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Different membrane behaviour and cellular uptake of three basic arginine-rich peptides

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ABSTRACT

Cell penetrating peptides (CPPs) are peptides displaying the ability to cross cell membranes and transport cargo molecules inside cells. Several uptake mechanisms (endocytic or direct translocation through the membrane) are being considered, but the interaction between the CPP and the cell membrane is certainly a preliminary key point to the entry of the peptide into the cell. In this study, we used three basic peptides: RL9 (RRLLRRLRR-NH_2), RW9 (RRWWRRWRR-NH_2) and R9 (RRRRRRRRR-NH_2). While RW9 and R9 were internalised into wild type Chinese Hamster Ovary cells (CHO) and glycosaminoglycan-deficient CHO cells, at 4 °C and 37 °C, RL9 was not internalised into CHO cells. To better understand the differences between RW9, R9 and RL9 in terms of uptake, we studied the interaction of these peptides with model lipid membranes. The effect of the three peptides on the thermotropic phase behaviour of a zwitterionic lipid (DMPC) and an anionic lipid (DMPG) was investigated with differential scanning calorimetry (DSC). The presence of negative charges on the lipid headgroups appeared to be essential to trigger the peptide/lipid interaction. RW9 and R9 disturbed the main phase transition of DMPG, whereas RL9 did not induce significant effects. Isothermal titration calorimetry (ITC) allowed us to study the binding of these peptides to large unilamellar vesicles (LUVs). RW9 and R9 proved to have about ten fold more affinity for DSPG LUVs than RL9. With circular dichroism (CD) and NMR spectroscopy, the secondary structure of RL9, RW9 and R9 in aqueous buffer or lipid/detergent conditions was investigated. Additionally, we tested the antimicrobial activity of these peptides against *Escherichia coli* and *Staphylococcus aureus*, as CPPs and antimicrobial peptides are known to share several common characteristics. Only RW9 was found to be mildly bacteriostatic against *E. coli*. These studies helped us to get a better understanding as to why R9 and RW9 are able to cross the cell membrane while RL9 remains bound to the surface without entering the cell.

1. Introduction

For the past 15 years, cell penetrating peptides (CPPs) have attracted much attention. Indeed, their unique ability to cross the plasma membrane designates them as potentially very powerful vectors to transport molecules that would otherwise never penetrate into cells such as heavily negatively charged polynucleic acids or some small molecules with therapeutic interest [1]. Since the discovery of penetratin, the third helix of the Antennapedia homeodomain [2,3], and of the HIV Tat peptide [4,5] in the 1990s, there has been extensive work to develop new synthetic and powerful CPPs such as polyarginines [6,7]. Many of these peptides share common features, namely a high density in basic residues and an amphipathic secondary structure in membrane environments. Together with the design of new peptides, there has been massive investigation on the internalisation mechanisms of CPPs. Indeed, a good understanding of the internalisation routes is essential for the development of efficient vectors. Cellular uptake mechanism is highly controversial, and both

Abbreviations: APA, Amino pentanoic acid; CD, Circular dichroism; CHO, Chinese hamster ovary; CPP, Cell-penetrating peptide; CS, Chondroitin sulfate; CSD, Chemical shift deviation; D8PG, Dioctanoyl phosphatidylglycerol; DiPoPE, Dipalmitoleoyl phosphatidylethanolamine; DLS, Dynamic light scattering; DMPC, Dimyristoyl phosphatidylcholine; DMPG, Dimyristoyl phosphatidylglycerol; DPC, Dodecyl phosphocholine; DSC, Differential scanning calorimetry; DSPG, Distearoyl phosphatidylglycerol; HS, Heparan sulfate; HSQC, Heteronuclear single quantum correlation; ITC, Isothermal titration calorimetry; LDH, Lactate dehydrogenase; LUV, Large unilamellar vesicle; MIC, Minimal inhibitory concentration; MLV, Multi lamellar vesicle; NOE, Nuclear Overhauser effect; NOESY, NOE spectroscopy; PA, Phosphatidic acid; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PG, Phosphatidylglycerol; PI, Phosphatidylinositol; PS, Phosphatidylserine; SDS, Sodium dodecyl sulfate; TOCSY, Total correlation spectroscopy

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endocytosis and direct translocation through the membrane are being discussed. Independently from their internalisation mechanisms, the interaction between the CPP and the cell membrane should be a preliminary key point to be taken into account. In this perspective, the study of the interaction of peptides with model lipid membranes can provide precious information about the phenomena that may occur at the cell surface. Cellular membranes can be simulated by very simple objects such as detergent micelles or lipid monolayers, or by more complex models such as multi-lamellar vesicles (MLVs) or large unilamellar vesicles (LUVs). The diversity of membrane models gives access to a very large array of biophysical techniques to study peptide/lipid interactions.

In this study, we chose to investigate the membrane behaviour of three arginine-rich peptides: the well known nona-arginine R9 peptide (RRRRRRRRR-NH_2) and two amphipathic peptides with a similar charge distribution, RW9 (RRWWRRWRR-NH_2) and RL9 (RRLLRRLRR-NH_2). The RW9 and RL9 peptides derive from the 16-mer sequences RW16 (RRWRRWWRRWWRRWRR-NH_2) and RL16 (RRLRRLLRRLLRRLRR-NH_2) respectively, which were designed from penetratin [8]. The three studied peptides RW9, RL9 and R9 share a common high density of arginine residues, which confers nine positive charges to R9 and six positive charges to RW9 and RL9.

