Analytical Biochemistry 438 (2013) 1–10

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

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

Direct translocation of cell-penetrating peptides in liposomes: A combined mass spectrometry quantification and fluorescence detection study

Astrid Walrant [a], Lucrèce Matheron [a,b], Sophie Cribier [a], Stéphane Chaignepain [c], Marie-Lise Jobin [c], Sandrine Sagan [a,*], Isabel D. Alves [a,c,*]

[a] UMR 7203 CNRS, ENS, Laboratoire des Biomolécules, UPMC–Université Paris 06, 75005 Paris, France
[b] Plateforme de spectrométrie de masse et protéomique, UPMC, IFR 83, 75005 Paris, France
[c] CBMN-UMR 5248 CNRS, Université de Bordeaux, IPB, 33600 Pessac, France

ARTICLE INFO

Article history:
Received 11 September 2012
Received in revised form 6 March 2013
Accepted 11 March 2013
Available online 20 March 2013

Keywords:
Cell-penetrating peptides
Internalization mechanism
Direct translocation
Lipid vesicles
Mass spectrometry
Free radicals

ABSTRACT

Cell-penetrating peptides (CPPs) can cross cell membranes in a receptor-independent manner. Two main routes for their cellular uptake have been proposed: endocytosis and direct translocation through the cell membrane. The ability of a peptide to enter cells through direct translocation can be assessed by evaluating the amount of peptide crossing the membrane of liposomes. Most methods reported so far rely on the use of fluorescent probes, which, when attached to a CPP, often alter its physical/chemical properties. Herein, a matrix-assisted laser desorption/ionization time-of-flight MS-based method is described to quantify the amount of CPP taken up into lipid vesicles and to distinguish it from the amount that is bound or inserted in the membrane. For comparison, visualization of the uptake of the same, but fluorophore-labeled, peptides into giant vesicles and cells by fluorescence microscopy is also reported. We show that membrane charge density is an important factor for direct translocation. We also show that fluorophore-labeled peptides have a different translocation behavior and that they are more toxic to cells. Alternative methods to fluorescence, such as the one reported herein, should be favored when investigating the uptake mechanism of CPPs, as fluorescent dyes can alter short peptides' physical/chemical properties and their internalization capacities.

Cell-penetrating peptides (CPPs)[1] are defined as sequences of up to 40 amino acids possessing the ability to cross cell membranes and transport cargos such as peptides, proteins, and other bioactive molecules [1]. The understanding of their uptake mechanism has been a matter of study and debate since their discovery in the 1990s. Two major routes have been suggested: endocytosis and direct translocation through the cell membrane. Nowadays, it is mostly agreed that CPPs may take either one or both of these routes and that the mode of internalization can depend on several factors such as type and size of the transported cargo [2–4], peptide concentration [5–7], temperature [7], or cell type [8]. While the endocytotic route has been generally accepted, direct translocation through the cell membrane has been constantly questioned and over the years, many studies have reported contradictory results regarding the capacity of CPPs to directly cross the cell membrane.

To test CPP internalization by direct translocation, cell uptake studies are commonly conducted at 4 °C, as endocytic pathways are greatly inhibited at this temperature. Yet, the lowering of the temperature may also affect direct translocation as the membrane fluidity decreases with temperature. Thus, an alternative way to study translocation is to use liposomes that do not possess the endocytotic machinery and are therefore a good model to test for CPP direct translocation through the membrane.

Such studies have already been performed with penetratin. In 2000, Thoren and collaborators [9] showed by fluorescence microscopy that penetratin, labeled with carboxyfluorescein, was capable of crossing giant vesicles (GVs) composed of asolectin. In 2004, the same laboratory reported on the capacity of penetratin and analogs to translocate in GVs composed of soybean lecithin [10]. In that study, using confocal microscopy, they demonstrated that the uptake of the CPPs into the GVs was not an artifact of the fluorophore. They also reported that the same peptides were not able to cross the membrane of large unilamellar vesicles (LUVs) of varied composition. They attributed the different behaviors with the two lipid

* Corresponding authors. Address: CBMN-UMR 5248 CNRS, Université de Bordeaux, IPB, 33600 Pessac, France (I.D. Alves).
E-mail addresses: sandrine.sagan@upmc.fr (S. Sagan), i.alves@cbmn.u-bordeaux.fr (I.D. Alves).

[1] *Abbreviations used:* CHCA, α-cyano-4-hydroxycinnamic acid; CPP, cell-penetrating peptide; egg PC, egg yolk phosphocholine; GV, giant vesicle; LUV, large unilamellar vesicle; NBD, 4-*nitrobenzo*-2-oxa-1,3-diazole; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; Rh-DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl).

0003-2697/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2013.03.009

model systems to the dynamic behavior of GVs compared to LUVs. On the other hand, in 2003, Silvius and collaborators [11] reported that penetratin, hexalysine, and hexa-arginine, labeled with 4-nitrobenzo-2-oxa-1,3-diazole (NBD), were all able to cross the membrane of LUVs, especially in the presence of a transmembrane potential. The same year, contradictory results were published using the same quantification method (NBD-labeled peptide and dithionite quenching) [12]. Nonetheless one should note that in that study the incubation time of the CPP with LUVs was much shorter (10 min) than that reported by Silvius et al. (several hours) and therefore may not be sufficient to allow internalization to occur. In 2005 Dathe and collaborators failed to reproduce [13] the experiments performed the year before by Persson et al. [10]. They attributed the disparity of the results to membrane defaults in the GVs and questioned the use of this model system in CPP-uptake studies. Recently, Pooga and co-workers [14] showed by confocal microscopy that six classic CPPs, including penetratin, are able to enter into giant vesicles derived from the plasma membrane. The composition of these vesicles mimicked well the composition of the cell membrane from which they were prepared but lacked the machinery necessary for endocytosis.

