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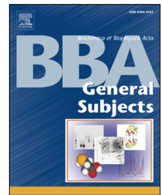

Photodamage of lipid bilayers by irradiation of a fluorescently labeled cell-penetrating peptide

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ABSTRACT

Background: Fluorescently labeled cell-penetrating peptides can translocate into cells by endocytosis and upon light irradiation, lyse the endocytic vesicles. This photo-inducible endosomolytic activity of Fl–CPPs can be used to efficiently deliver macromolecules such as proteins and nucleic acids and other small organic molecules into the cytosol of live cells. The requirement of a light trigger to induce photolysis provides a more spatial and temporal control to the intracellular delivery process.

Methods: In this report, we examine the molecular level mechanisms by which cell-penetrating peptides such as TAT when labeled with small organic fluorophore molecules acquire a photo-induced lytic activity using a simplified model of lipid vesicles.

Results: The peptide TAT labeled with 5(6)-carboxy-tetramethylrhodamine binds to negatively charged phospholipids, thereby bringing the fluorophore in close proximity to the membrane of liposomes. Upon light irradiation, the excited fluorophore produces reactive oxygen species at the lipid bilayer and oxidation of the membrane is achieved. In addition, the fluorescent peptide causes aggregation of photo-oxidized lipids, an activity that requires the presence of arginine residues in the peptide sequence.

Conclusions: These results suggest that the cell penetrating peptide plays a dual role. On one hand, TAT targets a conjugated fluorophore to membranes. On the other hand, TAT participates directly in the destabilization of photosensitized membranes. Peptide and fluorophore therefore appear to act in synergy to destroy membranes efficiently.

General significance: Understanding the mechanism behind Fl–CPP mediated membrane photodamage will help to design optimally photo-endosomolytic compounds.

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1. Introduction

Intracellular delivery of macromolecules such as proteins and nucleic acids is important for therapeutic applications and various cell biology applications. A strategy used for macromolecular delivery into live cells is photochemical internalization (PCI). PCI utilizes light-responsive and membrane disruptive chemical agents known as photosensitizers [1,2]. Typically, cells are first incubated with a photosensitizer and a cell-impermeable macromolecule of interest. During incubation, both the photosensitizer and macromolecule accumulate inside endosomes upon endocytic uptake by the cells. Following incubation, cells are irradiated with visible light and the photosensitizer causes disruption of the membrane of endosomes by generating reactive oxygen species (ROS) locally. The macromolecule present in the lumen of these organelles can subsequently escape into the cytosol of cells and exert a biological function [1]. One of the attractive aspects of this approach is that endosomal escape is induced only in the presence of a light-trigger. As a result, the delivery process can be temporally and spatially modulated and controlled [3]. More importantly, endosomal release, which is usually a limiting step in delivery strategies that utilize endocytosis as a route of cellular entry, is relatively efficient with the PCI technique.

Photosensitizers used for PCI have typically included phthalocyanine or porphyrin derivatives that localize within endocytic organelles [4]. When irradiated, these photosensitizers generate reactive oxygen species (ROS) which can then damage and lyse the membrane of endosomes [5]. Interestingly, fluorescently labeled cell-penetrating peptides (Fl-CPPs) have been shown to also possess a photo-endosomolytic activity. Upon endocytosis and irradiation, the cell-penetrating peptides (CPPs) TAT or R9 labeled with fluorescein, tetramethylrhodamine (TMR), Alexa fluors and Cy3 lyse endosomes and subsequently deliver proteins and nucleic acids to the cytosol of live cells successfully [3,6–8]. The porphyrin

Abbreviations: PCI, photochemical internalization; BMP, bis(monoacylglycero)-phosphate; CPP, cell-penetrating peptide; Fl–CPP, fluorophore/cell-penetrating peptide conjugate; LUV, large unilamellar vesicle; NBT, nitro blue tetrazolium; PBS, phosphate buffered saline; PC, phosphatidylcholine; PS, phosphatidylserine; PnA, cis-parinaric (9Z,11E,13E,15Z-octadecatetraenoic) acid; RB, rose bengal; RNO, p-nitrosodimethylaniline; ROS, reactive oxygen species; TAT, protein transduction domain of Human Immunodeficiency Virus 1 trans-activating transcriptional activator; TMPD, N,N,N′,N′-tetramethyl-1,4-phenylenediamine; TMR, tetramethylrhodamine

