membrane organization introduced by some CPPs [10,28,29]. The general conclusion from these studies is that cell-penetrating peptides bind to the membrane of LUVs, and depending on the lipid composition, are able to insert into one or both membrane leaflets. However, CPPs cannot translocate into the lumen of LUVs unless the transbilayer potential is created across the vesicle membrane [30,31]. It has been demonstrated that some CPPs can enter the GUVs but only up to the limit where the concentration of the peptide in the lumen of the vesicle equals with the concentration in the extravesicular environment and no accumulation takes place [32]. Since the results obtained with membranes that contained only a limited number of lipids and usually no proteins could hardly be extrapolated to cellular conditions — a membrane with a very complex composition — LUVs and GUVs have been used in CPP studies less frequently in recent years.

Giant plasma membrane vesicles (GPMVs), or blebs, represent a more natural membrane model than GUVs, because the former are derived from the cells’ plasma membrane and therefore their lipid composition resembles that of the parent cell [33,34]. The formation of GPMVs — “vesiculation” or “blebbing” — can be chemically induced, and at low temperatures lipids in their membranes segregate into liquid-ordered (Lo) and liquid-disordered (Ld) domains [35]. The separation of membrane components into two domains occurs due to the tighter packing of cholesterol with phospholipids containing long, saturated acyl chains than with phospholipids containing two or more double bonds in their acyl chains [36]. This partitioning leads to the formation of two phases with different lipid density: more tightly packed, cholesterol and sphingolipid-rich liquid-ordered, and loosely packed more dynamic liquid-disordered lipid phases. The cholesterol content of the formed vesicles determines the temperature at which the phases segregate [37], and the phases can be easily distinguished by microscopy after labeling with specific fluorescent probes [35], out of which some have the property to rise the temperature of phase miscibility [38]. The lumen of GPMVs contains cytoplasm but is free of cellular organelles and the actin cytoskeleton [39], and the content of intracellular membranes is low [40]. The lipid composition of GPMVs in general represents the lipid composition of the plasma membrane, having phospholipid/cholesterol ratio of approximately 2:1, and favoring Lo state [41]. However, the absence of cellular energy causes the GPMVs to lose the lipid asymmetry and localization of some phosphatidylserine to the outer membrane leaflet. The partitioning of a particular membrane component (protein or lipid) into specific lipid phase is determined by its intrinsic preference for the Lo or Ld environment, as well as by its interactions with other membrane constituents [36].

The membranes of living cells are likely to exhibit submicroscopic concentration fluctuations at physiological temperatures that lead to local differences in composition. These differences are thought to be related to Lo/Ld phase segregation and to subsequent larger scale phase separation and/or membrane reorganization, especially if induced to [35]. Thus, the partitioning of molecules between the fluid phases in GPMVs could provide data about the localization of these molecules in submicroscopic microdomains in the membranes of living cells.

In our study we used GPMVs derived from rat basophilic leukemia (RBL) and HeLa cells as a model membrane system with the aim of unraveling the first steps of membrane interaction for the six most characterized CPPs: pTat, Arg9, Transportan, TP10, MAP and Pene-tratin. The Lo and Ld domains were visualized with cholera toxin subunit (CtxB) and annexin V (AnV), respectively to specify the interaction loci at the membrane for studied CPPs. The amphipathic CPPs, like MAP and Transportan associated with the Ld domains of GPMVs. However, to our surprise, all the tested fluorescently labeled CPPs translocated into the lumen of GPMVs and accumulated there both at physiological and low temperature.

2. Materials and methods

2.1. Cell culture

Rat basophilic leukemia RBL-2H3 cells (ATTC CRL-2256) and human cervical carcinoma cell line HeLa (ATTC CCL-2) were cultured at 37 °C in humidified atmosphere containing 5% CO2 in MEM (enriched with Earle's salts and l-glutamine) and in IMDM with Pen/Strep and 15 or 10% of FBS, respectively.