Herein, we report a method to quantify the uptake of CPPs by LUVs that has been adapted from a protocol used to measure CPP uptake in cells by matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectrometry [15,16]. In this study we used two arginine-rich, amphipathic peptides: RW9 (RRWWRRWRR-NH_2) and RL9 (RRLLRRLRR-NH_2). Despite their close sequence and similar secondary structure, we previously showed that they have opposite cell uptake capacities: RW9 is able to enter cells by both endocytosis and direct translocation while RL9 binds and accumulates on cell membranes but internalizes very poorly [17]. These two peptides are therefore an ideal couple for liposome internalization studies. Translocation of the same peptides, labeled with Alexa 488, into GVs and Chinese hamster ovary (CHO) cells assessed by fluorescence microscopy is also reported herein as a comparison. Fluorescence is indeed the most classical technique to study such events and allows direct measurements with high sensitivity and easily accessible labeled peptides. However, in our case, MS proved more successful than fluorescence, because of difficulties and toxicity issues that arose from the use of labeled peptides.

Material and methods

Materials

The glycerophospholipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were obtained as a powder from Genzyme (Liestal, Switzerland). Egg PC was purchased from Avanti Polar Lipids (Alabaster, AL, USA) and asolectin from Sigma. 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rh-DOPE) was obtained as a 1 mg/ml solution in chloroform from Avanti Polar Lipids.

Biot-$(Gly)_4$-RL9, Biot-$([2,2\text{-}D_2]\text{-}Gly)_4$-RL9, and Biot$(O_2)$-$(Gly)_4$-Cys-RW9 were obtained from PolyPeptide Laboratories (Strasbourg, France). Biot(O_2)-$(Gly)_4$-RW9 was obtained from Cambridge Research Biochemicals (Billingham, UK). Biot(O_2)-$([2,2\text{-}D_2]\text{-}Gly)_4$-RW9 was synthesized using the Boc solid-phase strategy and Biot(O_2)-$(Gly)_4$-Cys-RL9 was synthesized using the Fmoc solid-phase strategy.

Alexa 488 C_5 maleimide was purchased from Life Technologies (Gaithersburg, MD, USA) and labeling of the peptides bearing a cysteine was performed as described by Vivès and Lebleu [18].

Liposome preparation

To prepare 100-nm-diameter LUVs, lipid films were formed by dissolving the appropriate amounts of lipids into chloroform/methanol (2/1 vol/vol). The solvent was then evaporated under a N_2 flow to deposit the lipids as a film on the wall of a test tube. Final traces of solvent were removed in a vacuum chamber during 2–3 h. Films were then hydrated by addition of the appropriate amount of buffer (50 mM Tris, 100 mM NaCl, pH 7.5) to reach a lipid concentration of 1 mg/ml and vortexed extensively at room temperature. The multilamellar vesicles thus obtained were then submitted to five freeze/thawing cycles and the homogeneous lipid suspension was passed 15 times through a miniextruder (Avanti Polar Lipids) equipped with a 100-nm-pore polycarbonate membrane above the phase transition temperature of the lipids.

To prepare GVs, 50 µl of a lipid solution (5 mg/ml; containing 1% Rh-DOPE) in chloroform/methanol (4/1 vol/vol) was spread on a rough Teflon surface and the solvent was evaporated for 1 h under vacuum. The lipid film was then rehydrated with a buffer solution (50 mM Tris, 100 mM NaCl, pH 7.5) containing 150 mM sucrose. The vesicles formed spontaneously after 24 h and could be harvested using a truncated pipette tip to avoid shear stress that could damage the vesicles.

Dynamic light scattering

The size distribution of liposomes following peptide addition (after 2 h incubation) was monitored by dynamic light scattering for POPG, POPG/POPC (3/1 mol/mol), egg PC, and asolectin at a peptide (P)/lipid (L) ratio of 1/100, which was close to the P/L ratios used for the liposome internalization experiments (P/L used was about 1/150). Measurements were performed on a Malvern Zeta-Sizer Nano ZS instrument with a detection angle at 90°. Mean hydrodynamic diameters and their size distributions were determined using a cumulative analysis method; each measurement was performed twice and experiments were repeated three times.

Calcein leakage

Calcein-containing LUVs were made using the same protocol used to make regular LUVs, except that the lipid film was rehydrated with buffer containing 70 mM calcein [19]. Free calcein was separated from the calcein-containing LUVs using size-exclusion column chromatography (Sephadex G-75) with 50 mM Tris, 100 mM NaCl, pH 7.5, as elution buffer. The concentration of lipids was estimated using a phosphorus assay [20]. For the calcein-leakage assay, the lipid concentration was set at 1 µM and peptide concentration was set for a P/L ratio of 1/100. All measurements were performed with a PerkinElmer LS55 spectrometer (Buckinghamshire, UK). Data were collected every second at room temperature using a λ_{exc} at 485 nm and λ_{em} at 515 nm with an emission and excitation slit of 2.5 nm. The fluorescence from calcein-encapsulated liposomes was measured for 10 min to allow stabilization (in absence of peptide); following that the peptide was added and data were acquired for 2 h. Finally, complete leakage of LUVs was achieved by adding 100 µl of 10% Triton X-100 solution, dissolving the lipid membrane without interfering with the fluorescence signal. The percentage of calcein release was calculated according to the following equation:

$$\%\text{calcein leakage} = (F_t - F_0)/(F_f - F_0) \times 100, \quad (1)$$

where the percentage of calcein leakage is the fraction of dye released (normalized membrane leakage), F_t is the measured fluorescence intensity at time t, and F_0 and F_f are the fluorescence intensity at time $t = 0$ and after final addition of Triton X-100, respectively. A dilution