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photosensitizer–CPP conjugates have been applied previously for PCI and other photodynamic therapy related applications [9–12]. While photosensitizers and Fl–CPPs share a similar photo-endosomolytic activity, the mechanisms involved in membrane disruption appear to be quite different. Like PCI photosensitizers, the fluorophores used to label CPPs can generate ROS upon irradiation. In particular, singlet oxygen can be formed upon irradiation by transfer of energy from the fluorophore in triplet excited state to dissolved molecular oxygen [13]. However, while photosensitizers generally generate singlet oxygen in relatively high yields, the fluorophores used to label CPPs typically have very poor singlet oxygen quantum yields [14,15]. Studies with membrane models such as the plasma membrane of red blood cells (RBCs) indicate that generation of ROS is indeed involved in the photolytic activity of Fl–CPPs [7,16]. Yet, these studies also suggest that the CPP moiety acts in synergy with the singlet oxygen-generating fluorophores to disrupt membranes efficiently [16]. For instance, RBCs irradiated in the presence of TMR–TAT undergo a dramatic shrinkage while RBCs irradiated with conventional photosensitizers such as hematoporphyrin do not. Moreover, the photolysis of RBCs mediated by a photosensitizer is greatly enhanced if unlabeled TAT or R9 is added during or after irradiation. Because these effects are not observed in the dark, these results suggest that CPPs destabilize biological membranes after photo-oxidation of membranous components takes place. The molecular details of this phenomenon remain however unclear. Moreover, the membrane components involved in the photolysis induced by Fl–CPPs have not been identified.

In this report, we use large unilamellar vesicles (LUVs) as simplified membrane models to evaluate the implication of lipids in TMR–TAT mediated photolysis. In particular, we test the hypothesis that TMR–TAT promotes lipid oxidation by local generation of ROS. We also test whether membrane destabilization by the peptide contributes to photolysis. We demonstrate that TMR–TAT destroys liposomes upon light irradiation. First, TAT brings TMR in close proximity to lipid bilayers by binding to negatively charged lipids. Excitation of TMR then causes singlet oxygen and superoxide formation followed by lipid oxidation. Importantly, lipid oxidation is not sufficient to account for liposomes destruction. Instead, the arginine-rich CPP promotes the aggregation of photo-oxidized LUVs and accelerates their lysis. Our results therefore reveal a unique synergy between oxidized lipids and CPPs that leads to enhanced photolysis.

2. Material and methods

2.1. Materials used

All peptide synthesis reagents were obtained from Novabiochem (EMD/Merck, Darmstadt, Germany). The fluorophores 5(6)-carboxytetramethylrhodamine and 5(6)-carboxyeosin Y were purchased from Novabiochem and Marker Gene Technologies (Eugene, OR) respectively. Eosin Y, tetramethylrhodamine, Rose Bengal, *p*-nitrosodimethylaniline (RNO), sodium azide, α-tocopheryl acetate, imidazole, and salts for buffer preparation were received from Sigma-Aldrich (St. Louis, MO). For liposome preparation, 1-stearoyl-2-oleoyl-sn-glycero-3-phospho-choline (PC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC'), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (PS), bis-(mono-oleoylglycero)-phosphate (BMP) and cholesterol were bought from Avanti Polar Lipids (Alabaster, AL).