2.2. Formation of giant plasma membrane vesicles

3 × 10^5 cells were plated per well in a six-well plate (Greiner Bio-One, Germany) 24 h before inducing formation of cell-free GPMVs. GPMVs were generated as described earlier (Holowka and Baird 1983) with minor modifications. Briefly, adherent cells were washed twice with buffer containing 2 mM CaCl2; 150 mM NaCl; 10 mM HEPES (pH 7.4) (GPMV buffer), and incubated in the same buffer supplemented with 25 mM formaldehyde (Fluka Chemie GmbH, Switzerland) and 2 mM DTT (AppliChem GmbH, Darmstadt; Germany) for 1 (RBL-2H3) or 2 h (HeLa) at 37 °C with mild shaking (300 r/min) (Thermomixer comfort, Eppendorf AG, Germany). In an alternative protocol, GPMV buffer supplemented with 2 mM N-ethylmaleimide (Fluka) was used to induce the formation of vesicles. After incubation, the supernatant with detached GPMVs was delivered into a flow cytometry tube (BD Biosciences, Belgium) where GPMVs were allowed to settle on ice for 30 min. After that about 20...

50% of the total volume from the bottom of the tube was collected into a separate tube. Before experiments, GPMVs were stored on ice.

### 2.3. Peptides

Transportan (GWTLSAGYLLGKINLALAKKLK-NH2), TP10 (AGYLLGKINLALAKKLK-NH2), MAP (KALKLALKLAKKLA-NH2), pTat (GRKKRRQRPRPQ-NH2), AnV-AF647 (RQIKIWFQNRRMKWKK-NH2), Argβ (RRRRRRRRR-NH2), a peptide from Perforin (82-96) (QHRVTRAKVSSTEAV) [12] and a peptide from C-terminus of β1 adrenergic receptor (β1; CSSLEPDGRGFSSESVK-NH2) were synthesized stepwise on a 0.1 mmol scale on an automated peptide synthesizer (Applied Biosystems Model 431A) using the tert-butyloxycarbonyl amino acids were coupled as hydroxybenzotriazole esters to a p-methybenzhydrylamine resin (Neosystem, Strasbourg, France) to obtain C-terminally amidated peptides. The final cleavage and purification of peptides was performed as described earlier [42]. Fluorescent labels (fluorescein for TP10, TP, MAP, Argβ, pAntp, pTat, Perforin (82-96), β1 adrenergic receptor peptide, and Oregon Green 488 for TP) were coupled manually to the side chain of Lys3 or Lys7 in Transportan and TP10, respectively, or to the N-terminus of MAP, pTat, pAntp, Argβ, β1A and Perforin (82-96). The molecular weights of fluorescenteinated peptides were determined by MALDI-TOF mass spectrometry (pO-TOF 2000, PE Biosciences) and calculated molecular weights were obtained each time.

### 2.4. Labeling of GPMVs with lipid phase markers

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 concentrations of 10 μg/mL, and AnV-AF647 at 1:100 dilution according to manufacturer’s instructions. Laurdan membrane dye (Invitrogen) was used at 8 μM concentration to visualize liquid-ordered domains in the membrane of GPMVs.

### 2.5. Confocal laser scanning microscopy

The fluorescently labeled CPPs at 1 μM concentrations were added to GPMV suspension shortly after labeling vesicles with phase marker proteins, and visualized at time points specified in the figure legends. DAPI and propidium iodide were used in concentration of 1 and 5 μg/mL, respectively, (MW: aluminum, peroxidase, Sweden) were used as controls of the intactness of the vesicles and were added to GPMV suspensions at concentrations of 1 μM after incubating with AnV-AF647 and CtxB-AF595. 50 μL of GPMV suspension was deposited onto glass slides (8-well, 0.7 cm2 of area per well, Nalge Nunc International, Rochester, NY) after the removal of the media chamber, and leaving the silicon frame in place, and covered with a glass coverslip (24×24 mm, No 1). During the low temperature imaging an ice cube was placed on the specimens and replaced if necessary. GPMVs were imaged with an Olympus IX81 inverted microscope equipped with the Fluoview 1000 confocal system using a 60× water-immersion objective and excitation at 405 nm (DAPI, Laurdan), 488 nm (fluorescein and Oregon Green 488), 559 nm (Alexa Fluor 594, propidium iodide) and 635 nm (Alexa Fluor 647).

