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Differential membrane perturbation caused by the cell penetrating peptide Tp10 depending on attached cargo

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Abstract **The membrane leakage caused by the cell penetrating peptide Tp10, a variant of transportan, was studied in large unilamellar vesicles with the entrapped fluorophore calcein. The vesicles were composed of zwitterionic 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine. A significant decrease in membrane leakage was found when the 55 kDa streptavidin protein was attached to Tp10. When a 5.4 kDa peptide nucleic acid molecule was attached, the membrane leakage was comparable to that caused by Tp10 alone. The results suggest that direct membrane effects may cause membrane translocation of Tp10 alone and of smaller complexes, whereas these effects do not contribute for larger cargoes.**

Keywords: Cell-penetrating peptides; Transportan 10; Cell membrane perturbation; Streptavidin; Peptide nucleic acid

1. Introduction

Cell-penetrating peptides (CPPs) have the ability to translocate through cell membranes with high efficiency [1–3]. When they are covalently linked to a larger cargo, such as polypeptides, oligonucleotides or proteins, they still retain their translocation properties [4]. These short, synthetic peptide based vectors are considered as biocompatible and economical candidates for delivery of hydrophilic drugs (especially in connection with gene therapy) [1,2,5,6]. Transportan is a chimeric CPP constructed from the (1–12) amino acid residues from the N-terminal part of the neuropeptide galanin linked with a lysine residue to the full length wasp venom mastoparan [7]. Transportan 10 (Tp10) was developed as an analog to transportan by deleting the first six N-terminal amino acids, in order to reduce toxicity [8]. Tp10 has the sequence AGYLLGKINLKALAALAKKIL [8]. Tp10 and transportan have remarkably good translocation capabilities and has successfully been used as delivery vectors for peptide nucleic acid (PNA) [9,10], peptides and proteins [11,12] but they have been shown to induce membrane leakage in model systems [13]. Previous studies have also shown that Tp10 is toxic to cancer cells (assayed as leakage of lactate dehydrogenase) and that the peptide induces hemolysis [14].

The CPPs are a variable group of peptides, and their mechanisms of action have been under intense debate. Only relatively recently [15] some consensus has appeared: Cargo delivery is mostly endocytosis-driven and very likely dependent on endosomal escape. Direct membrane interaction mechanisms seem to operate in parallel in some cases, as has been suggested for Tp10 [16]. Such interactions have been mainly associated with highly efficient CPPs, which however also display cytotoxic activities [14]. The modifying effects of different cargoes on the uptake mechanisms have also been reported [17,18].

Whereas it seems clear that cargoes affect the CPP uptake mechanisms in cells, less work has been done on how attachment of cargo may affect the membrane perturbations induced by CPPs. Of particular interest is then the group of highly efficient CPPs which are considered to be somewhat cytotoxic. The aim of the present work is to evaluate the membrane interactions of Tp10 carrying different biologically relevant cargoes. Tp10 and transportan are structurally similar to active antimicrobial peptides, since they contain the mastoparan sequence [19,20]. In this respect similar to the antimicrobial peptides magainin and buforin, Tp10 may also have more than one uptake mechanism, and the relative importance of these mechanisms may depend on the cargo [17,18].

We have compared the membrane perturbation effects of $Tp10_{bt}$ alone and in complex with a large and hydrophilic cargo such as streptavidin (55 kDa), or a smaller and hydrophobic cargo such as 18 bases long PNA (5.4 kDa). Streptavidin [21,22] was coupled to Tp10 via a biotin molecule attached to the Lys 7 of Tp10. The PNA was covalently attached to Tp10 by a disulfide bond. The membrane perturbation by the different constructs was studied by calcein leakage experiments in large unilamellar vesicles (LUVs) composed of phospholipids. Our hypothesis was that membrane perturbation (manifested as calcein leakage in a vesicle model system) should be strongly connected to CPP activity due to direct membrane interactions, as well as to the endosomal escape of efficient CPPs.

Phospholipid vesicles are artificial membrane systems which are suitable mimics of the properties of biological membranes. With phospholipid vesicles it is possible to manipulate the various parameters of the membrane system such as size or

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Abbreviations: CPP, cell-penetrating peptide; Tp10, transportan 10; PNA, peptide nucleic acid; LUVs, large unilamellar vesicles; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]; bt, biotin labeled

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charge, etc. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was used to create neutral vesicles, whereas POPC:-POPG mixtures were used in order to create vesicles with net negative surface charge. The membrane perturbing effect of $Tp10_{bt}$ and transportan on neutral vesicles was compared to the effect that they had on vesicles including 25% negatively charged phospholipids. We also compared the membrane leakage caused by transportan, Tp10 and mastoparan, respectively.

2. Materials and methods

2.1. Materials

Transportan was purchased from Neosystems (Strasbourg, France). Biotin labeled Tp10 was synthesized at the Department of Neurochemistry, Stockholm University. Mastoparan from the venom of wasp (zoological name) was obtained from NeoMPS (Strasbourg, France). The identity and purity were controlled by amino acid, mass spectrometric and HPLC analysis. 1-Palmitoyl-2-oleoyl-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (POPG) were purchased from Avanti Polar Lipids (Alabaster, Alabama), of the best quality, and were used without further purification. Calcein, a fluorescein derivative ($C_{30}H_{26}N_2O_{13}$, Mw = 622.5 g/mole), was purchased from Sigma-Aldrich (Stockholm, Sweden). Streptavidin was obtained from MP Biomedicals (Illkirch, France), and was used as purchased. The Tp10-PNA construct was prepared as previously described [23]. In brief, the PNA was attached with a disulphide bridge to the ε-amino group of Lys 7. The single stranded PNA used was an 18 bases long sequence (underlined), C- and N-terminally flanked with two lysines (K) on either side: (Cys)-KK**CCTCTTACCTCAGTTACA**KK (Mw = 5380.4 Da).

