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Peptide degradation is a critical determinant for cell-penetrating peptide uptake

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Abstract

Cell-penetrating peptide mediated uptake of labels appears to follow an equilibrium-like process. However, this assumption is only valid if the peptides are stabile. Hence, in this study we investigate intracellular and extracellular peptide degradation kinetics of two fluorescein labeled cell-penetrating peptides, namely MAP and penetratin, in Chinese hamster ovarian cells. The degradation and uptake kinetics were assessed by RP-HPLC equipped with a fluorescence detector. We show that MAP and penetratin are rapidly degraded both extracellularly and intracellularly giving rise to several degradation products. Kinetics indicates that intracellularly, the peptides exist in (at least) two distinct pools: one that is immediately degraded and one that is stabile. Moreover, the degradation could be decreased by treating the peptides with BSA and phenanthroline and the uptake was significantly reduced by cytochalasin B, chloroquine and energy depletion. The results indicate that the extracellular degradation determines the intracellular peptide concentration in this system and therefore the stability of cell-penetrating peptides needs to be evaluated.

Keywords: Cell-penetrating peptide; Protein transduction domains; Uptake; Degradation; Endocytosis inhibitor

1. Introduction

Cell-penetrating peptides (CPPs) or protein transduction domains (PTDs) are peptides that translocate across the plasma membrane of mammalian cells both in vitro [1,2] and in vivo [3,4]. CPPs are capable of delivering functional cargos into cells, such as oligonucleotides [5], peptide nucleic acids [6], plasmids [7] and liposomes [8]. The mechanism by which CPPs enter cells is not clear although many recent papers have indicated different forms of endocytosis.

A few studies have investigated the metabolic stability or the pattern of degradation of these peptides. In a paper by Elmquist and colleagues the enzymatic degradation of pVEC and its all-D analog was investigated in buffer containing physiological concentration of trypsin or carboxypeptidase A and B and the half-lives were found to be 10.5 and 44.6 min in respective buffer sample [9]. This was followed by another paper by Lindgren and colleagues who investigated transportan, TP10 and penetratin in contact with Caco-2 cells and found the stability of the peptides was in the order of transportan > TP10 > penetratin [10]. Moreover, Tréhin and colleagues investigated Tat, penetratin and several calcitonin analogs in MDCK, Calu-3 and TR146 cells and found that the levels of proteolytic activity varied highly among the cell lines tested [11].

Recently, we showed that the main mechanism by which CPPs, or rather the cargo attached to the CPPs, accumulate in the cellular interior is by the proteolytic processing of the CPP-cargo conjugate into membrane impermeable products [12]. Hence, it is of interest to characterize the degradation kinetics and products of CPPs in order to investigate how they relate to cargo-delivery efficiency. The understanding of the internalization and degradation kinetics of CPPs is important for the practical aspects of cargo delivery. In uptake experiments, with incubation time points up to 1 h or longer, the peptide needs to be stabile outside the membrane

Abbreviations: CPP, cell-penetrating peptide; PTD, protein transduction domain; fl-HPLC, fluorescence-HPLC; MAP, model amphipathic peptide; PBS, phosphate buffered saline; IC, intracellular; EC, extracellular; CHO, Chinese hamster ovarian

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Table 1
Names and sequences of the cell-penetrating peptides used in this work

Peptide	Sequence
MAP(KLAL) [16]	Fluo-KLALKLALKALKAALKLA-amide
Penetratin [19]	Fluo-RQIKIWFQNRRMKWKK-amide
Perforin 1–16 [15]	Fluo-PCHTAARSECKRSHKF-amide
Perforin 1–34 [15]	Fluo-PCHTAARSECKRSHKFVPGAWLAGEGVDVTSLRR-amide

and not degraded within that time, hence, evaluation of peptide stability is an important parameter. These studies are also essential in order to achieve a better understanding of the mechanism by which CPPs pass through membranes and enter cells.

In this study we investigate intracellular (IC) and extracellular (EC) peptide degradation products and kinetics of two structurally different CPPs, namely MAP and penetratin. The degradation products and kinetics of fluorescently labeled MAP and penetratin after incubation with Chinese hamster ovarian (CHO) cells were assessed by RP-HPLC equipped with a fluorescence detector. Data obtained from the experiments were used to generate a schematic of different pathways in uptake of the peptides. An attempt to inhibit degradation of the peptides was made by using several common protease inhibitors and the toxicity of these protease inhibitors and substrates was investigated in an LDH leakage assay. Wortmannin, cytochalasin B and nocodazole, which have previously been shown to inhibit different forms of endocytosis [13], were analyzed for their effect on uptake of MAP and penetratin. In addition, the uptake and degradation was investigated in cells treated with chloroquine and in energy depleted cells.

2. Materials and methods

2.1. Materials

5(6)-Carboxyfluorescein was purchased from Molecular Probes. All amino acids were purchased from Bachem, Switzerland. Cell culture medium DMEM-F12, Foetal Bovine Serum (FBS), Bovine Serum Albumin (BSA), Penicillin–Streptomycin (PEST) and Trypsin were purchased from GIBCO, USA. Other chemicals and reagents, when not specified were purchased from Sigma-Aldrich. Data evaluation was performed using the software GraphPad Prism 4.0 from GraphPad soft Inc.

