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SAP(E) – A cell-penetrating polyproline helix at lipid interfaces

Johannes Franz [a], Marco Lelle [a], Kalina Peneva [a,b], Mischa Bonn [a], Tobias Weidner [a,c,*]

[a] Max Planck Institute for Polymer Research, Molecular Spectroscopy Department, 55128 Mainz, Germany
[b] Institute of Organic Chemistry and Macromolecular Chemistry, Friedrich Schiller University, 07743 Jena, Germany
[c] Department of Chemistry, Aarhus University, Århus C DK-8000, Denmark

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ABSTRACT

Cell-penetrating peptides (CPPs) are short membrane-permeating amino acid sequences that can be used to deliver cargoes, e.g. drugs, into cells. The mechanism for CPP internalization is still subject of ongoing research. An interesting family of CPPs is the sweet arrow peptides – SAP(E) – which are known to adopt a polyproline II helical secondary structure. SAP(E) peptides stand out among CPPs because they carry a net negative charge while most CPPs are positively charged, the latter being conducive to electrostatic interaction with generally negatively charged membranes. For SAP(E)s, an internalization mechanism has been proposed, based on polypeptide aggregation on the cell surface, followed by an endocytic uptake. However, this process has not yet been observed directly – since peptide–membrane interactions are inherently difficult to monitor on a molecular scale. Here, we use sum frequency generation (SFG) vibrational spectroscopy to investigate molecular interactions of SAP(E) with differently charged model membranes, in both mono- and bi-layer configurations. The data suggest that the initial binding mechanism is accompanied by structural changes of the peptide. Also, the peptide-model membrane interaction depends on the charge of the lipid headgroup with phosphocholine being a favorable binding site. Moreover, while direct penetration has also been observed for some CPPs, the spectroscopy reveals that for SAP(E), its interaction with model membranes remains limited to the headgroup region, and insertion into the hydrophobic core of the lipid layer does not occur.

1. Introduction

Most drugs are designed to bind to either the extracellular binding domain of receptors to subsequently trigger or inhibit a signaling cascade in the cell or ion channels to regulate ion transport over the cell membrane. However, direct delivery of pharmaceutical agents to or into individual cell organelles can result in an increased therapeutic outcome and is even required for new, specific therapeutic approaches [1], e.g. deblocking of apoptosis in multidrug resistant cancer cells by directly targeting the mitochondrial or lysosomal apoptotic pathways [2–3]. From an energetic point of view, a molecule has to be in an intermediate range of lipophilicity to cross cell membranes. For other molecules that do not meet these requirements, a transportation mechanism is necessary: A very promising approach is covalent attachment of cargo molecules to a carrier that is non-toxic, non-immunogenic, biodegradable and recognized by the cell membrane structures involved in endocytic uptake. One family among these carriers are the so-called cell-penetrating peptides (CPPs), membrane-permeable, short amino acid sequences that can be used to deliver cargoes, e.g. drugs, into cells without damaging the cell membrane [4–8]. Today many different types of CPPs are known with a remarkable variety in charge, polarity and structure [9–10]. Their cell internalization mechanisms can be quite different and are still subject of ongoing research: Although "active" direct translocation of CPPs has also been described, most studies emphasize the relevance of endocytosis [11–18].

A particularly interesting CPP is the so-called sweet arrow peptide [SAP, sequence $(VRLPPP)_3$] that is derived from the *N*-terminus of γ-zein [sequence $VHL(PPP)_8$], a storage protein of maize, responsible for targeting the endoplasmatic reticulum [19–21]. Due to its large number of proline residues, the peptide adopts a highly flexible polyproline II helical structure (Scheme 1A), which has the ability to self-aggregate and also has been recognized as a major conformational element of proline-rich binding motifs and natively unfolded proteins [22–25].

In this study, we used SAP(E) [sequence *Ac*-CGGW$(VELPPP)_3$-NH_2, Scheme 1B], a chemically modified version of SAP, for which all arginine residues have been replaced by glutamic acid residues, rendering the peptide negatively charged [26]. Moreover, four amino acids have been added to the *N*-terminus of the SAP(E) primary structure (sequence *Ac*-CGGW) functionalizing the *N*-terminus as a binding domain that can be utilized for the immobilization of SAP(E) to surfaces or the attachment to biologically active substances [27]. Interestingly, it has been shown that the reversed net charge of SAP and SAP(E) does not affect the cellular uptake rate [26]. This is surprising as the cell membrane

* Corresponding author.
E-mail address: weidner@mpip-mainz.mpg.de (T. Weidner).

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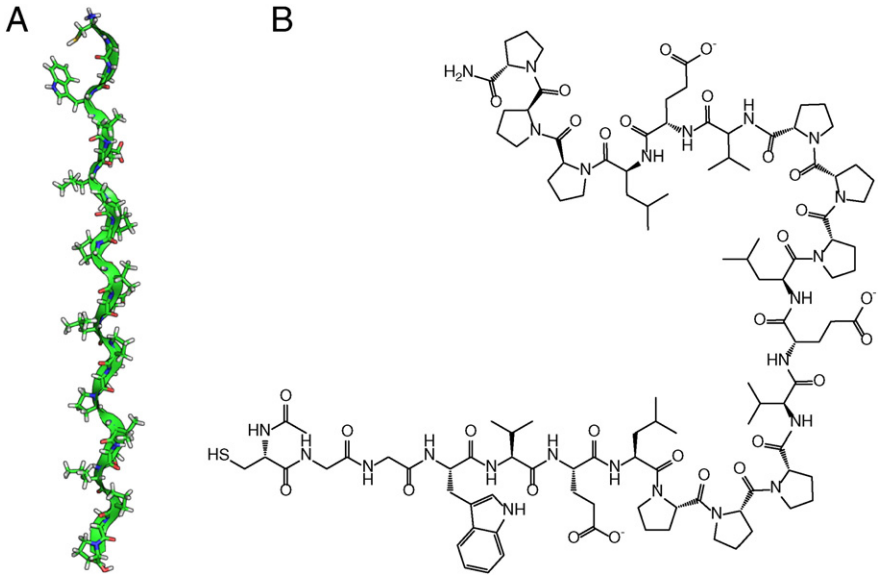

