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Extensive cellular uptake into endothelial cells of an amphipathic β-sheet forming peptide

Johannes Oehlke*, Eberhard Krause, Burkhard Wiesner, Michael Beyermann, Michael Bienert

Institute of Molecular Pharmacology, Alfred-Kowalke-Str. 4, D-10315 Berlin, Germany

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Abstract Extensive internalization into endothelial cells has been found for a water soluble amphipathic 26-mer β-sheet peptide (FLUOS-DPKGDPKGVTVTVTVTVTGKGDPKPD-NH_2; VT5). With the D-Val^{13},D-Thr^{14} di-D-amino acid analog of VT5 (DD-VT5), exhibiting an identical primary structure but no propensity to adopt a β-sheet conformation, only about 5% of the cellular uptake of VT5 was found. The mechanism of entry of VT5 into the cells remained unclear, but proved to be energy, temperature and pH dependent and, therefore, clearly distinct from that reported for helical amphipathic peptides. No detectable cytotoxicity, high solubility in water and the found extensive entry into endothelial cells make VT5 appear a good lead for developing new types of vectors for delivering oligonucleotides and peptides into intact cells.

Key words: Cellular uptake of peptides; Beta-sheet conformation; D-Amino acid replacement

1. Introduction

Peptides exhibiting the propensity to form amphipathic helices at water-lipid interfaces have repeatedly been shown to be capable of crossing plasma membranes of mammalian cells [1–4]. Such peptides could be translocated into the cell interior at low micromolar concentrations without any detectable impairment of the cell viability and could be exploited successfully as vectors for oligonucleotides and oligopeptides [2,5]. The mechanism for the translocation across the plasma membrane has remained unclear, so far [1,6].

Beta-sheet forming peptides appeared not to be good candidates for such an application due to their aggregation tendency in water and, therefore, very limited solubility. Recently, however, the synthesis of a water soluble amphipathic 26-mer β-sheet peptide has been reported by our group [7]. The solubility in water was achieved by attaching two unstructured charged hydrophilic octapeptides to a central $(Val\text{-}Thr)_5$ β-sheet sequence [7]. In the present study we report on the internalization into endothelial cells of a fluorescein labelled derivative of this water soluble β-sheet peptide (FLUOS-DPKGDPKGVTVTVTVTVTGKGDPKPD-NH_2; VT5) being comparably extensive to that observed with helical peptides. This finding introduces new aspects into the discussion about the still unclear mechanisms for the passage of structure forming peptides through biological membranes and probably opens the way for exploiting β-sheet peptides as vectors for delivering oligonucleotides and oligopeptides into the cell interior.

*Corresponding author. Fax: (49) (30) 51551 365/206.
E-mail: bienert@fmp-berlin.de

2. Materials and methods

2.1. Peptide synthesis

VT5 and its D-Val^{13},D-Thr^{14} double-D-amino acid analog were synthesized automatically by solid phase methods using standard Fmoc chemistry as described previously [7]. To introduce the fluorescence label, the peptides were N-terminally conjugated with 5(6)- carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS; Boehringer, Mannheim) (2 equiv. in DMF), and the final cleavage was performed with 95% trifluoroacetic acid (TFA)/5% water for 3 h.

2.2. Cell culture

Calf aortic endothelial cells (AEC), at the 12th to 20th subculture of a cell line established and characterized by Halle et al. [8], were seeded at an initial density 5×10^4 cells/cm^2 into 24-well culture plates. The cells were cultured at 37°C in a humidified 5% CO_2 containing air environment in minimal essential medium (MEM; Sigma GmbH, Deisenhofen) supplemented with 290 mg/l glutamine and 10% fetal bovine serum (Sigma). After 4 days without replacing the medium, the cells were used for the uptake experiments.

