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Structure–activity relationship study of the cell-penetrating peptide *p*VEC

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Abstract

The peptide *p*VEC is a recently described cell-penetrating peptide, derived from the murine vascular endothelial-cadherin protein. In order to define which part of this 18-amino acid long peptide is important for the cellular translocation, we performed a structure–activity relationship study of *p*VEC. Together with the L-alanine substituted peptides, the *retro*-*p*VEC, D-*p*VEC and the scramble *p*VEC are studied for comparison. The peptide analogues are labeled with carboxyfluorescein at the N-terminus for monitoring the cellular uptake into human Bowes melanoma cells with different efficacy. We show that all the Fl-*p*VEC analogues internalize in live Bowes melanoma cells. L-Alanine substitution of the five respective N-terminal hydrophobic amino acids significantly decreases the translocation property, while replacing of Arg^6, Arg^8 or Ser^{17} by alanine enhances the uptake. The uptake of *p*VEC is significantly reduced by treatment with an endocytosis inhibitor wortmannin. Treatment with heparinase III, nystatin and EIPA had no effect on the peptide uptake. The data presented here show that the N-terminal hydrophobic part of *p*VEC is crucial for efficient cellular translocation.
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Keywords: *p*VEC; Cell-penetrating peptide; VE-cadherin; Cellular uptake; L-Ala-scan, S.A.R study

1. Introduction

The main field of application for cell-penetrating peptides (CPPs) is the delivery of hydrophilic compounds to the cell interior [1,2]. The 18-amino acid-long peptide *p*VEC has previously been shown to be able to internalize into several cell lines in vitro [3]. *p*VEC is derived from the murine vascular endothelial-cadherin (VE-cadherin) protein which main function is to mediate physical contact between adjacent cells by homophilic dimerization. VE-cadherin also plays an active role in transferring information from the outside of the cell and for the control of vascular permeability and angiogenesis. Four Arg and two Lys residues in the primary sequence make *p*VEC highly positively charged when diluted in physiological buffer. Recently, we showed that the basic amino acids, besides adding positive charges to the peptide, make *p*VEC susceptible to protease degradation [4]. In general, the N-terminus of the *p*VEC sequence is hydrophobic, the middle part charged and the C-terminus hydrophilic. In previous cargo-delivery experiments, *p*VEC was able to carry the proteins avidin [5] and streptavidin [3] and a hexameric PNA oligomer into cells [3]. Further, *p*VEC uptake into several microbial species including gram-negative bacteria, gram-positive bacteria and fungi has been shown together with preferential killing of microbes over eukaryotic cells [6].

The uptake mechanism(s) for cell-penetrating peptides have been investigated over the years. At first, a temperature- and receptor independent uptake pathway was reported for several CPPs. Recent data have, however, revealed a possible common endocytotic internalization mechanism among arginine-rich basic peptides such as poly-Arg, Tat (48–60) and penetratin [7,8].

Here, we carried out a structure–activity relationship (S.A.R.) study of the *p*VEC sequence to define the importance of each residue and the effect of a single substitution on the translocation ability. The amino acids were one by one substituted with L-Ala,

Abbreviations: BMC, Bowes melanoma cells; CPP, cell-penetrating peptide; D-*p*VEC, *ent*-*p*VEC (all-D-enantiomer of *p*VEC); EIPA, 5-(N-ethyl-N-isopropyl)-amiloride; Fl, 5-(and-6) carboxyfluoresceinyl; HKR, HEPES-buffered Krebs–Ringer solution; LDH, lactate dehydrogenase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PI3K, phosphatidylinositol-3-OH kinase; *p*VEC, peptide vascular endothelial-cadherin; RP HPLC, reverse-phase HPLC; S.A.R., structure–activity relationship; VE-cadherin, vascular endothelial-cadherin

