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Uptake of cell-penetrating peptides is dependent on peptide-to-cell ratio rather than on peptide concentration

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Abstract

The influence of the peptide-to-cell ratio and energy depletion on uptake and degradation of the cell-penetrating peptides (CPPs) MAP (model amphipathic peptide) was investigated. The intracellular concentration of the CPPs, MAP and penetratin was monitored while varying the number of cells at fixed peptide concentration and incubation volume, or changing the concentration and incubation volume at fixed cell number. The uptake of CPPs was shown to be dependent on the peptide/cell ratio. At given peptide concentration and incubation volume, the intracellular concentration of peptide increased with lower cell number. At given cell number, doubling of the incubation volume increased intracellular peptide concentration to a similar extent as the doubling in incubation concentration. From a practical view, this means that the peptide/cell ratio has at least the same importance for the uptake of CPPs as the used peptide concentration. No influence of the peptide/cell ratio was found for the cellular uptake of peptide nucleic acid (PNA), or a non-amphipathic MAP analogue, investigated in parallel for comparison purposes.

Energy depletion resulted in significantly reduced quantities of intracellular fluorescence label. Moreover, we show that this difference is mainly due to a membrane-impermeable fluorescent-labelled degradation product, which is lacking in energy-depleted cells. The mechanism of its generation is not likely to be endosomal degradation of endocytosed material, as it is not chloroquine- or brefeldin-sensitive.

Keywords: Cellular uptake; Cell-penetrating peptides

1. Introduction

During the last 10 years, several peptides have been demonstrated to translocate across the plasma membrane of eukaryotic cells by a seemingly energy-independent pathway, i.e., they internalize even when cells are incubated at 4 °C or in energy-depleted cells. These peptides are collectively called cell-penetrating peptides (CPPs).

Cellular delivery using cell penetrating peptides offers several advantages over conventional techniques, as it is efficient for a range of cell types and can be applied to cells en masse. Thus, CPPs might be used as delivery vectors for pharmacologically interesting substances, such as antisense oligonucleotides, proteins and peptides. For review, see Lindgren et al. [1].

Penetratin, or antennapedia (43–58), is a 16-amino-acid-long peptide corresponding to the third helix of the *Antennapedia* homeodomain deprived of its N-terminal glutamate (Table 1). Penetratin and its analogues have been used for transmembrane delivery of a diversity of hydrophilic macromolecules; for review of penetratins, see Derossi et al. [2].

The cell-permeable α-helical model amphipathic peptide (MAP) (Table 1) has previously been stepwise modified with respect to hydrophobicity, hydrophobic moment and

Abbreviations: CHO, Chinese hamster ovary cells; CLSM, confocal laser scanning microscopy; DOG, 2-deoxy-D-glucose; DPBSG, Dulbecco's phosphate buffered saline supplemented with 1 g/l D-glucose; FLUOS-, 5(6)-carboxyfluoresceinyl; MAP, model amphipathic peptide (KLAL); PBS, phosphate buffered saline; PNA, peptide nucleic acid

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Table 1
Name and sequences of the CPPs used in this work

Name and sequence
Penetratin
fluos-RQIKIWFQNRRMKWKK-amide
MAP (KLAL)
fluos-KLALKLALKALKAALKLA-amide
KLGL
fluos-KLGLKLGLKGLKGGLKLG-amide

hydrophilic face as well as molecular size and charge [3]. Cellular uptake and membrane destabilizing activity of the resulting peptides were studied using aortic endothelial cells and HPLC combined with confocal laser scanning microscopy (CLSM). None of the investigated structural parameters proved to be essential for the passage of these peptides across the plasma membrane [4]. However, it was proposed that high amphipathicity decreased peptide outflow from preincubated cells. Membrane toxicity also showed no correlation to any of the parameters investigated and could be detected only at concentrations higher than 2 μM. The mode of uptake remains unclear and has been shown to involve both temperature-sensitive and -insensitive processes, indicating non-endocytic contributions.