2.3. Uptake experiments

After removal of the medium the cell layers were rinsed twice at 37°C with Dulbecco's phosphate buffered saline (DPBS; Biochrom KG, Berlin) and subsequently exposed, unless indicated otherwise, at 37°C for 30 min to the peptide solutions in DPBS. Thereafter the incubation solutions were removed, the cells were washed twice with ice-cold PBS, incubated with 250 μl of ice-cold PBS and treated with diazotized 2-nitroaniline as described previously [9] in order to modify surface bound 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 $NaNO_2$ was added. After standing for 5 min at ambient temperature, 5 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 again washed twice with ice-cold PBS and finally lysed with 0.2 ml 0.1% Triton X-100 containing 10 mmol/l TFA for 2 h at 0°C. The resulting lysate was used for HPLC analysis and for determining the protein amount according to Bradford [10]. The protein content of 10^6 cells assayed by this method amounted to 110 μg. The average volume of the used cells was determined to be 1.4 pl by means of a Coulter ZM counter (Coulter Electronics ltd., Luton, England).

2.4. HPLC analysis

HPLC was performed using a Bischoff HPLC- radient system (Leonberg, Germany) equipped with a Polyencap A 300, 5 μm column (250×4 mm I.D.), precolumns containing the same sorbent, a Rheodyne-RH 8125 injection valve with a 50 μl sample loop and a fluorescence HPLC Monitor RF-551 (Shimadzu).

70 μl of the cell lysates was mixed with 30 μl of 0.1 M TFA in acetonitrile/water 1/1 and injected without further pretreatment into the sample loop. Alternatively, to enhance the assayed peptide quantity, up to 200 μl of the lysates was concentrated on a Polyencap A 300 precolumn, which subsequently was connected with the HPLC system. The elution was carried out with 0.01 M TFA (A) and acetonitrile/water 9/1 (B) with gradients of 30–40% B (0–15 min) and 40–80% B (15–20 min) at a flow rate of 1.0 ml/min. Quantitation was performed by fluorescence measurement at 520 nm after excitation at 445 nm by means of calibration lines obtained with the parent peptides using analogous conditions.

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2.5. Confocal laser scanning microscopy (CLSM)

10^4 cells were plated on 22×22 mm coverslips glued (with Silicones RTV 615A/615B, Paul Hellermann GmbH, Pinneberg) above the hole (15 mm diameter) of punched plastic Costar culture dishes (35 mm diameter; Tecnomara Deutschland GmbH, Frankfurt/Main) and cultured, exposed to peptides and washed as described above. After subsequently covering the cells with about 200 μl PBS, microscopy was performed within 10 min at room temperature using a LSM 410 invert confocal laser scanning microscope (Carl Zeiss Jena GmbH, Jena, Germany). Excitation was performed at 488 nm by means of an argon-krypton laser and a dichroitic mirror (FT 510) for wavelength selection. Emission was measured upon 515 nm by use of a cut-off filter (LP 515) in front of the detector. For optical sectioning in the *z*-direction 16 frames with a thickness of 1 μm were made.

3. Results

3.1. Extensive internalization of VT5 into endothelial cells

Exposure of bovine AEC to the fluorescently labelled β-sheet model peptide VT5 resulted in high quantities of cell associated peptide. Treatment of the peptide exposed cells with diazotized *o*-nitroaniline, a reagent proven to modify the surface bound but not the internalized amino group containing compounds [9], reduced the HPLC peak of VT5 by about 30%, suggesting the remainder to be internalized. CSLM of the peptide treated cells supported this notion by revealing extensive cell internal fluorescence (Fig. 1). Considering the ratio of cell volume to protein content of about 15 μl/mg protein determined for AEC (see Section 2) the quantities of internalized peptide correspond to an up to sixfold enrichment within the cell interior (Figs. 2–4).

3.2. Role of the β-sheet forming propensity of VT5 for its entry into the cells

To elucidate the role of the β-sheet forming propensity of VT5 for its internalization into the cells, we exposed AEC in parallel to VT5 and DD-VT5, the D-Val[13],D-Thr[14] di-D-amino acid analog of VT5, exhibiting an identical primary structure but no propensity to adopt a β-sheet conformation [7]. With the di-D-amino acid analog only about 5% of the peptide quantity obtained with VT5 using identical conditions was found to be cell associated (not shown). This finding clearly demonstrates dependence of the predominant part of the VT5 internalization on the β-sheet forming propensity of this peptide.