The lasers were run in a sequential scanning mode to avoid the spectral overlap. Obtained images were processed with Adobe Photoshop CS4.

### 2.6. Cholesterol depletion from GPMVs

In order to abolish the formation of liquid-ordered phase, cholesterol was removed from the membrane of vesicles. GPMVs were collected as described above. For cholesterol extraction, the GPMV suspension was incubated with 5 mM methyl-β-cyclodextrin (MβCD; Sigma-Aldrich Chemie, Germany) at 37°C for 1 h. After MβCD treatment, the suspension was centrifuged in a Sorvall SS34 rotor at 27,000 g for 45 min at 4°C to recover the vesicles. After centrifugation, GPMVs were collected by removing about 20% of the volume from the bottom of the centrifuge tube.

### 2.7. Quantification of Fluo-CPPs in GPMVs by flow cytometry analysis

GMPVs were harvested as described above and the suspension was subjected to FACS analysis in GPMV buffer and the fluorescent CPP was added directly before the counting. In order to obtain results comparable with other used CPPs we used fluorescein-labeled TP instead of Transportan-Oregon Green 488. We used 1 μM peptide concentration per 1.5–2×106 of GPMVs; 104 events were counted for every sample using the constant voltage settings of forward and side scatter. DAPI and propidium iodide were used at concentration of 1 μg/mL. The results were analyzed by using FACS Diva and MS Excel software. Values on graphs represent the mean value of the histogram plot from three separate experiments. Error bars represent the standard deviation.

### 2.8. Studying the effect of CPPs from GMPVs

Fluorochrome-labeled CPPs were added to 250 μL solution of GPMVs (final concentration of peptides 1 μM) and the vesicles were allowed to adhere on a concanavalin A-coated glass coverslip (24×24 mm, No 1) on ice for 45 min. The buffer with free CPP and non-adhered GPMVs was removed and the adhered vesicles were carefully washed two times with GPMV buffer supplemented with 0.5 mg/mL heparin sodium salt (Sigma-Aldrich). The imaging was performed at low temperature in GPMV buffer in the presence of heparin and as indicated above. Image stacks (0.75 μm Z-step size) from different fields of CPP-containing GPMVs were collected during 1 h with a 10-minute interval with CPP-specific settings of laser and photomultiplier avoiding zero/saturated pixel values and keeping constant settings during the imaging period. For data analysis, four stacks from the equatorial plane of GPMVs were chosen for each time point (avoiding the proximity to the glass surface which revealed a high fluorescence signal due to the adhered CPP) and merged, followed by defining the interior of vesicles as regions of interest (ROIs). The representative data from one of three experiments is shown where intravesicular fluorescence from 7 to 10 GPMVs was recorded at each time point. Error bars represent standard deviation.

### 3. Results

#### 3.1. Giant plasma membrane vesicles

The RBL cells released GPMVs upon chemical induction as described earlier [35]. The vesicles had a spherical shape and varied in size from 1 to 10 μm in diameter (Fig. 1, Supplementary Fig. 1). Cholera toxin B subunit (CtxB-AF594) and annexin V (AnV-AF647) [34] marked the liquid-ordered (Lo) and liquid-disordered (Ld) domains on the vesicles, respectively. Both phases, LO and LD were present in the majority of vesicles notwithstanding the size, and segregated into microscopically resolvable domains at temperatures below 10°C. The labeled annexin V and cholera toxin B subunit were excluded from the interior of GPMVs analogously to the previous report [35]. The membranes of GPMVs were impermeable or permeable in a very low extent also for peptides with negative (β1 peptide, charge: −1) or low positive charge (Perforin 82–93, charge: +3) and for polySarcidin 20 with molecular mass 2–3 kDa (Supplementary Figs. 1 and 3). The nuclear stains DAPI and propidium iodide (PI) entered all vesicles and concentrated there at least during 1 h (Supplementary Fig. 1 and 5). This data corroborates the earlier findings showing the presence of...
3.2. CPPs interact with and penetrate into giant plasma membrane vesicles at low temperature