Recently, studies focusing on the CPP tat (48–60) have cast some doubt over the usefulness of CPPs and pointed out that several published observations concerning cell uptake may well be flawed due to methodological drawbacks, particularly in the case of the tat peptide [5] These studies have pointed out the importance of using methods that avoid bias of measurements of uptake by plasma membrane-bound peptides. Several such methods have been used, i.e., fluorescence correlation spectroscopy [6], diphtalate separation [7], fluorescence HPLC after diazo-modification of surface bound peptide [8] and fluorescence/quenching pairs [9]. Here we used the Fluorescence-HPLC approach previously developed in our laboratory [8], using diazotised 2-nitroaniline to modify the primary amino groups of the peptide.

So far, little information is available concerning the influence of cell state on CPP uptake and degradation of labelled peptides. Particularly, degradation could seriously affect uptake as well as intracellular distribution of the label. Therefore, we decided to examine the influence of culturing time and cell density of Chinese hamster ovary (CHO) cells on uptake and degradation pattern of CPPs.

Here we investigate the influence of the peptide-to-cell ratio and cell-culture age on the intracellular concentration of a MAP [8] (Table 1). For comparison, we used three substances: the well-studied CPP penetratin, a 12-mer peptide nucleic acid (PNA) [10], which is primarily taken up by endocytosis, and KLGL (Table 1), a non-amphipathic analogue of MAP [3], shown to be taken up much less extensively and, probably, by another mechanism than MAP.

2. Materials and methods

5(6)-Carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS) was purchased from Boehringer (Mannheim, Germany). Release of lactate dehydrogenase was assessed by means of LDH-L reagent from Sigma (Deisenhofen, Germany). Other chemicals and reagents, when not specified, were purchased from Sigma or Bachem (Heidelberg, Germany).

2.1. Peptide synthesis

Peptides were synthesized automatically (MilliGen 9050 peptide synthesizer) by solid phase methods using standard Fmoc chemistry in the continuous flow mode [TentaGel S RAM resin 0.22 mmol/g (Rapp Polymere, Tübingen), TBTU, 2 equiv. of DIEA, coupling time 20 min, deblocking with 20% piperidine in DMF for 10 min, final cleavage with 93% TFA/5% H_2O/2% triisopropylsilane for 3 h] as described previously [12]. To introduce the fluorescent label, the peptides were N-terminally conjugated with FLUOS (Boehringer) (2 equiv. in DMF), and the final cleavage was performed with 95% TFA/5% water for 3 h. Purification of 100-mg samples was carried out by preparative HPLC on PLRP-S 100A, 10 μ, 250×20-mm i.d. (Phenomenex, Hösbach, Germany) to give final products that were >95% pure by RP-HPLC analysis, which gave the expected $[M+H]^+$ mass peaks by MALDI-MS (MALDI II, Kratos, Manchester).

2.2. Cell culture

CHO cells were cultured in Eagle's Minimal Essential Medium with Glutamax-I (Life Technologies, Gaithersburg, MD) supplemented with 10% foetal calf serum, 1% nonessential amino acids, 1% sodium puryvate, 100 μg/ml streptomycin, 100 U/ml penicillin. Cells for uptake was seeded in 24-well plates (Life Technologies) at various densities (20,000–200,000 cells/well) in 2-ml medium. The cells were used for experiments 2, 4 or 6 days after seeding with medium changes every second day.

2.3. Uptake experiments

Cells for uptake experiments were incubated with 200-μl 1 μM fluos-MAP, 10 μM fluos-penetratin, 50 μM KLGL or 0.5 μM fluos-PNA in Dulbecco's phosphate buffered saline (DPBS; Biochrom KG, Berlin) supplemented with 1 g/l D-glucose (DPBSG) at 37 °C, unless otherwise indicated.

For the experiments with energy-depleted cells, cells were preincubated at 37 °C for 30 min in DPBS containing 25 mM 2-deoxyglucose and 60 mM sodium azide. Peptide incubation was performed in 200 μl of the same solution.