2.1.1. Peptide synthesis

The peptides TAT (GRKKRRQRRRG-NH_2), R9 (GRRRRRRRRR-NH_2) and K9 (KKKKKKKKK-NH_2) were prepared using Fmoc solid-phase chemistry on a 0.72 mmol scale using rink amide MBHA resin to obtain C-terminal amides. The amino acids Fmoc–Gly–OH, Fmoc–Arg(Pbf)–OH, Fmoc–Lys(Boc)–OH, Fmoc–Gln–OH, 5(6)-carboxy-tetramethylrhodamine and 5(6)-carboxyeosin Y were used to synthesize the required peptides. All reactions were performed at room temperature and with constant agitation using dry N_2 gas. The Fmoc on the peptide resin was first deprotected by addition of a 20% piperidine solution in DMF. The deprotection was performed twice for 5 min and 15 min respectively followed by DMF washes each time. Then, the amino acids were added on the resin using a coupling reaction. The coupling reactions were carried out using a mixture of the Fmoc amino acid (2.88 mmol), HBTU (1.06 g, 2.80 mmol) and DIEA (1.25 mL, 7.2 mmol) in DMF for 3 h. The resin was washed with DMF after each coupling step and the Fmoc-deprotected before each coupling reaction. After synthesis of the peptide on the resin corresponding to TAT, R9 or K9 sequence, 20% piperidine in DMF was added for 1×5 and 1×15 min to deprotect the Fmoc on the N terminal residue of the peptide while keeping the side-chain protecting groups on the amino acids intact. The fluorophores were then coupled onto the peptide by reacting the peptide with a mixture of 5(6)-carboxy-tetramethylrhodamine or 5(6)-carboxyeosin Y (2.88 mmol), HBTU (1.06 g, 2.80 mmol) and DIEA (1.25 mL, 7.2 mmol) in DMF overnight.

After assembly of the Fl–CPP on the solid support, the resin was treated with a solution of TFA containing 2.5% H_2O and 1% triisopropylsilane for 2 h in order to deprotect all the side chains on the peptide and cleave the Fl–CPP off the resin. The crude Fl–CPPs present in the TFA solution were then washed with cold anhydrous Et_2O to achieve peptide precipitates. The crude peptides were then dissolved in aqueous acetonitrile and lyophilized. Fl–CPPs were purified using semi-preparative HPLC and their purity was confirmed by mass spectrometry (MALDI-TOF) analysis. TAT expected mass: 1451.92 Da, observed mass: 1452.41 Da; R9 expected mass: 1478.96 Da, observed mass: 1479.52 Da; K9 expected mass: 1169.88 Da, observed mass: 1170.96 Da; TMR–TAT expected mass: 1865.07 Da, observed mass: 1866.1 Da; TMR-K9 expected mass: 1583.0 Da, observed mass: 1583.30 Da; and TMR-R9 expected mass: 1893.20 Da, observed mass: 1894.4 Da. The pure lyophilized peptides were dissolved in water to make 1 mM stock solutions that were diluted to desired working concentrations in PBS (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM; adjusted to pH 7.4) for experiments.

2.1.2. Preparation of liposomes

Lipids in chloroform were mixed in a glass vial at molar ratios of 7:3 PC:cholesterol for neutral liposomes and 4:3:3 PC:PS:cholesterol or 4:3:3 PC:BMP:cholesterol for negatively charged liposomes. Alternatively, neutral liposomes were prepared with a composition of 4:3:3 PC:PC':cholesterol, in order to generate neutral LUVs that contain the same amount of unsaturated lipids as their negatively charged counterparts. Lipid films were prepared by evaporating the solvent from the mixture using a flow of nitrogen gas, then removing trace solvent by freeze-drying. The films were hydrated with only PBS buffer or buffer solution of calcein (60 mM) by vigorous vortexing and then allowed swelling for 2 h at 10 °C under nitrogen to obtain multilamellar lipid vesicles. Liposomes were extruded through Nuclepore polycarbonate membranes (Whatman, Clifton, NJ) with pore sizes of 100 nm (21 passes; for fluorometric studies) or 200 nm (11 passes; for turbidimetry studies) using a Mini-Extruder device (Avanti Polar Lipids, Alabaster, AL). The respective size distributions of liposomes were on average 140 and 236 nm, as determined by dynamic light scattering using a Zeta Sizer device (Malvern instruments, Worcestershire, UK). The extruded large unilamellar vesicles (LUVs) were stored at 4 °C and used within two weeks of preparation.

When required for calcein-leakage experiments, calcein-loaded LUVs were separated from non-entrapped fluorophore by gel filtration on Sephadex G-50 (GE Healthcare, Pittsburgh, PA) column (2.5×14 cm). Additionally, for experiments performed with cis-parinaric acid (PnA), the latter was added in ethanol solution to the lipid mixtures listed above at 1% mol. to the total lipids. PnA-containing lipid mixtures were dried with a flow of nitrogen and freeze-dried. Lipids were then re-dissolved in a minimum amount of chloroform and dried again, in order to provide a homogeneous distribution of constituents. PnA-containing

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