We chose fluorescently labeled CPPs Tat peptide (pTat), non-arginine (R9), model amphipathic peptide (MAP), Penetratin (pAntp), Transportan (TP) and Transportan 10 (TP10) as the representatives of different subclasses of CPPs [14] to characterize their lipid phase preference on the phase-segregated GPMVs. We incubated GPMVs first shortly with Lα/Lβ phase markers, followed by the addition of fluorescently labeled CPPs at 1 μM concentration and the visualization by confocal microscopy at low temperature. All the tested peptides were found to efficiently interact with, and to our surprise, also internalize and accumulate into GPMVs (Fig. 1). The accumulation of CPPs was not dependent on the presence of lipid phase markers (Supplementary Fig. 2) and occurred in a similar manner when the vesicle formation was induced by N-ethylmaleimide (NEM) treatment [35] (Supplementary Fig. 3). The accumulation into the lumen of vesicles was characteristic only to peptides with confirmed cell-penetration ability but not for the control peptide. The fluorescently labeled 18-mer peptide corresponding to the C-terminus of rat β1-adrenergic receptor ([β1], a peptide lacking the cell-penetrating ability (Supplementary Fig. 1), either did not enter the GPMVs, or the intravesicular concentrations of the peptide were equalized in the case of some vesicles (Supplementary Fig. 1). CPPs internalized into the majority of both the phase-segregated and optically homogeneous vesicles, showing that low temperature-induced large-scale lipid phase segregation is neither the determinant nor the hindrance to the internalization process. When comparing the accumulation ratio of different CPPs into GPMVs at low temperature, pTat and R9 showed a gradual concentration into GPMVs at least during 2 h without a detectable fluorescence difference between the membrane and the lumen of the vesicle. The rest of the studied fluorescent CPPs — MAP, pAntp, TP and TP10, in spite of the clearly detectable accumulation into the GPMVs, concentrated in and stained more intensely the membranes than the lumen of the vesicles (Fig. 1). The higher membrane affinity correlates well with the amphipathic properties of these CPPs [14]. We also confirmed that at low temperature the CPPs analogously accumulated in the GPMVs obtained from HeLa (Supplementary Fig. 4), CHO and other cell lines (data not shown) suggesting that the discovered phenomenon is universal and not specific to GPMVs derived from RBL cells.

3.3. CPPs interact with and accumulate into giant plasma membrane vesicles at room temperature

At room temperature, the membranes of the majority of the GPMVs revealed an optically homogeneous lipid phase and the domains had segregated only in a few of the vesicles. Analogously to the results obtained at low temperature, fluorochrome-labeled CPPs entered both types of vesicles (Fig. 1). However, by visual estimation, the ratio of accumulation into the lumen of the vesicle at room temperature was higher for all CPPs compared to the low temperature experiments. The only exception was Penetratin that at longer incubation times (2 h) concentrated in the membrane of the vesicles with the concomitant decrease of the fluorescence from the lumen (data not shown). In addition, the accumulation of Penetratin interfered with the integrity of the membrane resulting in the fragmentation of some GPMVs into smaller vesicles while pAntp being still bound to the membrane (Figs. 1, 2 and 3). However, this phenomenon was detected only with GPMVs from RBL cells, and

Fig. 1. Accumulation of fluorescently labeled cell-penetrating peptides (CPPs) in giant plasma membrane vesicles (GPMVs) prepared from RBL cells. GPMVs derived from RBL cells were incubated with fluorescently labeled CPPs (1 μM concentration and the visualization) for 30 min and 45 min, respectively. Note the membrane outgrowths on GPMVs incubated with pAntp (red arrowheads). Lα phase is marked with Alexa Fluor (AF) 594-labeled cholera toxin B subunit (red) and Lβ phase with AF647-labeled annexin V (pseudo-colored as white). Scale bar 10 μM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

nucleic acids in the apoptotic vesicles [43]. It also suggested that GPMV membranes were in very low extent permeable to hydrophilic medium-sized molecules, but able to accumulate small aromatic-type nucleic acid probes that are usually pumped out from living cells.