The incubation solutions were aspirated and kept on ice until use for quantifying LDH leakage. Subsequently, the cells were washed twice with ice-cold PBS, incubated with

200 μl of ice-cold PBS and treated with diazotized 2-nitroaniline as described previously [8] in order to modify any surface bound peptide. In brief: 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 $NaNO_2$ was added. After standing for 5 min at ambient temperature, 4 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 twice with ice-cold PBS and finally lysed with 0.2-ml 0.1% Triton X-100 containing 10 mmol/l trifluoroacetic acid for 2 h at 0 °C. The resulting lysate was used for HPLC analysis and Bradford protein determination (Sigma) according to the manufacturers' instruction. The average protein content of 10^6 cells assayed by this method was 180 μg. The average volume of the cells was determined to be 1.2 pl by means of a Coulter-ZM counter (Coulter Electronics Ltd., Luton, England) and was not significantly dependent on cell density. To measure the pore-forming propensity of the peptides, an LDH quantification kit (Sigma)) was used according to the manufacturer's instruction. In brief: 50-μl incubation solution was mixed with 1-ml assay reagent and the evolution of signal was followed spectrophotometrically at 340 nm for 2 min. The maximum leakage was ascertained by incubating cells with 0.1% Triton X-100.

2.4. HPLC analysis

HPLC was performed using a Bischoff-HPLC-gradient system (Leonberg, Germany) equipped with a Polyencap A 300, a 5-μm column (250×4-mm i.d.), precolumns containing the same adsorbent and a Fluorescence HPLC-Monitor RF-551 (Shimadzu).

Up to 200 μl of the cell lysates was passed through a precolumn containing 60 mg of Polyencap (A 300, 5 μm), which thereafter was connected to the HPLC column. The elution was carried out with 0.01 M TFA (A) and acetonitrile/water 9:1 (B) at a flow rate of 1.0 ml/min with gradients from 30–45% B (0–10 min) to 45–80% B (15–20 min). Quantitation was performed by fluorescence measurement at 520 nm after excitation at 445 nm using calibration values obtained with the parent peptide under identical conditions.

2.5. Capillary electrophoresis

Capillary electrophoresis was performed using a P/ACE MDQ system with a P/ACE MDQ Laser-Induced Fluorescence Detector (Beckman Coulter, Fullerton, CA, USA) and a Phenomenex 31-cm neutral coated capillary 50-μm ID at 645 V/cm with 200 mM Tris/borate buffer, pH 7.5, containing 5 M urea and 0.1% SDS. Cells were washed and lysed using 0.1% Triton X-100/0.01 M TFA. After aspiration of the cell lysate (containing below 10% of taken up PNA), each 200 μl of 20 mM Tris/borate buffer, pH 7.5 with 5 M urea and 0.1% SDS was added to the wells of the culture plate containing attached nuclei and debris along with the predominant portion of the internalized PNA, followed by sonication for 5 min at 70 °C. These extracts were injected into the capillary for 5 s at 0.5 PSI and quantitation was performed by fluorescence measurement at 520 nm after excitation at 490 nm using calibration values obtained under identical conditions.

3. Results

Differentiation between surface bound and internalized peptide fractions was performed as described previously [8] using the membrane-impermeable, primary amino group modifying reagent diazotized 2-nitroaniline. The treatment results in a significant increase in retention time for the surface-bound peptide. In addition, peptide accumulating in dead cells is also modified [8]. Degradation products were easily recognized by HPLC separation (Fig. 1).