2.2. Peptide synthesis

Peptides (Table 1) were synthesized in a stepwise manner in a 0.1 mmol scale on a peptide synthesizer (Applied Biosystems model 431A, USA) using *t*-Boc strategy of solid-phase peptide synthesis. *t*-Boc amino acids were coupled as hydroxybenzotriazole (HOBt) esters to a *p*-methylbenzylhydrylamine (MBHA) resin to obtain C-terminally amidated peptides. The peptides were labeled with 5-(and-6)-carboxyfluorescein as HOBt ester at the N-terminus as described in Fischer et al. [14]. The peptides were finally cleaved from the solid phase with liquid HF at 0 °C for 1 h in the presence of *p*-cresol (and thiocresol 1:1 for penetratin). Purification of peptides with reverse-phase (RP) HPLC was carried out with a Supelcosil LC-18 preparative column 5 μm (250×21.2 mm) (Sigma Aldrich Chemie, Steinheim, Germany) using acetonitrile–water, both containing 0.1% trifluoroacetic acid, gradient from 20 to 100% acetonitrile. Correct masses of purified peptides were obtained using a Perkin Elmer prOTOF™ 2000 MALDI O-TOF mass spectrometer.

2.3. Cell culture

CHO-K1 cells were cultured in Dulbecco's Modified Eagle's Media (DMEM) F-12 with Glutamax-I (GIBCO, USA) supplemented with 10% foetal calf serum, 100 μg/ml streptomycin, 100 U/ml penicillin.

2.4. Cellular uptake experiments

CHO cells seeded in 24-well plates in 500 μl medium (200,000 cells/well) were used for experiments performed in triplicates 1 day after seeding. Cells for uptake experiments were washed 2 times with ice-cold PBS and incubated with 200 μl, 1 μM penetratin or MAP in PBS buffer supplemented with 1 g/l D-glucose at 37 °C for 5, 15, 30 or 60 min. As negative controls we used two sequences originating from the pore forming perforin protein, namely, amino acids 1–16 and 1–34 [15]. After each time point, the incubation buffer containing the peptides was removed, diluted with 0.1% Triton X-100 containing 0.1% trifluoroacetic acid and injected to HPLC where quantification was performed by fluorescence measurement at 524 nm after excitation at 445 nm using calibration values obtained with 10 pmol of the parent peptide analyzed under identical conditions. The cells were washed 2 times with ice-cold PBS, incubated with 500 μl of ice-cold PBS and treated with diazotized 2-nitroaniline as described previously [16] in order to modify any remaining extracellularly located peptide. Briefly: to 400 μl ethanol/water 1/1 v/v containing 2-nitroaniline (0.06 M) and HCl (0.125 M), 50 μl 0.6 M $NaNO_2$, were added. After standing for 5 min at ambient temperature, 10 μl of this reagent was added to the ice-cold PBS covering the cell layer and allowed to react for 10 min at 0 °C. After aspiration of the diazo reagent the cells were washed 2 times with ice-cold PBS and finally lysed with 200 μl 0.1% Triton X-100 containing 0.1% trifluoroacetic acid for 2 h at 0 °C. The resulting lysates were used for HPLC-analysis. The effects of the protease inhibitors, bacitracin (5 mg/ml), leupeptin (50 μM), phenanthroline (2 mM), PMSF (2 mM) were analyzed by incubating cells with MAP or penetratin together with each inhibitor or BSA (1%) at 37 °C for 1 h. In addition BSA and bacitracin were pre-incubated with the peptides 40 min prior to incubation with cells. In order to investigate the endocytosis inhibitors, the cells were pre-incubated with wortmannin (5 nM), nocodazole (5 μM) or cytochalasin B (5 μM) for 30 min, followed by incubation with peptides in the presence of inhibitors for 1 h at 37 °C. The same procedure was performed with chloroquine (50 μM) and sodium azide (60 mM) together with deoxyglucose (20 mM). Samples were taken and analyzed by RP-HPLC.

2.5. LDH leakage assay

The assay was performed using the In Vitro Toxicology Assay Kit, Lactic Dehydrogenase Based (Sigma). In the assay, CHO cells were seeded in 24-well plates in 500 μl medium (100,000 cells/well) and used for experiments performed in triplicates 1 day after seeding. Prior to the incubation with 100 μl peptide and 100 μl inhibitor at 37 °C, cells were washed with PBS buffer supplemented with 1 g/l D-glucose two times. After 1 h incubation, samples of 100 μl were added to 200 μl of an equal mixture of LDH Assay Substrate, Cofactor and Dye solution in a 96-well-plate and incubated at 21 °C. After 30 min the reaction was quenched with 1/10 of 1M HCl. The absorbance was measured at 490 nm and the background absorbance was measured at 690 nm and subtracted. Untreated cells and cells treated with LDH Assay Lysis Solution, were used as controls.

2.6. HPLC-analysis

HPLC was performed using a Gynkotek-HPLC-gradient system (Dionex, USA) equipped with a 5 μm (150×4.6 mm,) C_{18} column, a precolumn containing the same adsorbent and a fluorescence detector. The elution was carried out with water containing 0.1% TFA (A) and acetonitrile containing 0.1% TFA (B) at a flow rate of 1.0 ml/min with gradients from 100% A (5 min) 20% B (5–10 min) and 20–100% B (10–50 min). Quantification was performed by fluorescence measurement at 524 nm after excitation at 445 nm using calibration values obtained with 10 pmol of the parent peptide analyzed under identical conditions.