Fig. 2. Lipid phase preference of fluorescent CPPs. Giant plasma membrane vesicles derived from RBL cells were incubated with fluorescein-labeled (pAntp, MAP, and TP10) or Oregon Green 488-labeled (TP) CPPs at 1 μM concentration (green) at low temperature to detect phase preferences of peptides. Lα phase is marked with AF594-labeled cholera toxin B subunit (CtxB, red) and Ld phase with AF647-labeled annexin V (pseudo-colored as white). Scale bar 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vesicles from HeLa cells seemed to be more resistant to Penetratin-induced membrane reorganization (Supplementary Fig. 4).

3.4. Lipid phase preference of CPPs

MAP, pAntp and both Transportans concentrated more in the GPMV’s membrane than the interior of the vesicles, revealing high affinity to the Lα phase of lipid bilayer or the respective extracellular matrix components as indicated by the colocalization with the annexin V-positive domains (Fig. 2). The CPPs retained the localization in Ld phase also in the presence of Laurdan, a membrane dye concentrating in Lα phase (Supplementary Fig. 2). Furthermore, the phase preference of the four peptides at low temperature was detected also in the absence of all phase markers (Supplementary Fig. 2), corroborating that accumulation of CPPs at the Ld domain was not induced by the addition of phase markers. In a small fraction of GPMVs the lipid phases had segregated but peptides still distributed uniformly in the membrane and did not reveal a clear preference for a particular lipid phase (data not shown). The preference for Lα phase, however, was not observed for any tested CPPs. As cholesterol has been demonstrated to be essential for the formation of the Lα phase [44,45], we presumed that cholesterol in GPMV membranes could hinder the association and internalization of CPPs.

3.5. Removal of cholesterol from the giant plasma membrane vesicles facilitates the internalization of CPPs

To ascertain the preference of amphipathic CPPs for Lα phase, GPMVs from RBL cells were depleted of cholesterol by the treatment with 5 mM methyl-β-cyclodextrin (MβCD) at 37 °C for 1 h. Under these conditions, 75 ± 10% of cholesterol from the membranes of GPMVs is removed [46] and the lipid phase segregation abolished, since the removal of cholesterol leads to the disruption of lipid rafts [47]. We observed a slight decrease of the size of GPMVs after MβCD treatment, and no optically resolvable phase segregation was detected in GPMVs after MβCD treatment. In addition, the membranes of vesicles stained with CtxB at very low level (Fig. 3). These results are in concordance with the report by Gidwani et al. demonstrating the decrease in the membrane order of GPMVs after cholesterol depletion [46]. As expected, CPPs penetrated into the cholesterol-depleted GPMVs more efficiently and all vesicles stained at a rather uniform level. This suggests that the disordered lipid phase of the plasma membrane could also be the portals for CPPs’ entry into cells.

3.6. Quantification of the interaction of CPPs with giant plasma membrane vesicles

We included flow cytometry analysis to obtain more quantitative data about the accumulation of fluorescent CPPs in the vesicles. We chose pTat, R9 and Transportan for the kinetics studies, and in order to obtain comparable results, included fluorescein-labeled Transportan (TP-Fluo) in addition to Oregon Green 488-labeled TP (TP-OG) in the studies. The incubation of the vesicles with the β1 peptide at low temperature revealed that the peptide associated with the GPMVs but the interaction was rather weak and reached saturation in 10–20 min (Fig. 4). pTat and R9 interacted with GPMVs to a much higher extent and revealed a similar kinetic profile — the quick interaction (slower for pTat compared to R9) at the beginning slowed down after 30–40 min but did not reach saturation either at the 0.5 or 1 μM concentrations within 1 h (Fig. 4). Furthermore, the peptides continued to slowly accumulate into vesicles at least during 3 h (data not shown). The accumulation profile of pTat and R9 into GPMVs was comparable with that of the propidium iodide and DAPI (Supplementary Fig. 5A), although it should be noted that the fluorescence of the
However, no evident decrease in signal intensity was observed for R9 labeled Transportan, if heparin was present in extravesicular medium. A slight decreasing trend of intravesicular fluorescence signal was detected for both Oregon Green 488 and 470 labeled peptides (R9, pTat, TP) and visualized after the removal of the unbound CPP. When we quantified the intravesicular fluorescence intensity of CPP-loaded and subsequently washed GPMVs by CLSM, we noted that the fluorescence intensity of vesicles recorded during 1 h did not change markedly. A slight decreasing trend of intravesicular fluorescence signal was detected for both Oregon Green 488 and fluorescein-labeled Transportan, if heparin was present in extravesicular medium. However, no evident decrease in signal intensity was observed for R9 and pTat (Fig. 5), suggesting a negligible or very slow (if any) efflux of these CPPs from the intravesicular environment and confirming the lack of pores permeable to peptide-sized molecules.