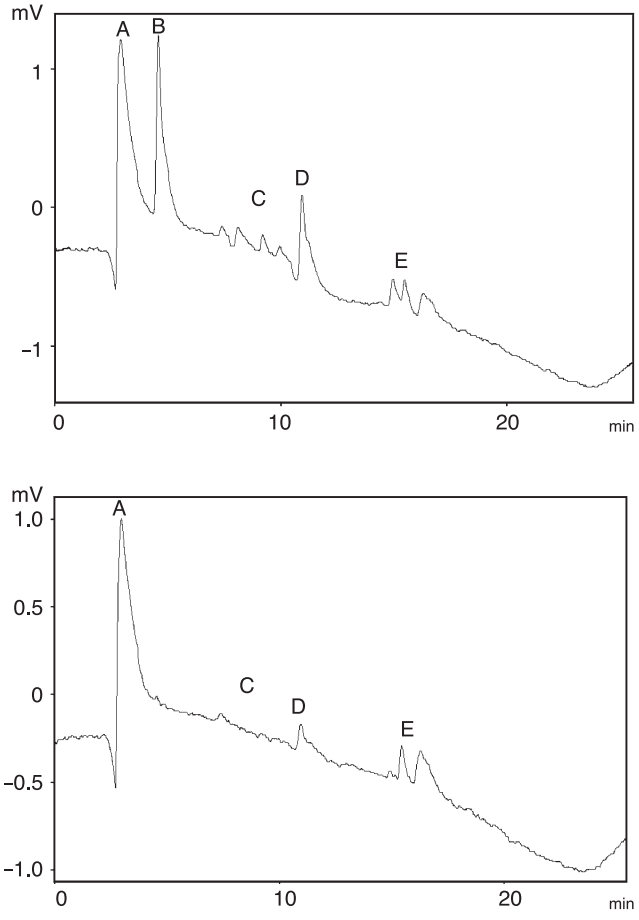

Fig. 1. Representative HPLC chromatograms of control (upper panel) and energy-depleted (lower panel) CHO cells incubated in 200-μl 1 μM Fl-MAP for 1 h. Peaks correspond to A: cell-intrinsic fluorescence, B: FLUOS-Lys, C: largely energy independently generated peptide fragments, D: FLUOS-MAP, E: modified FLUOS-MAP. Cells were energy-depleted by preincubation at 37 °C for 60 min in DPBS containing 25 mM 2-deoxyglucose and 10 mM sodium azide. Peptide incubation was performed in 200 μl of the same solution.

First we examined how cell-culture age and energy depletion changed the uptake of fluos-MAP. CHO-cells at 2, 4 or 6 days after splitting were incubated for 1 h in 1 μm MAP. It was apparent that prolonged culturing of the cells drastically reduced the intracellular concentration from 24 μM in 2-day-old cultures to 8.5 μM in 6-day-old cultures (Fig. 2). For energy-depleted cells the difference was even more drastic: from 20 μM at 2 days to 0.6 μM at 6 days.

In order to rule out that the cell-cycle stage of the cells was responsible for the change in uptake, cell were synchronized either by 24-h serum starvation or 96-h preincubation in 1.5% DMSO, which have been shown to induce G1 arrest [11]. Here, there was no significant difference in uptake of MAP as compared to control cells of the same density (data not shown).

In order to investigate whether the age or density of the cultures was the deciding factor, cells for uptake were seeded at different split ratios and treated with 200-μl 1 μM MAP, 0.5 μM PNA, 50 μM KLGL and 10 μM penetratin for 1 h. For the CPPs, a negative correlation of uptake with the number of cells became apparent (Fig. 3). The label concentration in the cells changed from approximately 40 μM at around 200 000 cells/well to 2 μM at 1 300 000 cells/well (Fig. 3a). At 1 μM penetratin, no uptake could be detected, therefore we decided to use 10 μM penetratin throughout the experiments. Here, the label concentration decreased from 250 μM at 300,000 cells to 120 μM at 1 million (Fig. 3b). The intracellular concentration of PNA and KLGL was not significantly dependent on the number of cells but remained around 0.3 μM for PNA and 1.5 μM for KLGL (data not shown). In no case could damage of the cells be observed as judged by LDH release.

In order to investigate if the confluency (and hence available membrane area) was responsible for the found dependency of the CPP uptake on the split rate, cells with identical density were incubated in different volumes of 1 μM MAP and 10 μM penetratin. Here the uptake demonstrated a linear increase with incubation volume (Fig. 4), rendering the available membrane area less important. For penetratin, at the highest incubation volume, a deviation from linearity can be seen. This could possibly be due to either saturation of the uptake or saturation of intracellular peptide degradation (see below).