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except the two original alanine residues that were changed to D-Ala (Table 1), and the internalization of the fluoresceinyl derivatives was studied in live human Bowes melanoma cells. Additionally, we included the D-enantiomer of *p*VEC (D-*p*VEC), consisting only of the D-amino acids, the *retro*-*p*VEC, with reversed primary sequence, and the scramble *p*VEC peptide (Table 1) to the study. The cellular uptake of the Fl-*p*VEC analogues was quantified in Bowes melanoma cells. The *p*VEC and D-*p*VEC cellular uptake was further quantified in cells, pretreated with known endocytotic inhibitors: wortmannin, nystatin, heparinase III or EIPA. In addition colocalization with tetramethylrodamine (TAMRA) labeled transferrin as a marker of endocytosis was performed, to confirm the quantification studies. The peptide uptake at low temperature was quantified for *p*VEC and D-*p*VEC. The effect of the peptides on cellular toxicity was measured by determining the release of lactate dehydrogenase (LDH).

2. Materials and methods

2.1. Peptide synthesis and labeling

The peptides (Table 1) were separately 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. *tert*-Butyloxycarbonyl amino acids were coupled as hydroxybenzotriazole esters to a *p*-methylbenzylhydrylamine resin (Neosystem, Strasbourg, France) to obtain C-terminally amidated peptides. The peptides were labeled with 5-(and-6)-carboxyfluorescein succinimidyl ester (Molecular Probes, Leiden, Netherlands) at the N-terminus as described previously [3]. Deprotection of the dinitrophenol group was carried out by treatment with 20% thiophenol in DMF for 1 h at room temperature. The peptides were finally cleaved from the solid phase with liquid HF at 0 °C for 1 h in the presence of *p*-cresol.

2.2. HPLC purification and analysis

Purification of peptides with reverse-phase (RP) HPLC was carried out with a Supelcosil LC-18 preparative column (25 cm × 21.2 mm, 5 μm) (Sigma Aldrich Chemie, Steinheim, Germany) using water–acetonitrile, both containing 0.1% CF_3COOH, gradient from 20 to 100%. The purity of the peptides was >95%, according to HPLC analysis performed as described below. Correct molecular masses of purified peptides were obtained by using MALDI-TOF (Voyager-DE STR, Applied Biosystems) mass spectrometer.

The purity and retention times of Fl-peptides (Table 1) were determined using Dionex P680 HPLC gradient system (Idstein, Germany) with Discovery BIO Wide pore C18 RP column (150 × 4.6 mm) (Sigma Aldrich Chemie, Steinheim, Germany) and Dionex RF2000 fluorescence HPLC monitor (Idstein, Germany). 100 μl of Fl-peptide solution in water was subjected to HPLC analysis. The elution was carried out with water (A) and acetonitrile (B), both containing 0.1% CF_3COOH, at a flow rate 1.0 ml/min with gradient of 20–100% B (1–21 min). Fluorescence at 520 nm was recorded after excitation at 445 nm. Average of at least two separate measurements was taken as a retention time with error limits of one standard deviation.

2.3. Cell culture

Human Bowes melanoma cells (BMC, American Type Culture Collection CRL-9607) were cultivated in Minimal Essential Medium (MEM) with glutamax, supplemented with 10% foetal bovine serum, 1% penicillin-streptomycin solution, 1% non-essential amino acids and 1% sodium pyruvate.

2.4. Qualitative cellular uptake of fluoresceinyl-peptides

2.4.1. Fluorescence microscopy

Bowes melanoma cells were seeded at the density of 20 000 cells per well on round glass coverslips in 24-well-plate. One day post-seeding, the culturing media was exchanged into serum-free (SF) media and 10 μM of Fl-peptide was added. In some experiments 50 nM of wortmannin or 50 μg/ml of nystatin were added 30 min prior to adding the peptide. After 1 h of incubation at 37 °C, cells were treated with 0.05% trypsin for 3 min and washed twice with PBS. Coverslips were mounted using Vectashield mounting media (Vector