4. Discussion

Since the discovery of cell-penetrating peptides the definition of penetration has had a number of implications. Several models have been proposed for the translocation process of peptides across the plasma membrane [48–50] and, up to now, actually none of the proposed mechanisms has been completely excluded as unlikely. The only model that has been hypothesized to describe the translocation process of one of the most well-known CPP, Tat peptide, at molecular level [24], is based on a computer simulation and it takes into account only a small number of lipids and peptides. If to consider that the cell's plasma membrane contains a vast variety of lipids, proteins, polysaccharides, and even more — that different energy-driven processes are active at physiological conditions, this model system is far too simplified. However, this and other experiments performed with different artificial systems have given valuable information about how the CPPs might interact with and position themselves in the lipid bilayer. Combination of data from these models with results obtained with living cells should lead us closer to the understanding of the mechanisms of CPPs' cellular entry.

In this study we have exploited a model system of giant plasma membrane vesicles (GPMVs) [33,34] to assess the interactions of CPPs with the cell's plasma membrane components in conditions not interfered by endocytic processes. The GPMVs have been used earlier to study the lipid phase dynamics and the phase preference of different plasma membrane proteins [35,38,51]. Since the membrane of GPMVs has composition very close to the plasma membrane of...
cells, we initially aimed to obtain more information about cell-entry portals and the lipid phase preference of different CPPs by using this model system. However, in parallel with the results that the primary and secondary amphipathic CPPs clearly preferred the Ld phase when interacting with plasma membrane components, we also detected the translocation and accumulation of all the tested CPPs into GPMVs at temperatures below the physiological, corroborating the ability of CPPs to cross the biological membrane without using endocytic processes. The membrane (voltage) potential that is considered a driving force for the passage of cationic CPPs across the plasma membrane of cells [52], is probably missing in GPMVs. Furthermore, the accumulation of CPPs was not impaired in buffer containing KCl instead of NaCl and used for depolarization of cells, suggesting that translocation could also take place in the absence of membrane potential (data not shown).

The CPPs in tested concentrations revealed no detectable membrane-disturbing activities to GPMVs except for pAntp. However, the fluorescence microscopy analysis of GPMVs incubated with Transportan revealed the presence of smaller and more numerous segregated phases compared to other used CPPs (data not shown), suggesting that Transportan could rearrange the membranes. Arsov and colleagues have demonstrated earlier that Cys-Transportan changed the

Fig. 4. Flow cytometry analysis of the interaction of fluorescent CPPs with giant plasma membrane vesicles prepared from RBL cells. Fluorescein-labeled CPPs were added to GPMVs either at 4 °C or room temperature and the fluorescence signal in vesicles was quantified at different time points within 1 h. Different concentrations of peptides are indicated as: filled squares — 0.01 μM, empty squares — 0.1 μM, filled triangles — 0.5 μM, empty triangles — 1 μM. Note the lower values of Y-axis in graphs presenting the data of β1 peptide.
lipid ordering in model membranes with moderate cholesterol concentration but at higher cholesterol content no effect was observed [53], indicating that the peptide preferentially bound to more loosely packed membrane areas and increased their ordering. One possible explanation for the observed phase-inducing property of TP might arise from its inherent affinity towards membranes, both negatively charged and neutral ones [9,14,54], resulting in a potentially more stable membrane binding and the subsequent stabilization of underlying lipid phase. The addition of the membrane-staining probes cholera toxin B subunit and annexin V has been shown to increase the stability of phase separation in GPMVs at room temperature [38]. One might speculate that the binding of Transportan could also influence the segregation of lipid phases in GPMVs in analogy with the abovementioned agents.

