Biochimica et Biophysica Acta 1828 (2013) 1457–1470

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

The enhanced membrane interaction and perturbation of a cell penetrating peptide in the presence of anionic lipids: Toward an understanding of its selectivity for cancer cells

Marie-Lise Jobin [a], Pierre Bonnafous [a], Hamza Temsamani [a], François Dole [b], Axelle Grélard [a], Erick J. Dufourc [a], Isabel D. Alves [a,*]

[a] CBMN-UMR 5248 CNRS, Université de Bordeaux, IPB, Allée Geoffroy St. Hilaire, 33600 Pessac, France
[b] CRPP, Centre de Recherche Paul Pascal, 115 Avenue Schweitzer, 33600 Pessac, France

ARTICLE INFO

Article history:
Received 10 December 2012
Received in revised form 29 January 2013
Accepted 15 February 2013
Available online 24 February 2013

Keywords:
Cell penetrating peptide
Peptide/lipid interaction
Lipid model system
Anticancer activity

ABSTRACT

Cell penetrating peptides (CPPs) are usually short, highly cationic peptides that are capable of crossing the cell membrane and transport cargos of varied size and nature in cells by energy- and receptor-independent mechanisms. An additional potential is the newly discovered anti-tumor activity of certain CPPs, including RW16 (RRWRRWWRRWWRRWRR) which is derived from penetratin and is investigated here. The use of CPPs in therapeutics, diagnosis and potential application as anti-tumor agents increases the necessity of understanding their mode of action, a subject yet not totally understood. With this in mind, the membrane interaction and perturbation mechanisms of RW16 with both zwitterionic and anionic lipid model systems (used as representative models of healthy vs tumor cells) were investigated using a large panoply of biophysical techniques. It was shown that RW16 autoassociates and that its oligomerization state highly influences its membrane interaction. Overall a stronger association and perturbation of anionic membranes was observed, especially in the presence of oligomeric peptide, when compared to zwitterionic ones. This might explain, at least in part, the anti-tumor activity and so the selective interaction with cancer cells whose membranes have been shown to be especially anionic. Hydrophobic contacts between the peptide and lipids were also shown to play an important role in the interaction. That probably results from the tryptophan insertion into the fatty acid lipid area following a peptide flip after the first electrostatic recognition. A model is presented that reflects the ensemble of results.

1. Introduction

Cell-penetrating peptides are short peptides that have the ability to translocate into cells in an energy- and receptor-independent manner. CPPs are proven to be vehicles for the intracellular delivery of macromolecules such as oligonucleotides, peptides and proteins, nano-particles and liposomes [1]. Therefore these molecules present a great potential in therapeutics and diagnosis. Indeed, the number of applications using CPPs is quickly increasing, with so far more than 300 studies from in vitro to in vivo using CPP-based strategies [2–6]. Since their discovery in the 1990s an important number of research groups have focused in the understanding of their mode of action with the final attempt of improving their internalization and specificity. It is now mostly agreed that their uptake occurs through both endocytotic and non-endocytotic pathways but the molecular requirements for an efficient internalization are not fully understood [7]. It appears that their uptake ability depends on their amino acid sequence and spacing [8]. Despite their uptake being endocytotic or not, the first barrier that these peptides encounter is the plasma membrane which prevents direct translocation of macromolecules.

Herein, we have focused in the understanding of the membrane interaction and perturbation by a CPP derived from penetratin SAR (structure–activity relationship) studies. The peptide is RW16 (RRWRRWWRRWWRRWRR), a 16 residue amphipathic peptide shown to be a good CPP [9]. Moreover this peptide was reported to possess anti-tumor activity, namely, to decrease the mobility and proliferation of cancer cells (EF cells), this without being cytotoxic (up to 20 μM; in both NIH 3T3 and EF cells) [10]. The mechanism of action of this peptide is not well understood. Lamaziere et al. have shown that RW16 induced GUVs (Giant Unilamellar Vesicles) adhesion and vesicle aggregation for certain lipid compositions. Aside from these liposome modifications