2.2. Determination of peptide concentrations

The peptide concentrations in the stock solutions were determined by light absorption on a Cary 4 spectrophotometer using cuvettes with a 1 cm light path. All the spectra were baseline corrected. A molar absorptivity of 5600 $M^{-1}\,cm^{-1}$ and 1280 $M^{-1}\,cm^{-1}$, at 280 nm, for tryptophan and tyrosine, respectively, were applied in the calculations. Mastoparan does not include any tryptophans or tyrosines and its concentration was therefore determined by amino acid analysis at Protein Analysis Center, Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm.

2.3. Determination of PNA concentration

PNA concentration in the stock solution was determined by light absorption on a Cary 4 spectrophotometer using cuvettes with 1 cm light path. The spectra were baseline corrected. A molar absorptivity of 164.3 $mM^{-1}\,cm^{-1}$ at 260 nm was used for this PNA.

2.4. Sample preparations

Large unilamellar vesicles were prepared by initially dissolving the phospholipids (POPC or POPC:POPG 3:1 mixtures, respectively) at the desired concentration in methanol, to ensure complete mixing of the components, and then removing the solvent by placing the sample under a stream of nitrogen or argon to create a thin lipid film. Further solvent was removed by vacuum drying for 3 h. The dried lipids were then dispersed in 50 mM potassium phosphate buffer (pH 7.0). The solution was vortexed to form large multilamellar vesicles (LMVs). The dispersion was run through a repeated freeze–thaw cycle 10 times, which resulted in decreased lamellarity, and then extruded through two polycarbonate filters (0.1 μm pore size) 20 times in an Avanti manual extruder. This process gave unilamellar vesicles with a size of 100 nm.

2.5. Fluorescence spectroscopy

Fluorescence was measured on a Perkin–Elmer LS 50B luminescence spectrometer with FLWINLAB software. All measurements were made in 4 ml plastic cuvettes (1 × 1 cm) at an ambient temperature of approximately 20 °C. The fluorescence intensity of calcein was measured at 512 nm (excited at 490 nm). Scans were recorded with 2.5 nm excitation and emission bandwidths and a scan speed of 800 nm/min.

2.6. Peptide-induced calcein release

LUVs with entrapped calcein were prepared by hydrating a lipid film of desired composition with 70 mM calcein present in the buffer (the final pH was adjusted to 7.0 by addition of NaOH from a 1 M stock solution). Calcein gives low fluorescence intensity at 70 mM because of self-quenching and the intensity increases considerably upon dilution. Free calcein was separated from the LUVs on a Sephadex-G25 column purchased from Amersham Biosciences (Uppsala, Sweden). Increasing concentration of peptides was added to LUVs composed of 100 μM phospholipids. After 5 min incubation, release of calcein from the LUVs was monitored by an increase in the fluorescence intensity at 512 nm.

The maximum fluorescence intensity corresponding to 100% leakage was determined by lysing the vesicles with 10% triton X-100. The % leakage was then calculated according to the following equation:

$$\%\text{Leakage} = 100\{(F - F_0)/(F_{max} - F_0)\}$$

where F_0 represents the fluorescence intensity of the intact vesicles, F and F_{max}, the intensity before and after the addition of the detergent, respectively.

3. Results

3.1. Peptide-induced calcein release from LUVs

The membrane perturbing effect of $Tp10_{bt}$ was investigated through calcein-release experiments in LUVs. Fig. 1 depicts the % leakage induced by $Tp10_{bt}$ with neutral POPC LUVs, after 5 min of incubation, at room temperature. The results show that $Tp10_{bt}$ causes membrane leakage at quite low concentrations.

3.2. Membrane perturbations with streptavidin as cargo

Fig. 1 also depicts the calcein leakage effect of $Tp10_{bt}$ when attached to streptavidin (55 kDa). Streptavidin can bind up to four molecules of $Tp10_{bt}$. Two and four times excess concentration of streptavidin to $Tp10_{bt}$ was used, respectively. For both cases a similar and drastic decrease in the membrane leakage could be seen compared to $Tp10_{bt}$ alone. Streptavidin was used in excess to ensure complete utilization of $Tp10_{bt}$ in complex formation. This complex was found much less membrane perturbing than $Tp10_{bt}$ alone. The leakage was reduced almost

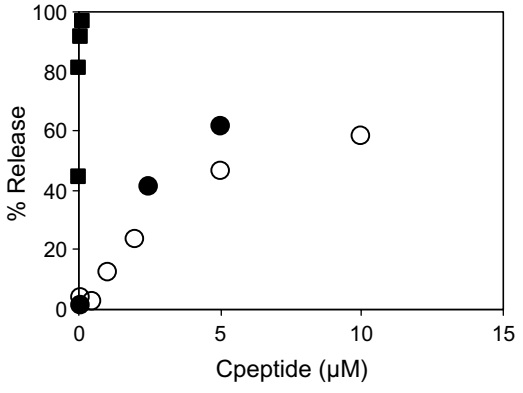

Fig. 1. Calcein leakage from LUVs induced by $Tp10_{bt}$ (black squares), $Tp10_{bt}$ pre-incubated with two times excess of streptavidin (black circles) and $Tp10_{bt}$ pre-incubated with four times excess of streptavidin (open circles) respectively. Increasing concentrations of the peptide and peptide + streptavidin solutions were added to LUVs composed of 100 μM POPC with 70 mM calcein entrapped. The % calcein release after 5 min at room temperature and pH 7.0 was plotted as a function of peptide concentration.