The membrane-active properties of pAntp were corroborated by the observed formation of short-living outgrowths and sporadical fragmentation of GPMVs after a prolonged incubation of vesicles with this CPP. It has been reported earlier that pAntp is able to induce invaginations in giant unilamellar vesicles comprised of lipids preferring the disordered phase whereas the liquid-ordered phase was resistant to membrane deformation by the peptide [29]. Since in our experiments Penetratin also favored Lα phase, it might indicate that membrane fluidity or the lower packing density is the critical parameter for the membrane deformations to occur after CPP binding. The detected membrane outgrowths could be driven by the ability of pAntp to insert into the leaflets of the membrane bilayer in an asymmetrical mode [28], resulting in the bending or fragmentation of the membrane.

The preference for the Lα phase has been reported earlier also for the Tat peptide [55]. Although, when by microscopical observations performed at sub-physiological temperatures the fluorescence signal of pTat was more intense in the lumen of GPMVs, at 37 °C the peptide was occasionally detected accumulating in the Lβ phase (especially in GPMVs from CHO cells, data not shown). The preference of all the membrane-affine CPPs, TP, TP10, pAntp and MAP, for the Lα phase in GPMVs suggests that in live cell, these peptides might also interact favorably with the membrane areas of more dynamic and less densely packed nature. We also demonstrated that the phase segregation that is characteristic to GPMVs at low temperature [35] is not a prerequisite for the internalization of CPPs into vesicles, since GMPVs with optically homogeneous membranes, either at low or room temperature, were able to accumulate the CPPs. Moreover, at higher temperatures where lipids are more dynamic and different phases do not segregate, the translocation into vesicles was more efficient for the non- amphipathic pTat and Rα. In addition, the depletion of cholesterol from the GPMVs led to more uniform internalization of all CPPs, suggesting that the large densely packed membrane areas on the vesicles rather inhibit than potentiate the translocation of CPPs into the lumen of GPMVs. This correlates with data obtained on live cells, where the depletion of cellular cholesterol increased the accumulation of nona-arginine into HeLa cells [2]. On the other hand, the depletion of cholesterol from the plasma membrane has been shown to affect the cellular uptake of CPP-cargo constructs at physiological temperature conversely by decreasing it [22,56,57]. The uptake of CPP-cargo complexes by cells has been shown to mostly take place by different types of endocytosis: via lipid rafts or caveolae [58–60], clathrin-coated pits [61] or macropinocytosis [22,57,62]. As all these pathways are shown to be more or less inhibited by the removal of cholesterol from the plasma membrane, it suggests that the optimal conditions for the cellular delivery of cargo molecules by CPPs using endocytosis, and for direct penetration of peptide across membranes differ profoundly. The putative mechanism for this dual phenomenon was proposed very recently. A high concentration of nona-arginine was demonstrated to induce the activity of cellular acidic sphingomyelinase, which lead to the formation of ceramide in the plasma membrane and subsequent rapid uptake of CPP via so-called nucleation zones [26]. Although the sphingomyelinase-sensitive uptake occurred only at high peptide concentrations and it was dependent on the size of the transduced compound, this still could explain the contradicting effect of cholesterol depletion on the CPP uptake by cells. Since cholesterol directly inhibits the acid sphingomyelinase activity [63] and competes with ceramide for the association with plasma membrane, its removal could facilitate the rapid uptake of CPPs induced by formed ceramide platforms. On the other hand, the inhibition of endosomal pathways by cholesterol depletion suppresses the cellular uptake route that functions in case of big cargoes or low concentrations of CPPs.

