The Antennapedia peptide penetratin translocates across lipid bilayers – the first direct observation

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Abstract The potential use of polypeptides and oligonucleotides for therapeutical purposes has been questioned because of their inherently poor cellular uptake. However, the 16-mer oligopeptide penetratin, derived from the homeodomain of Antennapedia, has been reported to enter cells readily via a non-endocytotic and receptor- and transporter-independent pathway, even when conjugated to large hydrophilic molecules. We here present the first study where penetratin is shown to traverse a pure lipid bilayer. The results support the idea that the uptake mechanism involves only the interaction of the peptide with the membrane lipids. Furthermore, we conclude that the translocation does not involve pore formation.

Key words: Antennapedia homeodomain; Penetratin; Membrane translocation; Delivery vector; Uptake

1. Introduction

Biological membranes are generally impermeable to large hydrophilic molecules. This complicates the employment of such compounds in a wide range of biomedical applications and necessitates research on improving their cellular uptake [1,2]. A promising strategy is to conjugate the molecule of interest to an entity that is efficiently internalized by cells. In recent years, several peptides with the ability to deliver polypeptides and oligonucleotides to the cytoplasm and nucleus of living cells have been discovered [2–6]. The most thoroughly studied one, penetratin, is a 16 residues long fragment derived from the homeodomain of the *Drosophila* transcription factor Antennapedia and has proven to be an excellent transport vector [4,7–9]. This peptide enters cells through an apparently non-endocytotic and receptor- and transporter-independent pathway [10,11], even when conjugated to large hydrophilic molecules such as oligonucleotides containing up to 55 bases [4,12]. The fact that penetratin is able to deliver molecular cargoes to the cytoplasm and the nucleus rather than to endosomal compartments [10] makes its transport properties particularly interesting. A full understanding of the mechanism of uptake is still lacking, but, based on numerous cell internalization experiments and spectroscopic studies in micellar and liposomal systems, a model in which the peptide enters the cell through interaction with the membrane lipids has been put forward [4,12,13].

In this paper, we have used fluorescence microscopy to assess the membrane translocating capability of penetratin in a model system (liposomes) in order to find support for a mechanism involving only peptide–lipid interactions. We have also investigated the influence of the peptide on the membrane integrity by studying induced leakage of vesicle-entrapped material with fluorescence spectroscopy. Interestingly, penetratin is found to translocate across a pure lipid bilayer via a non-pore-forming mechanism.

2. Materials and methods

2.1. Chemicals

Standard Fmoc-protected amino acids were obtained from Nova Biochem (Arg, Lys, Met, Trp, Phe), Alexis Corporation (Gln, Asn) and Perseptive Biosystems (Ile).

L-α-Phosphatidylcholine (type II-S) from Sigma was partially purified by acetone extraction [14]. Such preparations have been shown [14] to be composed of phosphatidylcholine (40%), phosphatidylethanolamine (33%) phosphatidylinositol (14%), lyso-phosphatidylcholine (5%) and cardiolipin (4%).

Potassium phosphate, Trizma base, HEPES, Triton X-100 and melittin (from bee venom) were purchased from Sigma. Magnesium sulfate, EDTA (titriplex III) and potassium dihydrogen phosphate were obtained from Merck. Glycerol from J.T. Baker and deionized water from a Milli-Q system (Millipore) was used. FM 1-43, 5-(and 6-)carboxyfluorescein (CF) and CF succinimidyl ester were purchased from Molecular Probes.

2.2. Peptide synthesis

Penetratin (Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys) was synthesized by Fmoc solid-phase synthesis using an Fmoc-PAL-PEG-PS support on a Pioneer Peptide synthesizer (Perseptive Biosystems), resulting in an amidated carboxyl terminus. The peptide used in the leakage experiments was capped with a $-COCH_3$ group at the amino terminus. In the case of CF-labeled penetratin, this end was left open for reaction with CF succinimidyl ester. For the labeling reaction, a onefold excess of the ester was added along with the resin-bound peptide to dimethylformamide and the reaction container was subjected to 24 h of gentle shaking. The peptides were cleaved from the resin with trifluoroacetic acid:1,2-ethanedithiol:water:triisopropylsilane (94:2.5:2.5:1), precipitated by addition of cold ether, collected by centrifugation, washed twice with ether, dried and dissolved in water. Preparative reversed-phase HPLC (Kromasil C8 column, Eka Chemicals) was used to purify the lyophilized peptides (isocratic elution: water:isopropanol:trifluoroacetic acid volume ratios 78:22:0.1 and 74:26:0.1 for the unlabeled and labeled peptide, respectively). The identity of the peptides was confirmed by electrospray mass spectrometry.