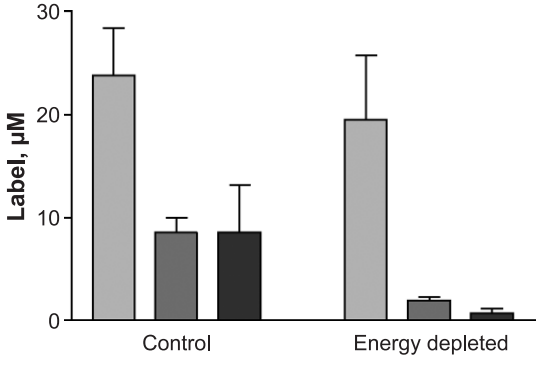

Fig. 2. The intracellular label content in CHO cell cultures grown for 2 (white), 4 (gray) and 6 (black) days after splitting incubated at 37 °C for 1 h in 200 μl of DPBSG containing 1 μM FLUOS-MAP. (Left panel) Prolonged culturing drastically reduced the uptake in control cells. For the energy-depleted cells, the change in label content between was even more drastic. (Right panel) Cells were energy-depleted by preincubation at 37 °C for 60 min in DPBS containing 25 mM 2-deoxyglucose and 10 mM sodium azide. Peptide incubation was performed in 200 μl of the same solution.

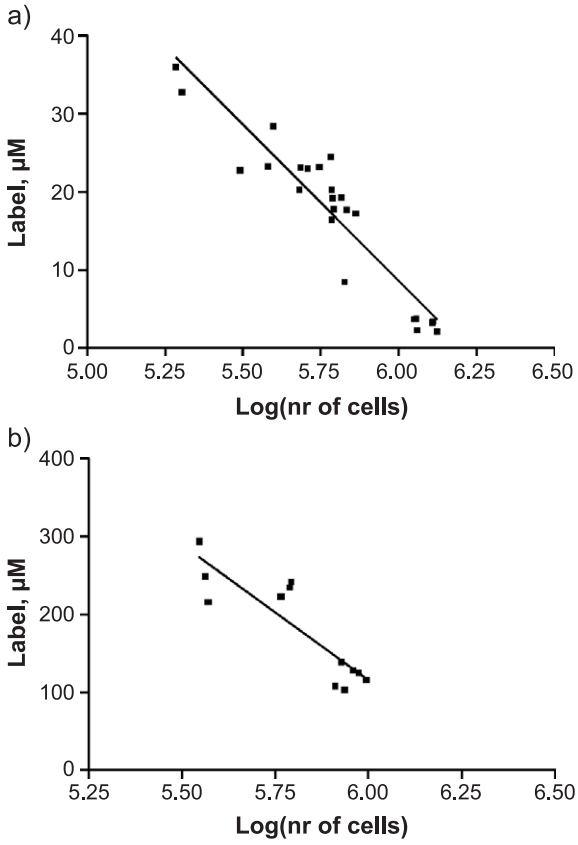

Fig. 3. The intracellular label concentration in 2-day CHO cell cultures seeded at different split ratios. The cells were incubated at 37 °C for 1 h in 200-μl DPBSG containing 1 μM MAP (a) or 10 μM penetratin (b). Uptake decreases rapidly with the number of cells and is nearly undetectable for MAP at the highest cell numbers.