Table 1
Properties of *p*VEC analogues

Sequence	Name	Molecular mass (Da)	Rt (min ± S.D.)	Positive charges
LLIILRRRIRKQAHAHSK	*p*VEC	2208.7	22.1 ± 0.2	7
ALIILRRRIRKQAHAHSK	[Ala1]*p*VEC	2166.7	21.1 ± 0.9	7
L**A**IILRRRIRKQAHAHSK	[Ala2]*p*VEC	2166.7	20.9 ± 0.2	7
LL**A**ILRRRIRKQAHAHSK	[Ala3]*p*VEC	2166.7	21.5 ± 0.3	7
LLI**A**LRRRIRKQAHAHSK	[Ala4]*p*VEC	2166.7	21.7 ± 0.2	7
LLII**A**RRRIRKQAHAHSK	[Ala5]*p*VEC	2166.7	21.9 ± 1.4	7
LLIIL**A**RRIRKQAHAHSK	[Ala6]*p*VEC	2123.6	23.0 ± 0.4	6
LLIILR**A**RIRKQAHAHSK	[Ala7]*p*VEC	2123.6	22.0 ± 0.7	6
LLIILRR**A**IRKQAHAHSK	[Ala8]*p*VEC	2123.6	25.5 ± 0.2	6
LLIILRRR**A**RKQAHAHSK	[Ala9]*p*VEC	2123.6	21.5 ± 0.8	7
LLIILRRRI**A**KQAHAHSK	[Ala10]*p*VEC	2123.6	16.3 ± 0.9	6
LLIILRRRIR**A**QAHAHSK	[Ala11]*p*VEC	2151.6	22.3 ± 0.2	6
LLIILRRRIRK**A**AHAHSK	[Ala12]*p*VEC	2151.7	21.9 ± 0.2	7
LLIILRRRIRKQ*A*HAHSK	[D-Ala13]*p*VEC	2208.7	21.6 ± 0.1	7
LLIILRRRIRKQA**A**AHSK	[Ala14]*p*VEC	2142.7	21.6 ± 0.5	7
LLIILRRRIRKQAH*A*HSK	[D-Ala15]*p*VEC	2208.7	16.4 ± 0.0	7
LLIILRRRIRKQAHA**A**SK	[Ala16]*p*VEC	2142.7	22.4 ± 1.0	7
LLIILRRRIRKQAHAH**A**K	[Ala17]*p*VEC	2192.7	18.5 ± 3.2	7
LLIILRRRIRKQAHAHS**A**	[Ala18]*p*VEC	2151.6	17.6 ± 1.3	6
KSHAHAQKRIRRRLIILL	*retro*-*p*VEC	2208.7	16.5 ± 4.4	7
LLIILRRRIRKQAHAHSK	D-*p*VEC	2208.7	16.6 ± 0.0	7
IAARIKLRSRQHIKLRHL	scramble *p*VEC	2208.7	10.2 ± 0.01	7

All sequences listed, were C-terminally amidated. Molecular masses are given as calculated for unlabeled peptides. Retention times (Rt) are obtained for Fl-peptides. Number of positive charges is given at physiological pH, together with N- and C-terminal charges.

Laboratories, USA) and fluorescence images were obtained using Leica DM IRE2 fluorescence microscope at 63 times magnification (Leica Microsystems, Stockholm, Sweden) and processed in Photoshop 7.0 software (Adobe Systems Inc, Ca, and USA).

2.4.2. Confocal LASER scanning microscopy

Bowes melanoma cells were seeded at the density of 50,000 cells per well in 8-well chambered coverglasses (Nalge Nunc International, Rochester, NY). Experiment was the conducted as described above, except that cells were co-incubated with 10 μM of Fl-peptide and 50 ng/ml of TAMRA-transferrin in SF media. After 1 h incubation at 37 °C the media with the complexes was removed, the cells were rinsed twice with PBS, covered with PBS and the serial images were recorded using the laser confocal scanning microscope Zeiss Axiovert 200 (Carl Zeiss, Göttingen, Germany), equipped with PerkinElmer Ultra-View ERS Rapid Confocal Imager (PerkinElmer Ltd., Beaconsfield, UK).