2.3. Preparation of GUVs

Giant unilamellar vesicles (GUVs) were prepared as described elsewhere (Karlsson, M. et al., Analytical Chemistry, in press). In brief, a droplet (5 μl) of an aqueous lipid dispersion (1 mg/ml) of acetone-

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purified asolectin in PBS buffer (5 mM Trizma base, 30 mM K_3PO_4, 30 mM KH_2PO_4, 1 mM $MgSO_4$, 0.5 mM EDTA, pH 7.8) containing 1% v/v glycerol was placed on a borosilicate cover slip and dehydrated in a vacuum desiccator. When the lipid film was completely dry, it was carefully rehydrated with PBS buffer. Within a few minutes, several hundreds of cell-sized unilamellar and multilamellar vesicles were formed.

2.4. Peptide translocation assay

Peptide translocation was studied using an inverted microscope (Leica DM IRB) equipped with a Leica PL Fluotar 40× objective. Fluorescence imaging was achieved by sending the output of an Ar^+-laser (Spectra-Physics 2025-05, 488 nm) through a 488 nm line interference filter followed by a spinning disc to break the coherence and scatter the laser light. The laser light was collected by a lens and was sent through a fluorescein filter (Leica I-3) into the objective to excite the fluorescent dyes. The fluorescence was collected by the objective and detected by a three chip color CCD camera (Hamamatsu) and recorded on VHS (Panasonic S-VHS AG-5700). Digital image editing was performed using an Argus-20 system (Hamamatsu) and Adobe Photoshop graphic software.

2.5. Preparation of large unilamellar vesicles (LUVs)

Vesicles with encapsulated CF were prepared by dispersion of a dried asolectin film in a solution of 10 mM HEPES, 50 mM CF, 10 mM NaCl, 148 mM NaOH and 1 mM EDTA (pH 7.4). The multilamellar vesicles were subjected to five freeze–thaw cycles before extrusion 21 times through two 100 nm polycarbonate filters on a LiposoFast-Pneumatic extruder (Avestin) to obtain LUVs. A homogeneous size distribution around 100 nm was confirmed by dynamic light scattering analysis, using a Malvern Instrument Series 7032 Multi-8 correlator and a PCS100 spectrometer (Malvern Instruments). The non-entrapped dye was separated from the vesicles on a Sephadex G-50 column (Amersham Pharmacia Biotech), using an isoosmolar buffer (10 mM HEPES, 107 mM NaCl, 5.3 mM NaOH and 1 mM EDTA, pH 7.4). Lipid concentrations were determined by static light scattering at 600 nm using a standard curve.

2.6. Induced leakage assay

The efflux experiments were performed at 25°C on a Spex Fluorolog τ-3 spectrofluorometer (JY Horiba). Peptide-induced leakage of vesicle-entrapped dye was monitored using excitation and emission wavelengths of 490 and 520 nm, respectively. The liposomes were diluted with HEPES buffer in a 1×1 cm quartz cuvette before addition of peptide. In order to minimize penetratin adsorption to the cuvette walls, these were modified by adsorption of a cationic polymer (under patent consideration) before the experiment. Furthermore, only a few seconds of rapid mixing, enough to achieve a homogeneous solution, was effected immediately after peptide addition.

Starting with a self-quenching concentration of CF inside the liposomes, the leakage of the dye can be detected as an increase in the fluorescence signal. The extent of leakage was taken to be equal to the corresponding increase in fluorescence according to

$$\text{Leakage} = \frac{I(t)-I_0}{I_T-I_0}$$

where $I(t)$ is the fluorescence at time t, I_0 is the fluorescence before peptide addition and I_T is the fluorescence after the vesicles have been lysed with Triton X-100. The self-leakage of the dye was found to be negligible and was not affected by the modification of the cuvette walls.