The penetration and accumulation of all the tested CPPs into the giant plasma membrane vesicles was the highest in the case of pTat and Rα. Based on our results, we suggest that the variable uptake of tested CPPs into the lumen of GMPVs depends on the balance between the overall positive charge and the hydrophobicity of the peptide. A higher positive charge results in a more pronounced accumulation in the lumen of vesicle, as seen for pTat and especially Rα while the peptides with a negative (p1) or low positive charge (Perforin 82–96) reveal no or very low accumulation to the GPMVs. CPPs that contain hydrophobic amino acids (TP, TP10, pAntp, and MAP), distribute in the GPMVs in a manner that in the confocal plane the fluorescence signal from the membrane is higher than that from the lumen of the vesicle in spite of the high positive charge of these CPPs.

The accumulation kinetics of pTat and Rα into GPMVs was analogous with the translocation of propidium iodide and DAPI into vesicles. It has been demonstrated that cell-derived microparticles contain DNA and different types of RNA [43]. pTat in turn is known to have high affinity towards heparan sulfate proteoglycans and nucleic acids [64]. It is feasible that the presence of these compounds in the lumen of GMPVs and the respective chemical gradient drive the translocation of propidium iodide and DAPI into the GPMVs preloaded with these.

The rapid interaction of Transportan with GPMVs detected by flow cytometry reaching to the plateau level of fluorescence intensity just in 5–15 min corroborates the affinity of this CPP to membranes [54]. Different fluorescence intensity of fluorescein- versus Oregon Green 488-labeled Transportan might be explained by the stronger quenching of fluorescein in the membranous environment or in the vicinity of anions. The quenching of fluorescein emission at low pH has been
The continuous accumulation of fluorescent pTat, R6 in GPMVs at least during an hour suggests that the studied CPPs are rather stably associated with membrane and/or intravesicular (e.g. cytoplasmic) contents. Even after the removal of the extravesicular CPP, the signal of intravesicular fluorescence does not follow the kinetics of the diffusion down the concentration gradient but remains negligible. In addition, the rearrangement of lipids in the GPMV membrane as a possible hindrance for efflux cannot be excluded either, as evidenced by the membrane organizing capacity of TP. However, we could not determine the amount of CPP that attached to the glass surface during loading the GPMVs with peptides. The possible dissociation of fluorescently labeled peptide from glass after washing step, and its contribution to the fluorescence signal from the extravesicular space (or even to the intravesicular fluorescence while the conditions favor internalization) are very difficult to quantify and could require a more detailed analysis.

In conclusion, GPMVs is a useful model for studying the phenomenon of some cationic peptides to translocate across the biological membranes. The absence of cortical cytoskeleton, endocytosis and processes powered by cellular energy on one hand, while retaining the sophisticated complexity of the plasma membrane with its lipids, proteins and extracellular matrix components, on the other, grants the GPMV model with several advantages over artificial lipid vesicles and live cells. However, it has to be considered that in living cells the efficacy of a direct passage across the plasma membrane is rather low [66], unless it is potentiated by using the unfavorable conditions for cellular homeostasis, like interfering with the highly orchestrated organization of the plasma membrane by the removal of cholesterol [23] or applying high concentration of peptide [2,3,5,7]. Still, the direct translocation across the membranes could have a biological relevance if the extracellular matrix assists in creating a high local concentration of membrane-active macromolecules to be followed by the penetration. This might also occur in intracellular vesicles after accumulation of CPPs to a particular endosomal compartment, where the high local concentration of peptides could induce leakage or phase transition of membrane lipids followed by the escape of the peptide into cytosol. It has been demonstrated recently that the presence of late endosome-specific lipid lysosomotropic phosphatidyl acid in LUVs favors the lipid phase transition and supports Tat peptide-mediated vesicle leakage, and lipid mixing [68]. Although the lipid composition of GPMVs resembles plasma membrane [41] rather than that of the endosomal membrane, our results support the direct membrane translocation of CPPs and hopefully shed light to the possible determinants in this process.

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