2.5. Quantitative cellular uptake of fluoresceinyl-peptides

Cells were seeded in 6-well plates at the density of 3×10^5 cells / well. After 72 h, the cells were washed once with SF media and treated with 10 μM of Fl-peptide in SF media for 1 h at 37 °C. After the incubation, the cells were washed once with trypsin (0.25%), twice with HKR buffer and lysed with 400 μl of 0.1 M NaOH for 10 min at room temperature. The cell lysates were centrifuged (14 000×*g* for 5 min) and the fluorescence intensity of the supernatant (100 μl) measured at 494/518 nm in a Spectramax Gemini XS plate-reader (Molecular Devices, USA). The peptide concentration was calculated from a standard curve of the respective peptide in 0.1 M NaOH. The cellular protein content was determined by the method of Lowry [9] using a Sigma-Aldrich protein assay kit (Sigma-Aldrich, Sweden) with bovine serum albumin (BSA) as standard. The cellular uptake of *p*VEC and its analogues is expressed as picomole peptide per mg of total cellular protein.

To investigate the endocytotic component in the uptake, cells were treated with wortmannin (50 nM), nystatin (50 μg/ml), heparinase III (0.5 units/well) (E.C. 4.2.2.8) or EIPA (50 μM) (all from Sigma-Aldrich, Sweden). The pretreatment was conducted in serum-free (SF) MEM at 37 °C for 30 min, except for the nystatin treatment, which was in HKR supplemented with 0.2% BSA. After 30 min, the heparinase treated cells were rinsed with SF media and then incubated with SF media containing 10 μM of Fl-peptide, added directly to the wortmannin, nystatin and EIPA treated cells. The experiment was then conducted as described above.

2.6. Lactate dehydrogenase release assay

To assess the possible impairment of the cellular plasma membranes during peptide passage, the lactate dehydrogenase (LDH) release assay [10] was performed by using a CytoTox-ONE™ membrane integrity assay kit (Promega), according to the instructions of the manufacturer of the kit. BMC were seeded at a density of 100,000 cells/well in 24-well plates. One day post-seeding, the cells were rinsed with SF media and incubated with SF media containing 10 μM of Fl-peptide. The plate was incubated at 37 °C and 300 rpm in a thermo mixer for 10 min. Background value is obtained from untreated cells and maximum LDH release by treating cells with 0.18% Triton X-100. 100 μl of the cell media was transferred to a black 96-well-plate and 100 μl of the substrate mix was added to each well. After 10 min of incubation at room temperature, the enzymatic reaction was stopped and the fluorescence measured at 560/590 nm in a Spectramax Gemini XS plate-reader (Molecular Devices, USA). The cell toxicity is calculated as: % toxicity = 100 × ((experimental-background) / (maximum-background)).

3. Results

3.1. Quantification of the cellular peptide uptake

Fl-derivatives of *p*VEC were used to quantify the cellular uptake in Bowes melanoma cells after 1 h incubation with 10 μM of peptide at 37 °C.

By substituting any of the five hydrophobic amino acids in the N-terminus to L-alanine the cellular uptake dropped significantly with between 50 and 75% compared to the original *p*VEC sequence (Fig. 1). A significant reduction in fluorescence was also detected when substituting the Ile[9] or His[14] to L-Ala or when applying a *retro*-pVEC. The least efficient uptake was detected for scrambled sequence, where the N-terminal hydrophobic residues are randomly placed into the sequence (Table 1, Fig. 1). In contrast, an increase in cellular uptake was shown when changing the Arg[6], Arg[8] or Ser[17] to L-Ala.

Together our data suggest that the hydrophobic N-terminus is important for peptide uptake, while the rest of the *p*VEC sequence can be modestly changed without impairing the translocation efficacy.