Scheme 1. The cell-penetrating peptide SAP(E). (A) Cartoon representation of the polyproline II helix and (B) structural formula of SAP(E).

itself is also negatively charged and leads to the assumption that in contrast to other reported CPPs, the electrostatic interaction between CPP and the glycocalyx of the cell membrane is not crucial for the uptake mechanism. Based on colocalization studies, an internalization mechanism of SAP and SAP(E) has been proposed involving aggregation of multiple peptides on the cell surface followed by an endocytic uptake mediated by caveolae [28]. These protein-coated invaginations in the cell membrane are capable of trapping membrane-bound but yet mobile molecules [29–30]. On model membranes and in the absence of presumably uptake-relevant caveolae-structures, the peptide should only sit on top of the model membrane without showing any insertion into the hydrophobic core of the lipid layer. However, the interaction of SAP(E) with membranes has not yet been observed directly – likely because of the intrinsic difficulty of monitoring peptide interaction with interfaces. We have recently used a variety of surface-science techniques to localize SAP(E) on model membranes in vacuum [31]. Here, we extend our studies to physiologically more relevant conditions: we investigate the cell-penetrating peptide SAP(E) in four distinct systems: in bulk, at the air/water interface, and at two model membrane interfaces: lipid mono- and lipid bilayer systems. Sum frequency generation (SFG) vibrational spectroscopy, a surface-sensitive technique that is inherently well-suited to study protein-membrane interactions, was used to glean information about structural transitions, binding affinity and model membrane perturbation of SAP(E) in situ.

2. Materials & methods

2.1. Chemicals

All solvents, chemicals, and reagents were bought from commercial sources and used without further purification. SAP(E) (*Ac*-CGGWVELPPPVELPPPVELPPP-NH_2, purity >95%) was purchased from Genosphere Biotechnologies (Paris, France). All lipids used in this study [1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1, 2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (DPPG), 1, 2-dipalmitoyl-3-trimethylammonium-propane (DPTAP), 1,2-dipalmitoyl-d62-*sn*-glycero-3-phosphocholine (d62-DPPC) and 1,2-dipalmitoyl-d62-*sn*-glycero-3-phosphocholine-1,1,2,2-d4-*N*,*N*,*N*-trimethyl-d9 (d75-DPPC)] were purchased from Avanti Polar Lipids (Alabama, USA).

2.2. Lipid monolayers

Lipid monolayers were prepared in a homemade trough with a volume of 20 mL. Surface pressure was monitored with a Kibron DeltaPi tensiometer (Helsinki, Finland). Lipids were dissolved in chloroform and spread on an aqueous subphase [phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M KCl and 0.137 M NaCl, pH 7.4) tablets (Sigma Aldrich, Munich, Germany) dissolved in Millipore water or deuterium oxide (99.8 at% D, Carl Roth, Karlsruhe, Germany), respectively]. The initial surface pressure was set to 20 mN/m. SAP(E) dissolved in PBS buffer was injected into the subphase to reach a final concentration of 0.2 mg/mL.

2.3. Lipid bilayers

Equilateral CaF_2-prisms (12.7 × 12.7 mm, Eksma Optics, Vilnius, Lithuania) were partially coated with chromium (1.5 nm) and gold (40 nm) in a vacuum coater (Edwards Auto 306, Crawley, England). Lipid bilayers were prepared on the prisms with a Langmuir-Blodgett/Langmuir-Schaefer deposition. The first layer was prepared at 34 mN/m with a Langmuir film balance (KSV NIMA 5000, Biolin Scientific, Stockholm, Sweden). For the second layer, a lipid monolayer was spread on an aqueous subphase in a homemade trough monitored with a Kibron DeltaPi tensiometer (Helsinki, Finland). At 34 mN/m the dipping process was initiated to form the second leaflet of the bilayer. The bottom of the prism carrying the bilayer was connected to a flow cell. A 0.2 mg/mL solution of SAP(E) in PBS buffer was injected into the flow cell.

2.4. Efflux

DPPC dissolved in chloroform was dried down in a nitrogen flow to prepare a lipid film and rehydrated in a 50 mM calcein (Sigma Aldrich, Munich, Germany) solution. Vesicles were prepared in an extruder (Avanti Polar Lipids, Alabama, USA) equipped with 50 nm filters. Calcein filled vesicles were separated from the surrounding calcein solution by size exclusion chromatography in a PD-10 column. Fluorescence emission intensity was measured in a Hitachi F4500 fluorimeter (Chiyoda, Japan) at 515 nm after excitation at 495 nm at room temperature. The integrity of the vesicles was proven by injection of the surfactant Triton X-100 to destroy the liposomal structures on purpose. The thereby reached fluorescence intensity was then normalized. In a second experiment,