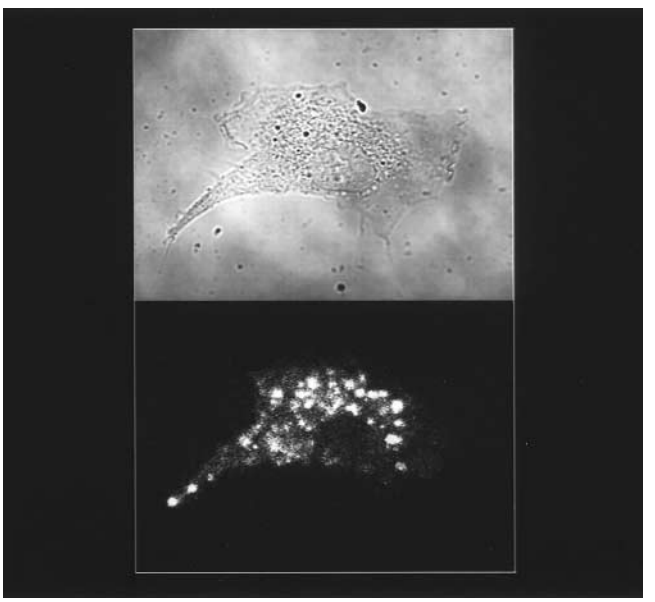

Fig. 1. CLSM image of a 1 μm thick central horizontal optical section through AEC exposed before to 8 μM VT5 in DPBSG for 30 min at 37°C. Insert: the same section viewed in transmission mode.

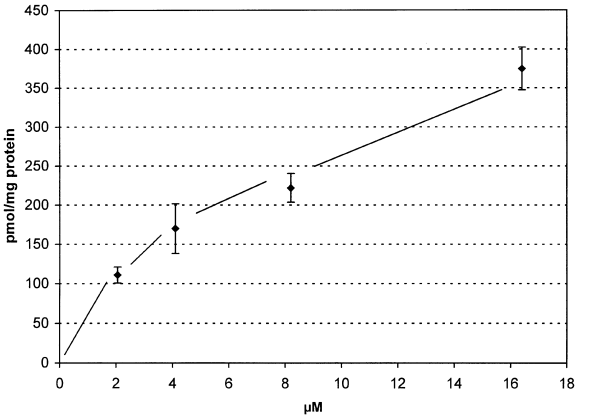

Fig. 2. Quantity of cell associated peptide after exposing AEC to different concentrations of VT5 for 30 min at 37°C. Each point represents the mean of three samples ± S.D.

Surprisingly, with respect to number and fluorescence intensity of the fluorescent vesicles, the CLSM image of the cells treated with DD-VT5 appeared to be comparable to that exposed to VT5 (displayed in Fig. 1). The only difference that could be additionally found in the VT5 treated cells was a faint fluorescence of the entire cytosol. This finding suggests comparable uptake into endocytic vesicles for DD-VT5 and a portion of VT5 corresponding to the amount of taken up DD-VT5 (about 5% of cell associated VT5, see above). It appears likely that this portion of internalized peptide is accounted for by non-specific adsorptive endocytosis mediated by electrostatic interactions between the negatively charged phospholipid head groups of the plasma membrane and the protonated amino groups being identical in both peptides. Likewise such interpretation suggests the predominant, β-sheet dependent, quantity of the internalized VT5 to be taken up by a mechanism other than non-specific adsorptive endocytosis.

Attempts to investigate effects of an increased β-sheet forming propensity by examining the cellular uptake of VT5 analogs possessing six and seven central Val-Thr β-sheet forming repeats failed, since extensive self aggregation around pH 7 prevented precise uptake measurements with these peptides.

3.3. Investigations into the mechanism of uptake

The concentration dependence of the VT5 uptake into the cells exhibited a complex course suggesting an overlay of saturable and unsaturable processes (Fig. 2).

To obtain further information about the mode of uptake, particularly to address the question of whether the internalization of this β-sheet peptide proceeds in a non-endocytic way as reported for α-helical amphipathic peptides [1,4,6], we investigated effects of several agents known to affect endocytosis.