3.2. Effect of endocytosis inhibitors on the pVEC uptake

To investigate the role of endocytosis in the uptake of *p*VEC, we treated the BMC with various endocytosis inhibitors. Cells were treated with wortmannin (50 nM), nystatin (50 μg/ml), heparinase III (0.5 units/ml) or EIPA (50 μM) in serum-free media before incubation with Fl-*p*VEC or Fl-D-*p*VEC. The uptake of both peptides was then quantified as described above in Materials and methods. Wortmannin is known to affect the fusion of early endosomes by inhibiting the phosphatidylinositol-3-OH kinase (PI3K) [11], nystatin to inhibit caveolae

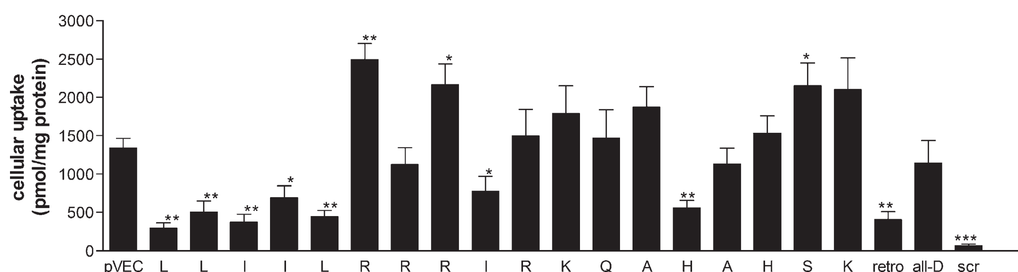

Fig. 1. Quantitative uptake of Fl-*p*VEC analogues in BMC. The cells were treated with 10 μM of Fl-peptide in SF media for 1 h at 37 °C. After the incubation, the cells were washed once with trypsin, twice with HKR buffer and lysed with 0.1 M NaOH. The peptide uptake was determined by measuring the fluorescence intensity of the supernatant and calculated from a standard curve of the respective peptide. The cellular uptake of *p*VEC and its analogues is expressed as picomole peptide per mg of total cellular protein. Results are expressed as mean ± S.E.M. from three independent experiments where each value was determined in duplicates. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ (two-way ANOVA followed by Bonferroni post-test) significantly different from *p*VEC.

formation by binding and precipitating cholesterol from cellular membrane [12,13], heparinase III to enzymatically cleave cell surface sulfated polysaccharides [14] and EIPA to disturb endocytosis via macropinocytosis [15].

Here, wortmannin decreased *p*VEC uptake significantly, by 25%, revealing an endocytotic component of the peptide uptake mechanism (Fig. 2). Treatment with nystatin, heparinase III or EIPA did not affect the *p*VEC uptake to the same extent. Further, none of the four reagents significantly affected the cellular uptake of D-*p*VEC.

3.3. Fluorescence microscopy of peptide uptake

Cells were incubated with 10 μM of the respective peptide at 37 °C for 1 h in serum-free media, followed by treatment with trypsin, to digest the extracellularly bound peptide. All the peptides tested were internalized into live BMC. L-Ala substitutions in the N-terminal hydrophobic domain obviously hindered the uptake, while substitutions in charged or C-terminal region did not visibly affect the uptake or even increased it. Live cell images in Fig. 3 represent L-Ala substitutions in the N-terminal domain—[Ala^{1}]*p*VEC, middle region—[Ala^{6}]*p*VEC and C-terminus—[Ala^{18}]*p*VEC.

To further investigate the role of hydrophobic domain in the uptake of *p*VEC, we introduced two additional sequences; one shortened N-terminally by 3 amino acids and another where entire hydrophobic domain was replaced by L-alanine. Deletion of 3 hydrophobic residues completely abolished uptake (Fig. 5d). Also, when all 5 hydrophobic residues were replaced by L-alanines in the N-terminus, the uptake of peptide was not detected (Fig. 5g).

To confirm the data from the experiment of endocytosis inhibitors effect on the uptake of pVEC we treated Bowes melanoma cells with endocytosis inhibitors 50 nM wortmannin (W) and 50 μg/ml nystatin (N), for 30 min prior the treatment with peptides and examined the cells by fluorescence microscopy (Fig. 3). No difference was detected with microscopy in the uptake of Fl-*p*VEC analogues when treated with different endocytosis inhibitors.