It is well established that polyarginines such as R9 are very potent CPPs [6]. Different studies also showed that RW9 is efficiently internalised into cells [9,10]. Surprisingly, we found, using mass spectrometry quantification methods [11], that RL9 is very poorly internalised by cells. Therefore, the behaviour of these three peptides was studied in the presence of membrane models (MLVs and LUVs) in an attempt to correlate their different cellular uptake with a distinct behaviour at the level of lipid/peptide interactions. Herein, the thermodynamics of peptide interaction with lipid model systems was investigated, both in terms of affinity and peptide induced perturbation of the lipid phase transition using isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC), respectively. Additionally, the peptide structural changes accompanying lipid interaction were monitored by circular dichroism (CD) and NMR spectroscopy. Finally, since CPPs and antimicrobial peptides share common features [12], the antimicrobial activities of these peptides were investigated against *Escherichia coli* and *Staphylococcus aureus*.

2. Materials and methods

2.1. Materials

DMPC, DMPG and DSPG were obtained as a powder from Genzyme (Switzerland). DiPoPE and D8PG were purchased from Avanti Polar Lipids (Alabaster, AL). DPC-d_{38} was purchased from Eurisotop (Saint-Aubin, France). Biot(O_2)-Apa-RW9, Biot(O_2)-Apa-R9, Biot-$(Gly)_4$-RL9 and Biot-([2,2-D_2]-$Gly)_4$-RL9 were obtained from PolyPeptide Laboratories (Strasbourg, France). Biot(O_2)-Apa-RL9, Biot(O_2)-$(Gly)_4$-RW9 and Biot(O_2-([2,2-D_2]-$Gly)_4$-RW9 were synthesised using the Boc-solid phase strategy. Peptides used in most experiments have the Biot(O_2)-Apa N-terminal extension, except in quantification studies where the peptides have the Biot(O_2)-$(Gly)_4$ N-terminal extension (Section 2.4).

2.2. Cell culture

Wild type Chinese Hamster Ovary CHO-K1 cells and xylose transferase-deficient CHO-pgsA745 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), penicillin (100,000 IU/L), streptomycin (100,000 IU/L), and amphotericin B (1 mg/L) in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Cytotoxicity and membrane integrity

Cytotoxicity was measured using the Dojindo Cell-Counting Kit 8 based on the reduction of a reagent into a coloured product by viable cell dehydrogenases. CHO-K1 cells were seeded in a 96-well plate 1 day before treatment (4000 cells per well). Cells were treated with 0.1 μM, 1 μM, 5 μM and 20 μM peptide for 1 h, 3 h, 6 h, 16 h and 24 h. Untreated cells were defined as zero (negative control), and 1% SDS as maximum cytotoxicity (positive control).

Membrane perturbation was measured using the Promega CytoTox-ONE kit based on the quantification of lactate dehydrogenase (LDH) leakage. CHO-K1 cells were seeded in a 96-well plate (10,000 cells per well) 1 day before treatment. The same conditions as for cytotoxicity assays were used. Maximum LDH release was induced by treatment with 0.2% Triton X-100.

2.4. Measure of cellular uptake and quantification of membrane-bound peptide

Cellular uptake was quantified using the method described by Burlina et al [13]. In this protocol, the studied peptide bears a tag composed of four glycine residues together with a biotin moiety for purification purposes. After 1 h incubation of 7.5 μM peptide and washing, a protease is added (0.05% Pronase in Tris–HCl buffer, 100 mM pH 7.5) in order to detach the cells and to degrade all the non-internalised or membrane-bound peptide. This avoids overestimating the quantity of internalised peptide due to the presence of peptides attached to the outer leaflet of the membrane. The cells are then lysed (0.3% Triton) and boiled and the cell lysate is then incubated with streptavidin-coated magnetic beads to extract the peptide from the lysate. For membrane-bound peptide quantification, the same experimental conditions were used except that no protease was added and the cells were directly lysed. The peptides are eluted from the streptavidin-coated magnetic beads with HCCA matrix and spotted on the MALDI plate. Mass spectrometry is not a quantitative method *per se*, therefore an internal standard is added to the lysis solution. This standard peptide has the same sequence as the one to quantify except that it bears a tag composed of four deuterated glycine residues instead of four glycine residues. This allows the quantification of internalised and membrane-bound peptide. The samples were analysed by MALDI-TOF MS (positive ion reflector mode) on a Voyager DEPRO mass spectrometer (Applied Biosystems).

2.5. Antimicrobial activity

Gram-positive eubacteria (*S. aureus* RN4220) and Gram-negative eubacteria (*E. coli* ML35p) were grown in Luria–Bertani (LB) broth at 37 °C. The minimal inhibitory concentrations (MICs) of peptides were determined in 96-well microtitration plates by growing the bacteria in the presence of 2-fold serial dilutions of peptide ranging from 0.2 to 100 μM. Aliquots (10 μL) of each serial dilution were incubated for 16 h at 37 °C with 90 μL of a suspension of a midlogarithmic phase culture of bacteria at a starting absorbance $A_{630} = 0.05$ in Poor Broth nutrient medium (1% Casein peptone and 1% NaCl, w/v). Growth inhibition was assayed by measuring the absorbance at 630 nm. The MIC was defined as the lowest concentration of peptide that inhibited the growth of 99% or more of the bacteria. 0% inhibition corresponded to untreated bacteria (negative control), 100% inhibition corresponded to bacteria treated with 0.2% formaldehyde (positive control).

If a MIC could be determined, then 2 μL of the bacterial suspension that was incubated with a concentration of peptide corresponding to the MIC and the concentration just above the MIC were taken, plated out on solid culture medium containing 1% noble agar and incubated overnight at 37 °C. If the bacteria were able to re-grow, the peptide was described as bacteriostatic, otherwise the peptide was described as bacteriolytic.