The uptake exhibited strong energy, temperature and pH dependence (Fig. 3) and could be prevented quantitatively by the presence of hyperosmolar sucrose (450 mM) in the incu-

bation solution. Such behavior is commonly regarded as characteristic of endocytic uptake [11,12].

On the other hand, the time course of uptake differed strongly from that determined for lucifer yellow, commonly considered to be a membrane impermeable marker for fluid phase endocytosis [13] (Fig. 4). For uptake by adsorptive endocytosis, however, irrespective of quantitative differences a time course parallel to that of fluid phase endocytosis is to be expected. A peculiar behavior of our endothelial cell line appears unlikely to account for this discrepancy since the found uptake of lucifer yellow agreed well with that reported by Guillot et al. [14].

No significant efflux of peptide out of preloaded cells (30 min, 37°C, 10 μM peptide) could be observed during 30 min at 37°C (not shown). This finding also contradicts an endocytic mechanism, since the high cellular uptake observed within 30 min, if accounted for by a corresponding extensive endosomal turnover, should result in a measurable efflux within the same period.

A lack of influence upon the cellular VT5 uptake of brefeldin A (5 μM), a reagent which perturbs the endosomal transport [15], points in the same direction (Fig. 3).

Vincristine (20 μM), an inhibitor of the transport of lipophilic peptides by the multidrug resistance transporter gp 170 [16], did not affect the uptake so that the involvement of mdrp-like peptide transporters in the translocation of VT5 appears unlikely (Fig. 3).

Addition of 10% fetal bovine serum remained without influence on the uptake suggesting a negligible influence of nonspecific protein binding for entry and escape into or from the plasma membrane, respectively.

3.4. Toxicity and metabolic breakdown of VT5

Perforation of the plasma membrane could be ruled out to account for the observed peptide internalization within the investigated concentration range by trypan blue exclusion and a lack of liberation of lactic dehydrogenase. The MTT viability test [17] resulted in identical values for controls and cells treated with VT5 under the conditions used for the uptake studies.

No metabolic breakdown exceeding 10% of the peptide amount could be detected in either the incubation solution or the cell interior.

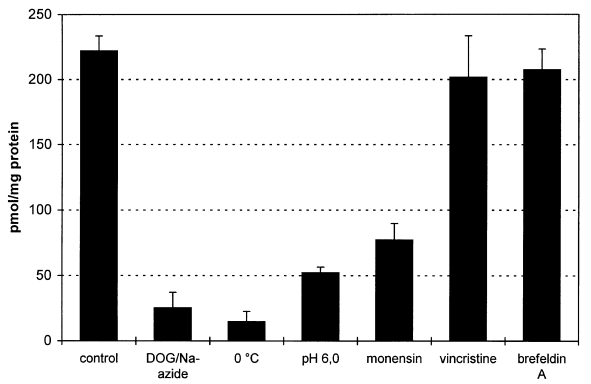

Fig. 3. Quantity of cell associated peptide after exposing AEC for 30 min to 8 μM VT5 in DPBSG at 37°C (control), at 37°C in DPBS containing 25 mM 2-deoxyglucose/10 mM Na-azide (DOG/Na-azide), in DPBSG adjusted with HCl to pH 6.0 (pH 6.0), in DPBSG containing 20 μM monensin (monensin) or 20 μM vincristine (vincristine) or 5 μM brefeldin A (brefeldin A), and in DPBSG at 0°C (0°C). Before exposure to the peptide the cells were incubated for 60 min at 37°C in DPBSG or 25 mM 2-deoxyglucose/10 mM Na-azide in DPBS or in DPBSG at 0°C. Each bar represents the mean of three samples ± S.D.

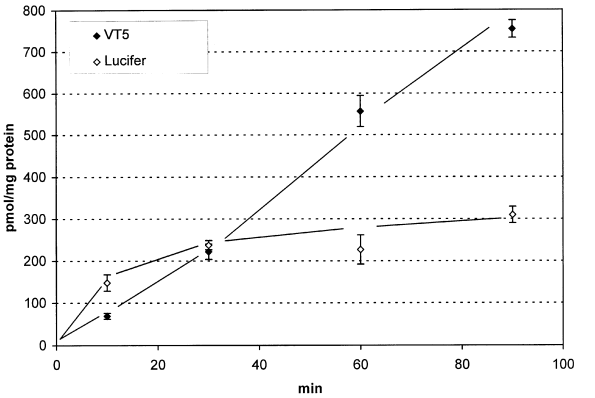

Fig. 4. Quantity of cell associated peptide after exposing AEC to 8 μM VT5 or 1 mM lucifer yellow in DPBSG at 37°C for different periods of time. Each point represents the mean of three samples ± S.D.