Abbreviations: AMP, Anti-microbial peptide; APA, Amino pentanoic acid; CD, Circular dichroism; CHO, Chinese hamster ovary; CPP, Cell-penetrating peptide; DLS, Dynamic Light Scattering; DMPC, Dimyristoyl phosphatidylcholine; DMPG, Dimyristoyl phosphatidylglycerol; DOPC, Dioleoyl phosphatidylcholine; DOPE, Dioleoyl phosphatidylethanolamine; DOPG, Dioleoyl phosphatidylglycerol; DOPS, Dioleoyl phosphatidylserine; DPPC, Dipalmitoyl phosphatidylcholine; DSC, Differential Scanning Calorimetry; GUV, Giant Unilamellar Vesicle; ITC, Isothermal Titration Calorimetry; LUV, Large unilamellar vesicle; MIC, Minimal inhibitory concentration; MLV, Multilamellar vesicle; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PG, Phosphatidylglycerol; PS, Phosphatidylserine; SAR, Structure–activity relationship

* Corresponding author. Tel.: +33 5 40 00 68 49.
E-mail address: i.alves@cbmn.u-bordeaux.fr (I.D. Alves).

0005-2736/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamem.2013.02.008

they have found that RW16 induced calcein leakage without being lethal to the cells [11]. It is interesting to notice that recent studies have shown that some CPPs preferentially accumulate in cancer cells [12–14]. This selectivity toward cancer vs healthy cells might be directly correlated with the richness in anionic components in their cell membranes. Indeed several studies indicated that cancer cell membranes overexpress certain proteoglycans such as glypicans and syndecans which are implicated in several aspects of tumorigenesis such as cell adhesion, growth and motility [15–18]. Additionally, when cells become apoptotic their transmembrane asymmetry is strongly perturbed with an increase in the levels of phosphatidylserine (PS) in the outer membrane leaflet, of up to 9% [19]. These components in the cancer cell membranes render them additionally anionic relative to healthy cell membranes which can be favorable for the interaction with cationic molecules such as CPPs.

In the present study the structural characterization and interaction mechanism of RW16 with different membrane models (composed of zwitterionic and/or anionic lipids) was performed. The zwitterionic lipid system was employed as a mimic of the outer leaflet of a healthy eukaryotic cell and the anionic system to model the enhanced anionic character of apoptotic cells. The interaction, affinity, perturbation of the lipid model systems upon RW16 interaction as well as the structural changes occurring in the peptide upon this interaction was investigated. Studies were performed using several biophysical techniques and lipid model systems to mimic healthy and cancer cell membranes. CPPs and antimicrobial peptides share common features namely their cationic nature [20] and in the particular case of RW16 the amphipathicity. Additionally several cell penetrating peptides have been reported to possess antimicrobial activity, including penetratin and its analogues [21–25]. Therefore the antimicrobial activities of RW16 were investigated against *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae*. Furthermore efficient antimicrobial peptides have also been shown to possess anticancer activity [26–28].

The analysis of the results obtained was complicated by the fact that RW16 alone autoassociates at increasing peptide concentrations, a property arising from its amphipathic character. Overall the studies indicate an enhanced interaction and perturbation of RW16 with anionic vs zwitterionic lipids which is specially marked when the peptide is in its oligomerized form. While this would indicate that electrostatic interactions are important in the P/L interaction, which is rather expected, the studies show that important hydrophobic interactions take place. Such interactions can be explained by a peptide flip, following the initial electrostatic contacts, probably around the Arg with the insertion of Trp residues in the fatty acid chain core. A model is proposed to explain the markedly distinct behavior of RW16 in interaction with anionic vs zwitterionic lipids that also takes into account the peptide oligomerization state. This preferential interaction and perturbation in membranes enriched in anionic components may explain the reported anti-tumor activity of this peptide and CPPs in general.

2. Materials & methods

2.1. Materials

All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). The calcein was obtained from Sigma. Biot(O_2)-Apa-RW16-NH_2 (RRWRRWWRRWWRRWRR) synthesis and purification was performed using Boc solid phase strategy. The bacterial strains were kindly provided by the laboratory of Pierre Nicolas.