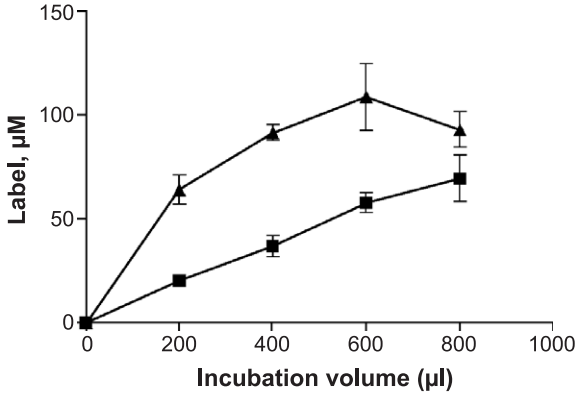

Fig. 4. The intracellular label concentration in 2-day CHO cell cultures incubated at 37 °C for 1 h in various incubation volumes of DPBSG (200, 400, 600, and 800 μl) containing 1 μM MAP (boxes) or 10 μM penetratin (triangles).

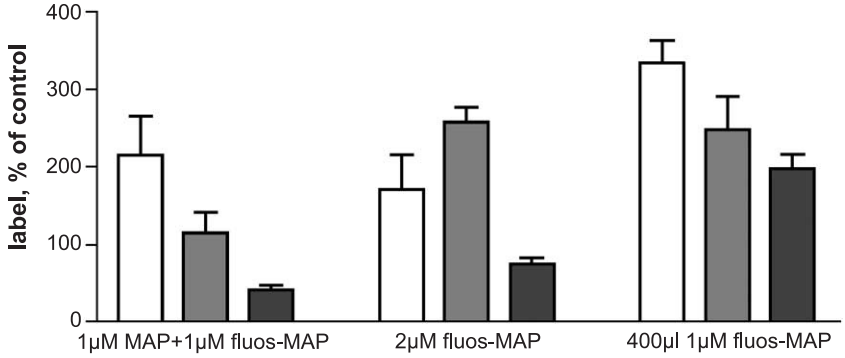

Fig. 5. The relative contribution of intact (white), energy-independent metabolized MAP (gray) and energy-dependent metabolized MAP (black) to the intracellular label content in 2-day CHO cell cultures compared to control (incubation in 200-μl 1 μM labelled MAP). The cells were incubated at 37 °C for 1 h in 200-μl 1 μM labelled and 1 μM unlabelled MAP (left panel), 200-μl 2 μM labelled MAP (middle panel) or 400-μl labelled MAP (right panel). In the control cells, the ratio between intact and energy-dependent metabolites was approximately 2.2 and 1.8 for the energy-independent metabolite.

We then compared uptake and degradation of MAP in cells incubated with 200-μl 1 μM Fluos-MAP, 200-μl 2 μM Fluos-MAP, 200-μl 1 μM MAP+1 μM Fluos-MAP or 400-μl 1 μM fluos-MAP (Fig. 5). We could classify degradation products produced either by energy-dependent (Fluos-Lys) [8] or energy-independent (>10 Fluos-peptide fragments) processes (Fig. 1). Here, increasing the incubation volume yielded more intracellular label than doubling the peptide concentration. Again, no damage of the cells could be detected by the LDH test, even at the highest uptake levels. This was surprising because corresponding intracellular peptide levels generated by incubation in 2 μM MAP yielded a slight LDH release (below 10% of total). Adding 1 eq of unlabelled peptide increased the amount of intact peptide but decreased the amount of energy-dependent metabolite in the cells.

