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Cationic cell-penetrating peptides induce ceramide formation via acid sphingomyelinase: Implications for uptake

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ABSTRACT

Cationic cell-penetrating peptides (CPP) are receiving increasing attention as molecular transporters of membrane-impermeable molecules. Import of cationic CPP occurs both via endocytosis and — at higher peptide concentrations — in an endocytosis-independent manner via localized regions of the plasma membrane. At present, this endocytosis-independent import of cationic CPP is not well understood, but has been shown to be sensitive to various pharmacological inhibitors, suggesting a role of an unidentified enzymatic activity. Here, we demonstrate that the direct translocation of cationic CPP depends on a CPP-induced translocation of acid sphingomyelinase (ASMase) to the outer leaflet of the plasma membrane and ceramide formation. The involvement of ASMase in uptake was confirmed by a pharmacological inhibition of ASMase by imipramine and a subsequent rescue of uptake through external addition of sphingomyelinase, and by using ASMase-deficient cells. We also found that the threshold for direct CPP translocation can be lowered through addition of sphingomyelinase and that sphingomyelinase enhances the translocation of R9 coupled to low-molecular weight cargos, but not high-molecular weight cargos. In conclusion, we show that a previously poorly understood mechanism of cationic CPP import depends on the ASMase-dependent formation of ceramide on the outer leaflet of the plasma membrane. To our knowledge, this is the first illustration that a class of delivery vectors operates through the induction of an enzymatic activity that changes the lipid composition of the plasma membrane.

1. Introduction

Interest in cell-penetrating peptides (CPP) as tools to deliver membrane-impermeable therapeutic molecules continues to grow. This development is illustrated by the increasing efforts of the scientific community to elucidate the chemical, physical and biological principles underlying the activity of CPP and CPP-based delivery systems [1–4] and the steep rise in the number of therapeutic strategies that are being pursued with these systems [5,6]. It is becoming progressively clear that instead of a single mechanism that is valid for all CPP, multiple modes-of-action exist with respect to the route of internalization and intracellular trafficking, that depend on the cell line [7], the CPP [8], and the cargo [9,10]. Although endocytosis is now considered the major internalization route of cationic CPP [4,11–14], direct translocation for cationic CPP has also been observed, mainly at high [8,15–17], but also at low concentrations [18,19].

For arginine-rich CPP the potential for direct translocation is thought be related to the ability of the guanidinium moieties of arginines to form bidentate hydrogen bonds with membrane lipids [20] and is underscored by the ability of the arginine-rich TAT peptide to induce pores in artificial membranes, as was observed by small angle X-ray scattering [21]. Moreover, molecular-dynamics simulations indicate the potential of arginine-rich peptides to form transient pores in membranes in the presence of an electrochemical gradient and provide a mechanistic hypothesis for direct membrane translocation [22].

Nevertheless, biological details remain poorly understood and it is not clear to which degree in vitro models manage to reflect the complex molecular environment of a cell. At higher concentrations, direct cytoplasmic import of cationic CPP has been shown to depend on spatially confined nucleation zones. This rapid import is sensitive to the PKCδ inhibitor rottlerin and to chlorpromazine [8]. Available evidence indicates that the plasma membrane remains intact.

Abbreviations: ASMase, acid sphingomyelinase; BSA, bovine serum albumin; bSMase, bacterial sphingomyelinase; CPP, cell-penetrating peptide; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; HBS, HEPES-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hLF, human lactoferrin; HPMA, N-(2-hydroxypropyl) methyl acrylamide; HPLC, high-performance liquid chromatography; PI, propidium iodide; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; R9, nona-arginine; RT, room temperature; WT, wild-type.

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In a search for a common denominator of the observed pharmacological profile, we discovered that both molecules act as inhibitors of acid sphingomyelinase (ASMase) activity [23–25]. This finding provided us with a rationale for investigating a possible involvement of ASMase in the CPP uptake via direct translocation.

Acid sphingomyelinase has traditionally been known in relation to Niemann–Pick disease, a lysosomal storage disorder [26]. More recently, an important role for this enzyme in ceramide-mediated signal transduction pathways has emerged, linking its activity to a variety of common diseases, among which are cancer, cardiovascular diseases and diabetes [27]. A major function of this enzyme is the hydrolysis of sphingomyelin to ceramide and phosphorylcholine following a trigger-initiated translocation from lysosomes to the outer leaflet of the plasma membrane. The details of the translocation mechanism are unknown at present. Ceramide is a lipid with a well-established role in the induction of antiproliferative and apoptotic responses in a variety of cancer cells [28]. As a part of this role, ceramide greatly affects the structure and properties of cellular membranes. In the plasma membrane ceramide-enriched membrane domains modulate signaling [29,30]. In the mitochondrial outer membrane ceramide increases permeability [31]. Here, we provide evidence for a role of ceramide formation by ASMase at the plasma membrane in the rapid cytoplasmic import of cationic CPP. This finding gives rise to the concept that cationic CPP enhance their own uptake by initiating a positive feed-back loop that involves an enzymatic alteration of the lipid composition of the plasma membrane.

