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Molecular characterization of the interaction of crotamine-derived nucleolar targeting peptides with lipid membranes

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ABSTRACT

A novel class of cell-penetrating, nucleolar-targeting peptides (NrTPs), was recently developed from the rattlesnake venom toxin crotamine. Based on the intrinsic fluorescence of tyrosine or tryptophan residues, the partition of NrTPs and crotamine to membranes with variable lipid compositions was studied. Partition coefficient values (in the 10^2–10^5 range) followed essentially the compositional trend POPC:POPG ≤ POPG < POPC ≤ POPC:cholesterol. Leakage assays showed that NrTPs induce minimal lipid vesicle disruption. Fluorescence quenching of NrTPs, either by acrylamide or lipophilic probes, revealed that NrTPs are buried in the lipid bilayer only for negatively-charged membranes. Adoption of partial secondary structure by the NrTPs upon interaction with POPC and POPG vesicles was demonstrated by circular dichroism. Translocation studies were conducted using a novel methodology, based on the confocal microscopy imaging of giant multilamellar vesicles or giant multivesicular liposomes. With this new procedure, which can now be used to evaluate the membrane translocation ability of other molecules, it was demonstrated that NrTPs are able to cross lipid membranes even in the absence of a receptor or transmembrane gradient. Altogether, these results indicate that NrTPs interact with lipid bilayers and can penetrate cells *via* different entry mechanisms, reinforcing the applicability of this class of peptide as therapeutic tools for the delivery of molecular cargoes.

1. Introduction

Some specific cationic peptides and proteins, usually rich in lysine and arginine residues and with the ability to translocate cell membranes, have found use as intracellular delivery agents. Due to their translocating properties, they were named cell-penetrating peptides (CPPs) more than two decades ago [1–4]. CPPs can be covalently conjugated or complexed with a variety of payloads, such as nucleic acids, proteins, nanoparticles or quantum dots [5–7], enabling their use in a broad range of applications, including transfection, siRNA technology, organelle imaging or delivery of low-permeability drugs [8–10]. The most studied CPPs in terms of cell translocation and effectiveness as biomedical agents are the HIV-1 transcriptional activator (Tat) [1], the antennapedia homeodomain from *Drosophila* (Antp) [11], and the VP22 protein from the Herpes simplex virus [12]. More recently, Kerkis et al. [13] reported the cell-penetrating ability of crotamine, a 42-amino acid cationic defensin-like polypeptide from the venom of South American rattlesnake (*Crotalus durissus terrificus*), and described, among other properties, its uptake by proliferating active human and murine embryonic stem cells *in vitro* and mouse cells *in vivo*. The NMR solution structure of crotamine [14] revealed as main features a short N-terminal α-helix (residues 1–7) and two anti-parallel β-sheets (residues 9–13 and 34–38), giving rise to an αββ fold stabilized by three intramolecular disulfide bonds. This type of fold was previously identified on other membrane-active peptides, such as the human antimicrobial peptide β-defensin 2 [15], platypus defensin-like peptide [16], scorpion α-toxins [17] and anemone anthopleurin-B [18]. Based on such structural data, a molecular dissection of crotamine allowed to define a minimal CPP pharmacophore [19] involving the splicing of the 1–9 and 38–42 segments (Fig. 1) and giving rise to a novel class of CPPs whose unexpected localization led to their naming of nucleolar targeting peptides (NrTPs). NrTPs retain the cell-penetrating peptide property of crotamine

Abbreviations: Ahx, 6-aminohexanoic acid; AMP, antimicrobial peptide; CD, circular dichroism; CF, 5,(6)-carboxyfluorescein; CPPs, cell penetrating peptides; DLS, dynamic light scattering; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; Fmoc, 9-fluorenylmethoxycarbonyl; GUV, giant unilamellar vesicles; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, High-performance liquid chromatography; LUV, large unilamellar vesicles; NBD, nitro-2-1,3-benzoxadiazol-4-yl; NMR, nuclear magnetic resonance; NrTPs, nucleolar-targeting peptides; 5-NS, 5-doxyl-stearic acid; 16-NS, 16-doxyl-stearic acid; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(19-*sn*-glycerol); RhB, rhodamine B

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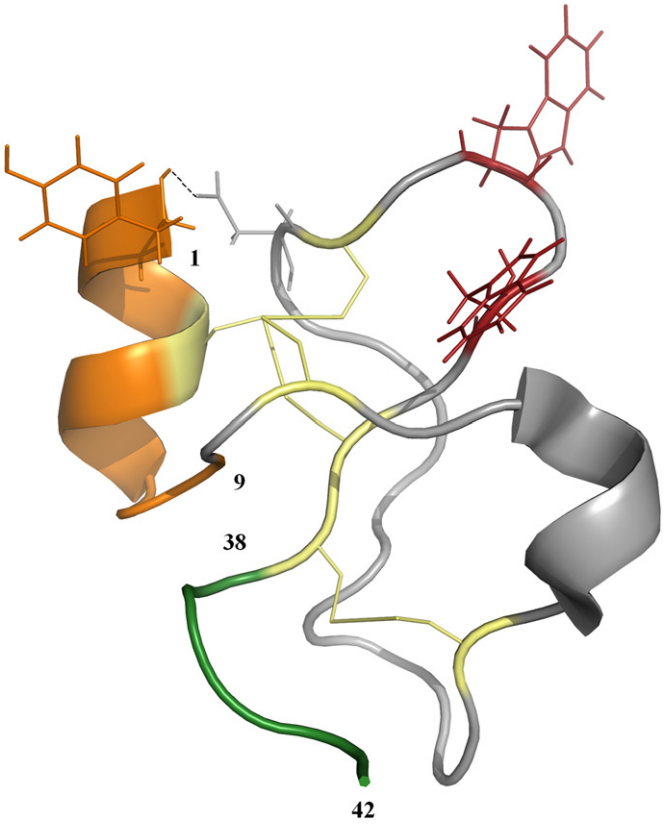