2.2. Antimicrobial activity

Gram-positive eubacteria (*S. aureus*) and Gram-negative eubacteria (*E. coli 363* and *K. pneumoniae*) were cultured as described previously [29]. 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. Aliquots (10 μL) of each serial dilution were incubated for 16 h at 37 °C with 100 μL of a suspension of a midlogarithmic phase culture of bacteria, at a starting absorbance $A_{630} = 0.01$ in Poor-Broth nutrient medium (1% bactotryptone and 0.5% NaCl, w/v) (peptide final concentrations ranged from 0.1 to 100 μM). Inhibition of growth was assayed by measuring the absorbance at 630 nm. The MIC was defined as the lowest concentration of peptide that inhibited the growth of about 99% of the cells. Bacteria that was incubated with the peptide corresponding to the MIC was plated out on solid culture medium containing 1% noble agar to distinguish between lytic and non-lytic effects. The peptide was considered to be lytic if after overnight incubation with the peptide (at the MIC concentration) the bacteria development was inhibited and non-lytic when the bacteria was able to re-grow upon peptide incubation. All assays were performed in triplicate plus positive controls without the peptide and negative controls with 0.7% formaldehyde.

2.3. Preparation of liposomes

All liposomes were prepared by initially dissolving the appropriate amount of phospholipids, to obtain the desired concentration, in chloroform and methanol (2/1 v/v) to ensure the complete mixing of the components. A lipid film was then formed by removing the solvent using a stream of N_2 (g) followed by 3 h vacuum. To form MLVs, the dried lipids were dispersed in buffer (either Tris 10 mM, 150 mM NaCl, 2 mM EDTA pH 7.4 or phosphate 10 mM, NaCl 150 mM, EDTA 2 mM pH 7.4 buffer depending on the technique used) and thorough vortexed. To form LUVs, the MLV dispersion was run through five freeze/thawing cycles and passed through a mini-extruder equipped with two stacked 0.1 μm polycarbonate filters (Avanti, Alabaster, AL).

2.4. Turbidity

Turbidity of LUVs was followed by measuring the absorbance at 436 nm upon addition of increasing peptide concentration (P/L ratios of 1/100, 1/50, 1/25, 1/10 were used). The measurements were acquired on a Jasco V-630 spectrometer at room temperature (~22 °C).

2.5. Dynamic Light Scattering (DLS)

DLS measurements were performed using an ALV laser goniometer, equipped with a 22 mW HeNe linearly polarized laser operating at 632.8 nm and an ALV-5000/EPP multiple τ digital correlator with 125 ns initial sampling time. Measurements were performed at a scattering angle of 90° and the intensity correlation functions were analyzed using the software provided by the company, to give the hydrodynamic radius (Rh) of the scattering particles. All measurements were performed at room temperature in phosphate buffer. To get an insight into the influence of RW16 on LUVs integrity, 100 μL of a 1 mg/mL LUV solution was analyzed by DLS, followed by addition of a small volume of RW16 (1 mM) to the LUV suspension to the desired P/L ratio (1/100, 1/50, 1/25, 1/10) and particle size again analyzed, immediately. The same measurements were performed in the absence of LUVs to determine whether the peptide auto-aggregates.

2.6. Tryptophan fluorescence

Small volumes of LUV suspensions (1 mM) were successively added to the peptide solution (0.5 μM) to obtain different P/L ratios: 1/10, 1/25, 1/50 and 1/100. After 2 min incubation, fluorescence spectra were recorded using an excitation wavelength (λ_{exc}) of 278 nm (5 nm bandwidth) and emission wavelength (λ_{em}) in the interval from 300 nm to 500 nm (10 nm bandwidth). The scan speed was 200 nm/min and the spectra were averaged over 10 accumulations. Fluorescence measurements were made on a Perkin Elmer LS55 spectrometer (Buckinghamshire, UK). The blue shift was plotted against the lipid concentration and fitted using a hyperbolic function being