2. Materials and methods

2.1. Materials

C-terminally amidated peptides were purchased from EMC microcollections (Tübingen, Germany). Fluorophore-labeled peptides were synthesized with an N-terminal carboxyfluorescein-label. Purity was evaluated by high-performance liquid chromatography (HPLC) and identity confirmed by mass spectrometry. If required, peptides were further purified to a purity of >95% using reversed-phase HPLC. The CPP hLF was oxidized before use as described previously [32]. Bovine serum albumin (BSA), glucose, imipramine, rottlerin and bacterial sphingomyelinase (from Bacillus Cereus, specific activity was 100 units/mg based on the Lowry protein assay) were from Sigma-Aldrich (Zwijndrecht, the Netherlands). Alexa-647 labeled annexin-V was from Invitrogen (Eugene, U.S.A.) and phenylmethylsulfonyl fluoride (PMSF) was from Research Organics (Cleveland, U.S.A.). Standard chemicals were from Sigma-Aldrich and Merck (Darmstadt, Germany). HPMA-R9 conjugates were prepared as described previously [33].

2.2. Cell culture

HeLa cells were maintained in RPMI 1640 (PAN Biotech, Aidenbach, Germany) supplemented with 10% fetal calf serum (FCS; PAN Biotech) and incubated at 37 °C in a 5% CO_2-containing, humidified incubator. Cells were passaged every 2 to 3 days. Primary mouse fibroblasts were obtained from lung or orbital tissue explants from wild-type (WT) and *Asm*$^{-/-}$ mice [34]. Mice were raised for 8–10 weeks before they were sacrificed. Primary fibroblasts were cultured in 6-cm dishes in minimum essential medium supplemented with 10% FCS, penicillin and streptomycin, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, and 2 mM L-glutamine (all from GIBCO, Invitrogen), at 37 °C in a 10% CO_2-containing humidified incubator and passaged every week. For experiments, passages 2 and 3 were used. For experiments with imipramine using primary cells, primary mouse feet fibroblasts were kindly provided by F. Valsecchi (Radboud University Nijmegen Medical Centre).

2.3. Confocal laser scanning microscopy

Confocal laser scanning microscopy was performed on a TCS SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with an HCX PL APO 63×N.A. 1.2 water immersion lens. Cells were maintained at 37 °C on a temperature-controlled microscope stage. Images of ASMase-deficient cells were also taken with an HCX PL APO 63×N.A. 1.4 oil immersion lens (Leica Microsystems) at room temperature (RT), as indicated. For all microscopy images, one confocal slice is shown.

2.4. Peptide uptake

For confocal microscopy, HeLa cells or primary mouse fibroblasts were seeded in 8-well microscopy chambers (Nunc, Wiesbaden, Germany) and grown to 75% confluence. Cells were incubated with peptides in RPMI 1640 supplemented with 10% FCS for durations and concentrations as indicated for the individual experiments. Imipramine pre-treatment to reduce ASMase activity was conducted by incubating cells for 30 min with 30 μM imipramine in RPMI 1640 without FCS. 30 μM imipramine and 10% FCS were included during peptide incubations. In experiments with bacterial sphingomyelinase, imipramine was only present before, but not during the peptide incubations. Cells were washed twice after the incubation and living cells were analyzed immediately by confocal microscopy.

Modifications for uptake experiments by flow cytometry were the use of 24-well plates (Sarstedt, Numbrecht, Germany) and a trypsinization step, which was followed by centrifugation and resuspension in 200 μl HEPES-buffered saline (HBS; 10 mM HEPES, 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, pH 7.4). Cellular fluorescence of 10,000 cells, gated on the basis of forward and sideward scatter, was measured using a BD FACScan flow cytometer equipped with a 488 laser (BD Biosciences, Erembodegem, Belgium) and analyzed using Summit software (Fort Collins, USA).

2.5. Immunofluorescence

HeLa cells were seeded in 8-well microscopy chambers and grown to 75% confluence. Cells were incubated with 20 μM R9 for 20 min at 37 °C, washed twice with pre-warmed HBS containing 0.1% (w/v) BSA and 5 mM glucose (HBS+) supplemented with 0.025% (v/v) Tween-20 and fixed for 15 min at RT with 3% paraformaldehyde in HBS. Then, cells were washed twice with HBS+ supplemented with 0.025% (v/v) Tween-20 and blocked for 15 min at RT with the same buffer. Incubation with the primary antibody took place for 1 h at RT in 150 μl HBS containing 0.1% (w/v) BSA (HBS/BSA) per well. The rabbit polyclonal IgG anti-ASMase antibody sc11352 (dilution 1:50, Santa Cruz Biotechnology, Santa Cruz, U.S.A.) was used for ASMase detection. A rabbit polyclonal IgG against NFκB was used as an isotype control (Santa Cruz Biotechnology). Ceramide was detected with a 1:50 dilution in HBS/BSA of a monoclonal mouse anti-ceramide IgM antibody 15B4 (Alexis Biochemicals, Lausen, Switzerland). A b allotype, anti-KLH mouse IgM antibody (BD Pharmingen, San Diego, U.S.A.) was used as an isotype control for ceramide detection. After incubation with the primary antibody, cells were washed 2×5 min with HBS+ supplemented with 0.025% (v/v) Tween-20. Incubation with secondary antibodies was for 45 min in HBS/BSA, after which cells were washed twice. Confocal images were taken immediately. For detection of ASMase and isotype control, an Alexa-633-conjugated goat-anti-rabbit IgG (H+L) antibody (Invitrogen, 1:100 dilution) was used. For detection of ceramide and isotype control, an Alexa-633-conjugated goat-anti mouse IgG (H+L) antibody (Invitrogen, 1:100 dilution) was used.