4. Discussion

The results of the present work reveal an extensive internalization into endothelial cells of a model peptide forming an amphipathic β-sheet at solid lipid/water interphases and in the presence of SDS without participation of helical structures [7]. A corresponding structure forming behavior of the peptide was observed in the presence of DOPC vesicles (Krause et al., unpublished). Because of analogy to the conditions used for the uptake experiments, a strong propensity to adopt a β-sheet can be expected for this peptide also at the interface between incubation solution and plasma membrane of the cells.

Comparable cellular uptake had been found before only for α-helical amphipathic peptides, some of which could be exploited successfully as vectors for translocating membrane impermeable peptides and oligonucleotides across the plasma membrane of mammalian cells [2,5]. VT5 also exhibited considerable helix forming propensity in 50% TFE/water [7]. This ability, however, appears not relevant in the context of the present cellular uptake experiments considering the peculiar structure inducing properties of TFE [18] and the poor analogy between the conditions in TFE/water and at lipid/water interphases.

The helical peptides were taken up to the greatest part in an uncleared, but energy and temperature independent and, therefore, non-endocytic way [1,4]. In contrast, strong energy and temperature dependence was found in the present study for the internalization of the β-sheet peptide VT5, implying a mode of uptake distinct from that of the α-helical amphipathic peptides. As a further difference from the helical peptides, no propensity to destabilize plasma membranes could be detected for VT5 within the low micromolar concentration range.

The mechanism for the internalization of VT5 remains unclear. Strong arguments exist for as well as against an endocytic mechanism.

Besides the observed energy and temperature dependence, the reduced VT5 internalization at pH 6 or in the presence of the acidifying reagent monensin [19] and the slow down of the uptake by hyperosmolar sucrose resemble effects commonly regarded to be characteristic of clathrin dependent endocytosis [11,12]. In the same way, the time course of VT5 uptake, approaching no equilibrium within 90 min, appears reconcilable with an endocytic mechanism, considering half-life times for plasma membrane recycling of various cell types in the range of 30 min to several hours [11,20].

On the other hand, several of our findings make it unlikely that endocytosis is the mechanism of the predominant part of peptide internalization and induce us to look for alternative interpretations. In particular, the observed up to sixfold enrichment of VT5 within the cell interior in relation to a total volume of the endocytic compartment of maximally about 10% of the cell volume [11,14], its retention within the cell interior and the discrepancy between the uptake kinetics of VT5 and that of the pinocytic marker lucifer yellow appear difficult to reconcile with an endocytic mechanism. Furthermore, in the case of non-specific adsorptive endocytosis, electrostatic interactions between the charged groups of the plasma membrane and that of the peptides, being identical for VT5 and its double-D analog, should predominate and, in strong contrast to our findings, result in comparable uptake of both peptides.

As an additional argument against an endocytic mechanism it should be noted that the aspects appearing characteristic of clathrin dependent endocytosis might also be explainable by translocation events mediated by carrier proteins or proceeding across protein channels, often found to exhibit pH and energy dependence [21–23]. In the same way, the effect of hyperosmolar sucrose might be interpreted, apart from slow down of endocytosis, simply by effects of membrane shrinking accompanying the hyperosmolar stress [24].

In conclusion, a comparably extensive cellular uptake combined with a reduced toxicity and a distinct mode of entry into the cell interior make VT5-like β-sheet peptides promising alternatives to α-helical amphipathic peptides to serve as vectors for translocating oligonucleotides and membrane impermeable peptides into mammalian cells. More detailed studies into the mechanism and to elucidate the possibility of exploiting β-sheet peptides as transport vectors for oligonucleotides and peptides are under way.

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