Fig. 1. Crotamine structure (PDB ID: 1Z99), showing the spliced regions, N-terminal residues 1–9 (orange) and C-terminal residues 38–42 (green), disulfide bonds (yellow), Trp_{32} and Trp_{34} (red), and H-bond between Tyr_1 and Asp_{29} (- - - -).

and present several improvements, such as reduced toxicity, preferential nucleolar localization and reduced synthesis cost [18,19]. We have recently reported their ability to deliver large cargoes (e.g., the 465 kDa β-galactosidase tetramer) into mammalian cells [20].

To gain insight into the biophysical aspects of the interaction of NrTPs with lipid bilayers, we carried out a systematic study of the molecular determinants underlying the CPP behavior of NrTPs, particularly concerning the involvement of specific types of lipids. The extent of NrTPs and crotamine partition into model membranes (including zwitterionic and anionic lipids), as well as their ability to translocate lipid bilayers, have been evaluated. The presence of aromatic amino acid residues on NrTPs and crotamine allowed the use of fluorescence spectroscopy to assess peptide:membrane interaction. Fluorescence quenching by acrylamide and lipophilic quenchers informed about peptide location on the membrane. Vesicle leakage was studied by monitoring the release of the fluorescent probe 5,(6)-carboxyfluorescein (CF) induced by NrTPs or crotamine. On the translocation studies, the uptake of rhodamine B-labeled NrTPs by giant lipid vesicles was examined by confocal microscopy.

2. Materials and methods

2.1. Materials

1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-nitro-2-1, 3-benzoxadiazol-4-yl (DPPE-NBD), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(19-*sn*-glycerol) (POPG) were obtained from Avanti Polar Lipids (Alabaster, AL). 5,(6)-Carboxyfluorescein, cholesterol, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), NaCl and Triton X-100 were from Sigma (St. Louis, MO). Acrylamide, tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl), L-tryptophan, L-tyrosine, sucrose and glucose were from Merck (Darmstadt, Germany). 5-Doxyl-stearic acid (5-NS) and 16-doxyl-stearic acid (16-NS) were obtained from Aldrich (Milwaukee, WI). All reagents were used without further purification.

2.2. Peptides

The synthesis of NrTP1 (YKQCHKKGGKKGSG), NrTP2 (with a 6-aminohexanoic acid spacer between GG and KK) and NrTP5 (as NrTP1, but with D-amino acid residues) was described earlier [19]. Similar protocols were used for the synthesis of NrTP6 (Cys residue replaced by Ser, see Table 1), NrTP7 (all 5 Lys residues changed to Arg) and NrTP8 (Tyr replaced by Trp). Amino acid sequences, molar extinction coefficients, quantum yields and molecular masses are also given in Table 1. All peptides were prepared both with free and rhodamine B (RhB)-labeled N-termini, the latter versions to be used in translocation experiments.

Synthetic crotamine in the native folding pattern was prepared from a linear precursor made by Fmoc solid phase synthesis methods as described before for similar multiple disulfide peptides [21]. Oxidative folding of the HPLC-purified hexathiol precursor, dissolved at 5 μM concentration in 0.1 M ammonium acetate, pH 7.8, was done in the presence of 1 M guanidinium chloride and reduced (GSH) and oxidized (GSSG) glutathione (1:100:10 peptide:GSH:GSSG molar ratio), under Ar atmosphere, for 48 h. A single oxidation product was obtained with the expected molecular mass and HPLC retention time of a natural crotamine sample. Further details are given as Supplementary Information.

2.3. Characterization of crotamine and NrTPs in solution

UV–vis absorption and fluorescence (both excitation and emission) spectral characterization of NrTPs (25–97 μM) and crotamine (7.7 μM) were carried out in 10 mM HEPES with 150 mM NaCl, pH 7.4. This buffer was used in all experiments, except when mentioned otherwise. Quantum yields were calculated as described by Fery-Forgues et al. [22].

2.4. Lipid vesicle preparation

Large unilamellar vesicles (LUV) with ~100 nm diameter were obtained by extrusion of multilamellar vesicles, as described elsewhere [23]. LUV of pure POPC and POPG, POPC:POPG 70:30 (mol%) and POPC: cholesterol 67:33 (mol%) were used on the peptide–membrane interaction and translocation studies. Giant unilamellar vesicles (GUV) were prepared by the electroformation method, as described elsewhere [24,25]. POPC and DPPE-NBD stock solutions were mixed to a final lipid concentration of 1 mM (1% NBD-DPPE) and hydrated with ~1 mL of 200 mM sucrose. After GUV preparation, the vesicles suspension was kept at room temperature in the dark until use. Independent of the method used to prepare GUV (gentle hydration or electroformation), a small percentage of non-unilamellar vesicles is formed [26]. These vesicles can be either multilamellar (when the entrapped vesicles are concentric; hereafter named giant multilamellar vesicles, GMLV) or multivesicular (when the entrapped vesicles are non-concentric and significantly smaller; hereafter named giant multivesicular liposomes, GMVL). Translocation experiments were conducted using these two types of non-unilamellar giant vesicles.

2.5. Extent of partition to membranes

Membrane partition studies were carried out using an Edinburgh Instruments steady-state fluorescence spectrophotometer Xe 900 (Livingston, UK). NrTPs and crotamine solutions concentrations were checked by UV/Vis absorption using the extinction coefficients on Table 1, and