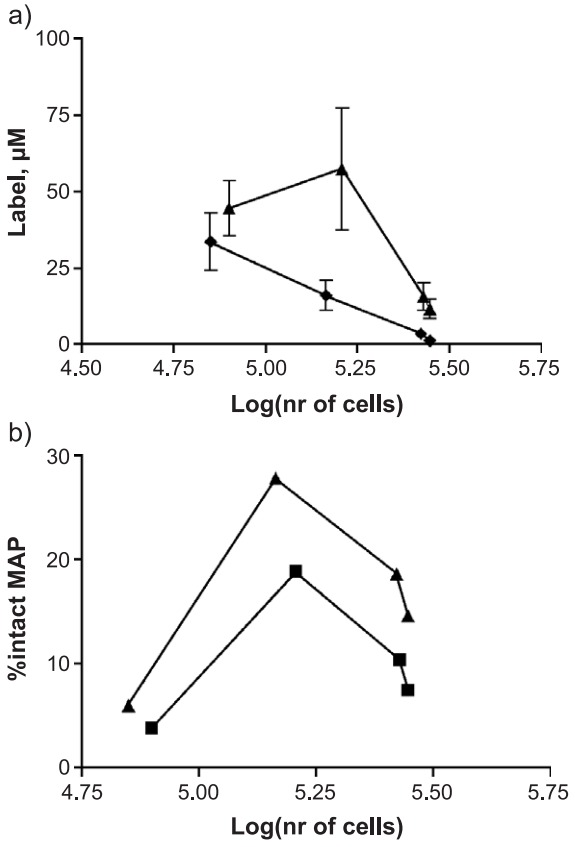

Fig. 6. (a) The intracellular label concentration in control (triangles) and energy-depleted (boxes) 2-day CHO cell cultures seeded at different split ratios and incubated at 37 °C for 1 h in 200-μl 1 μM MAP. (b) The relative contributions of intact MAP to the fluorescence signal, control (triangles) and energy-depleted (boxes) 2-day CHO cell cultures seeded at different spilt ratios and incubated at 37 °C for 1 h in 200 μl 1 μM MAP.

Next, we analyzed the relative contribution of intact and metabolized MAP to the fluorescence signal, for experiments with different cell numbers. After 1-h incubation in 1 μM Fluos-MAP, energy-depleted cells did indeed contain less label than untreated cells (Fig. 6a). While there was no significant difference in the content of energy-independent metabolites, there was a complete lack of the energy-dependent metabolite Fluos-Lys, in the energy-depleted cells (for representative HPLC chromatograms, see Fig. 1). In contrast to the energy-independent metabolites, this compound could not be detected in the supernatant indicating membrane impermeability for this metabolite [8]. In both control and energy-depleted cells, the relative contribution by intact peptide to the label content was low (<10%) in cultures of low and high density, while at intermediate density the contribution rose to approximately 20% (Fig. 6b). In order to ascertain that cell lysis did not release proteases that could be active even at the low pH used, Fl-MAP to a final concentration of 1 μM was added to lysis buffer prior to addition to cells. After 2 h at 0 °C and 20 h at ambient temperature, only one peak, corresponding to intact MAP, could be seen in the HPLC.

4. Discussion

In this work, we have investigated the uptake and degradation of CPPs in cells of different density and cell

cycle stage. Taken together, these data argue that the peptide/cell ratio is of critical importance for uptake. This conclusion is supported by a study conducted on penetratin internalization into lipid vesicles [12], in which the authors found that a critical amount of peptide per vesicle is needed for internalization to occur.

As many phenomena examined in the biophysics of peptide membrane interaction are described by the peptide to lipid ratio, we were inspired to investigate the effect of the peptide-to-cell ratio, rather than of the peptide concentration, on uptake. Thus, the intracellular concentration of CPP delivered label was monitored while changing the concentration and incubation volume at fixed cell number as well as changing the cell number at fixed incubation volume and peptide concentration. Indeed, doubling of the incubation volume at fixed cell number increased the intracellular peptide concentration more efficiently than doubling of external peptide concentration (Fig. 5). A possible reason for this is the increased membrane damage (and subsequent leakage of metabolites) seen with increased peptide concentration. Moreover, the uptake increases linearly with incubation volume (Fig. 4), indicating that the uptake of CPPs is correlated to the peptide-to-cell ratio rather than to the peptide concentration.

The differences in intracellular label of CHO cells grown for 2, 4 or 6 days in culture appear correlated to the peptide-to-cell ratio, due to different cell densities, rather than to cell-culture age. This could be caused by the different membrane composition or endocytosis activity of growing and G1 arrested cells. Another possibly is that access to the membrane decreases as confluency increases. However, we could not see a difference in uptake between synchronized and free-growing cells, arguing that the cell stage is in this case of minor importance. Cell age as the reason could further be rendered unlikely by experiments where cells were seeded at different densities and used 2 days later. The intracellular concentration of label in MAP and penetratin-treated cells here proved to be correlated to the cell number analogously as found in the above outlined experiments with cell cultures of different age (Fig. 4). An increase of cells from 200,000 to 1,000,000 led to a drop of intracellular label content from 400-fold accumulation to levels that are below detection with our method (approximately equilibrium concentration). Even the absolute amount of uptake, and not only the relative intracellular concentration, decreased with increasing cell number.