3. Results and discussion

In order to investigate whether penetratin is able to translocate across a model membrane, we studied the uptake of penetratin in giant vesicles. After formation of the vesicles (10–50 μm), CF-labeled penetratin was added to the sample

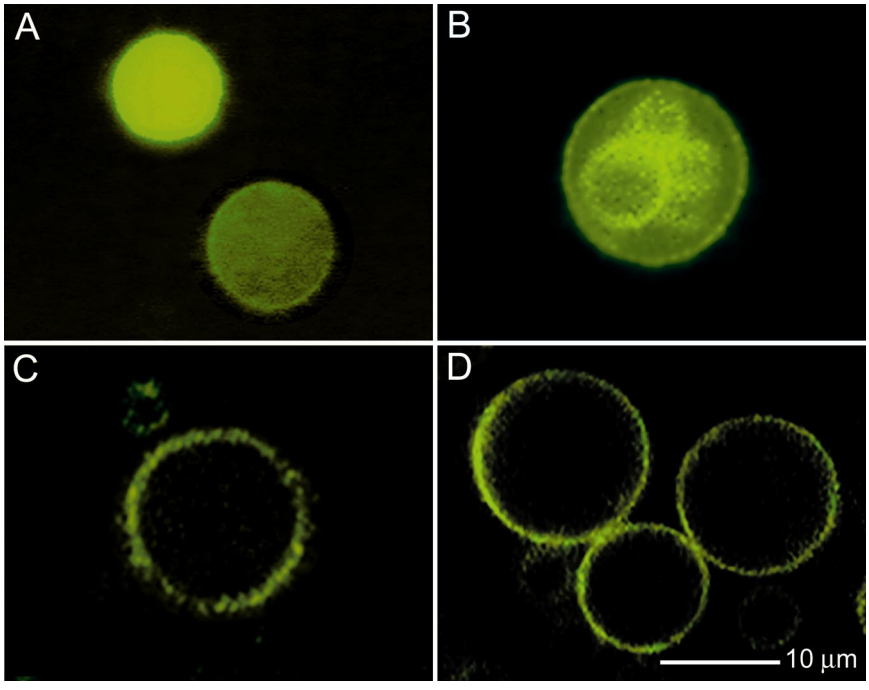

Fig. 1. Penetratin internalization monitored by fluorescence microscopy. A: Picture of CF-labeled peptide in multilamellar (top) and unilamellar (bottom) vesicles. B: GUV containing several smaller unilamellar vesicles. Peptide internalization is visualized by the strong fluorescence from the membranes of the interior vesicles. These pictures were taken 1 h after peptide addition (peptide:lipid molar ratio 1:46), but fluorescence from the interior of the vesicles is observed after less then 15 min. The external solution was diluted shortly before fluorescence imaging in order to reduce the background intensity. C: The signal from the lumen of the vesicles subsequently decreased over time, implying that the peptide is able to traverse the membrane in the outward direction as well. After 15 min, an appreciable reduction in fluorescence intensity was observed and after 2 h, when this picture was taken, no difference between the interior and the exterior of the GUVs could be discerned. D: Control experiment, where vesicles have been stained with the fluorescent membrane probe FM 1-43 in the absence of peptide. Asolectin (soybean phospholipids) was used in all vesicle preparations.

and the location of the peptide was monitored by fluorescence microscopy (Fig. 1). The strong fluorescence from the vesicle membranes shows that the positively charged peptide has a high affinity for the negatively charged lipid bilayer. Nevertheless, a significant fluorescence increase from the interior of the unilamellar vesicles is seen with time (Fig. 1A), indicating that penetratin is indeed able to translocate across the liposomal membranes. Furthermore, an intense and uniform fluorescence is obtained from the multilamellar vesicles, demonstrating that the peptide can penetrate the outermost bilayers and reach the inner lamellae. An even more evident observation of peptide translocation is presented in Fig. 1B, where several LUVs enclosed by a giant vesicle exhibit strong membrane fluorescence.

In the internalization experiments presented above, the background fluorescence was diminished by dilution of the sample with excess buffer shortly before fluorescence imaging. Interestingly, the fluorescence from the vesicles then decreased over time (Fig. 1C), implying that peptide translocation across the vesicular membrane occurs in both directions. Finally, only moderate fluorescence originating from membrane-associated peptide remained.

As a control experiment, liposomes were stained with the membrane probe FM 1-43 (Fig. 1D). The lack of fluorescence inside these vesicles indicates that the fluorescence from the interior of the unilamellar vesicle in Fig. 1A does not originate from membrane-bound peptide. In order to ensure that the vesicles are intact and non-leaky, free fluorescein was added to a liposome sample in a separate experiment. No fluorescence was observed inside the vesicles.