Increased degradation of the peptide in the incubation solution at increased cell number could be ruled out to be responsible for the observed uptake differences since analysis of the extracellular degradation products indicated comparable degradation in all cases. This indicates that the main site for degradation is intracellular. Moreover, the non-amphipathic KLGL and PNA showed an uptake that linearly increased with cell number whereby the degradation of KLGL proceeded comparatively to that of MAP while PNA remained completely intact.

It is possible that the importance of peptide-to-cell ratios can explain the large differences in accumulation of label seen by different authors, e.g., from equilibrium concentration up to hundred(s) fold accumulation.

Richard et al. [5] have pointed out that uptake seen with fluorescence microscopy might be in some cases an artifact stemming from fixation protocols. However, several methods using living cells have shown uptake of CPPs [4,13]. Moreover, from this study and from Waizenegger et al. [13], it is apparent that much of the label in the cell is associated with degradation products, raising the possibility that fixed and permeabilized cells might actually reflect the intracellular distribution of the peptide better than live cells, as labeled amino acids probably are washed out during the permeabilization procedure.

A decrease in label content in energy-depleted cells has typically been seen as indicative of an endocytotic component in the uptake of CPPs. However, we show that an additional cause of decrease of intracellular label content in energy-depleted cells is the elimination of the production of labeled membrane-impermeable metabolites. The metabolites appear after 5 min of incubation, which is consistent with the uptake kinetics for MAP, and steadily increases with time. Indeed, after 1-h incubation, some of our experiments show that more than 90% of the intracellular fluorescence is associated with degradation products (Fig. 6b). In this work, we have not analyzed the degradation pattern for penetratin; this, however, is the topic of an upcoming paper. Preliminary data show that the degradation of penetratin is comparable to that of MAP. Thus, we propose that CPPs are in equilibrium over the plasma membrane, but that an irreversible step, i.e., degradation, significantly contributes to intracellular labeling. As the degradation is partially energy-sensitive, it could be a consequence of endocytosis followed by degradation of the peptide in the lysosomes. In order to separate lysosomal degradation from other forms of degradation pathways, the lysosomatic reagent chloroquine is often used. It has previously been shown that MAP degradation is not significantly influenced by chloroquine nor by the endocytosis inhibitor brefeldin A nor by potassium depletion [8], arguing against lysosomal degradation of MAP. Moreover, adding 1 eq of unlabelled peptide doubled the amount of intact peptide and halved the amount of energy-dependent degraded metabolites in the cells, arguing that the energy-dependent degradation step can be outcompeted. The main route by which MAP is degraded is not clear, but energy-dependent, chloroquine-insensitive degradation is consistent with, e.g., proteasomal action. A recent report by Simeoni et al. [14] show similarly that the uptake of the CPP MPG, complexed with DNA, is independent of the endosomal pathway. Hariton-Gazal et al. [15] show that uptake of histone(s) functions in a similar manner.

In conclusion, we show that a large proportion of the internalized CPP is degraded, yielding a fluorescent labelled amino acid as product of an energy-dependent mechanism. This mechanism is not likely to be an endosomal

degradation of material taken up through classical endocytosis, as it is not sensitive to chloroquine, brefeldin-A or potassium depletion, and can be competitively inhibited. Thus, it cannot be excluded that the difference in concentration of the intracellular label in energy-depleted cells vs. untreated cells is mainly due to the production of membrane-impermeable degradation products rather than to the decrease of active uptake of the peptides. Moreover, we suggest that the uptake of CPPs could be increased, with subsequent improved delivery efficiency, by increasing the incubation volume.

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