Whereas the cell internalization experiments performed during the last couple of years have demonstrated that this peptide readily enters many different types of cells, it is not possible to conclude on the basis of cell studies alone that interaction with the membrane lipids is sufficient for producing membrane translocation. The results presented above strongly indicate that penetratin readily penetrates purely lipidic bilayers. How is this possible? Normally, such a large (~2300 Da) and heavily charged (+7) molecule is not expected to cross the lipid barrier. Even more puzzling is the ability to transport big and, in the case of e.g. DNA, polyanionic cargoes into cells. A conceivable explanation would be the formation of membrane-spanning pores, where the cargo could pass the membrane without being exposed to the hydrophobic interior of the bilayer. The interaction of several pore-forming peptides with lipid membranes has been studied extensively over the last decades [15–18]. For example, the bee venom peptide melittin has been shown to translocate across liposomal membranes by generating transient openings [19]. The pore formation can be analyzed by monitoring the leakage of vesicle-entrapped dye with fluorescence spectroscopy [20]. Fig. 2A displays a typical example of CF release through melittin pores.

We have performed a comparative study of penetratin and melittin acting on CF-containing liposomes, prepared from the same lipids as in the microscopy experiments (Fig. 2B). The melittin-induced release of CF was found to be significant even at low peptide:lipid ratios, in accordance with similar studies found in the literature [20]. On the other hand, the corresponding series of experiments with penetratin showed only a minute efflux of CF even at such a high peptide:lipid ratio as 1:10. It is noteworthy that this is not an effect of low affinity for the membrane. On the contrary, affinity studies have shown that penetratin binds strongly to lipid bilayers (Thorén and Persson, unpublished data). Therefore, the lack of leakage as penetratin interacts with the vesicles must imply that penetratin does not form pores in lipid membranes. This is also in agreement with an observed low toxicity in cell studies [4,7].

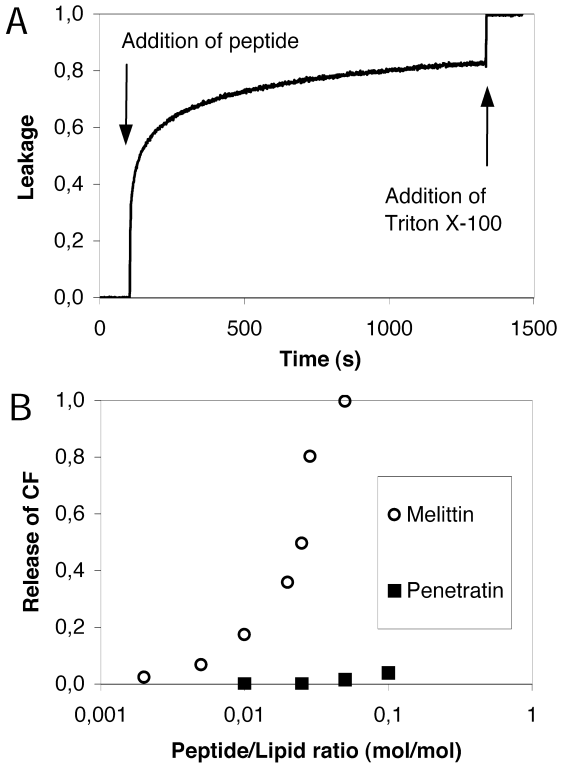

Fig. 2. Peptide-induced leakage of vesicle-entrapped CF. A: Typical release curve (melittin:lipid molar ratio 1:35) showing leakage (as defined in Section 2) as a function of time. The initial fluorescence is low due to the high, self-quenching concentration of CF inside the liposomes. As the dye is released, the fluorescence increases due to relief from self-quenching. B: Comparison of the permeabilizing ability of melittin and penetratin. Melittin- and penetratin-induced leakage of CF as a function of the peptide:lipid molar ratio. The leakage values were taken 15 min after peptide addition.

As is the case for other cell-penetrating peptides, such as transportan [5] and the protein transduction domain of HIV-1 Tat [21], a full understanding of the mechanism of membrane translocation still seems a distant goal. Although similar in their transporting efficiency, these peptides may well cross the cell membrane via different mechanisms. A notable common feature, however, is the importance of the basic residues in their amino acid sequences. There are reasons to believe that the interaction of these residues with negatively charged lipids in the bilayer plays a decisive role in the translocation process. Therefore, ongoing studies address the influence of lipid composition on the membrane interaction of penetratin.

In conclusion, the above results constitute the first direct evidence for penetratin translocation across a pure lipid bilayer. The mechanism of translocation is not necessarily the same in a liposomal system as in cells, but these findings show that penetratin internalization in cells via interaction with the membrane lipids is indeed possible.

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