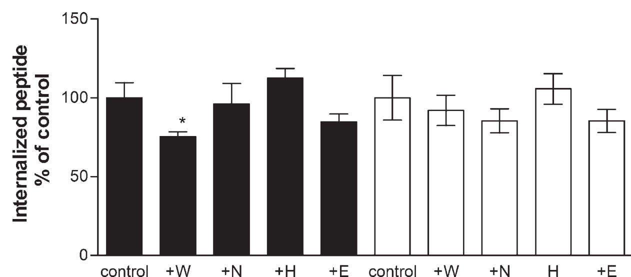

Fig. 2. Quantitative uptake of Fl-*p*VEC and Fl-D-*p*VEC in BMC pretreated with endocytosis inhibitors. BMC were treated with 50 nM wortmannin (W), 50 μg/ml nystatin (N), 0.5 units/ml heparinase III (H) or 50 μM EIPA (E). Thereafter, 10 μM of Fl-*p*VEC (solid bars) or Fl-D-*p*VEC (open bars) was added in SF media for 1 h at 37 °C. After the incubation, the cells were washed once with trypsin, twice with HKR buffer and lysed with 0.1 M NaOH. The peptide uptake was determined by measuring the fluorescence intensity of the supernatant and calculated from a standard curve of the respective peptide. The cellular uptake is expressed as percentage of uptake compared to untreated cells (control). Results are expressed as mean ± S.E.M. from three independent experiments where each value was determined in duplicates. *$P<0.05$; (two-way ANOVA followed by Bonferroni post-test) significantly different from *p*VEC or D-*p*VEC uptake in untreated control cells.

To further confirm the involvement of endocytotic component, the colocalization with transferrin was conducted using Confocal LASER Scanning Microscopy. In our experiments little colocalization with Fluorescein-labeled pVEC and TAMRA-labeled transferrin was detected, confirming the results obtained from uptake quantification experiments (Fig. 6).

3.4. Temperature effect on cellular uptake

Several investigators have shown that endocytosis has a threshold temperature of 10 °C [18,19]. Thus, the incubation of cells at temperatures lower than threshold temperature is an effective method to investigate the role of endocytosis.

Previously we have shown *p*VEC uptake at low temperature with fluorescence microscopy on fixed cells [3]. Here we quantified the uptake of *p*VEC and D-*p*VEC into live BMC at low temperature (+8 °C) by using the assay described above, which was not carried out in the previous *p*VEC studies. BMC were incubated with 10 μM of *p*VEC or D-*p*VEC at 37 °C or 8 °C to examine the temperature dependency of the peptide uptake. We did not detect any significant change in peptide uptake when lowering the incubation temperature to +8 °C (Fig. 4), which correlates well with previous observations.

3.5. Peptide toxicity

The differences recorded in cellular uptake of the *p*VEC analogues might be caused by impairment of the cell membranes that would cause a higher leakage and not a higher peptide uptake. Therefore peptide toxicity was detected by a cell leakage assay, which measures the release of lactate dehydrogenase (LDH) from cells. BMC were treated with the respective Fl-peptide (10 μM) and the released LDH was measured. None of the peptides used here caused cell leakage at 10 μM concentration, when the cells were treated for 10 min at 37 °C (data not shown).

Prolongation of the incubation time to 1 h did not increase the LDH leakage. Prolongation of the incubation time also increases the possibility of LDH breakdown and may, in turn lead to the underestimation of the toxicity [10]. Our results, however, show that the registered differences in cellular uptake are not caused by membrane toxicity.

3.6. Physico-chemical properties of pVEC analogues

The *p*VEC analogues displayed less than 2 min differences in RP HPLC retention times, with the exception of the inverted and the scramble analogues, [Ala^{10}]pVEC and [D-ala^{15}]pVEC (Table 1), all having significantly shorter retention times. Interestingly, D-isomer also showed shorter retention time. This was unexpected, but can be explained with different charge distribution of those peptides or, in case of [D-Ala15]pVEC, with conformational change of amino acid side-chain. Much shorter retention time of D-isomer was also unexpected,

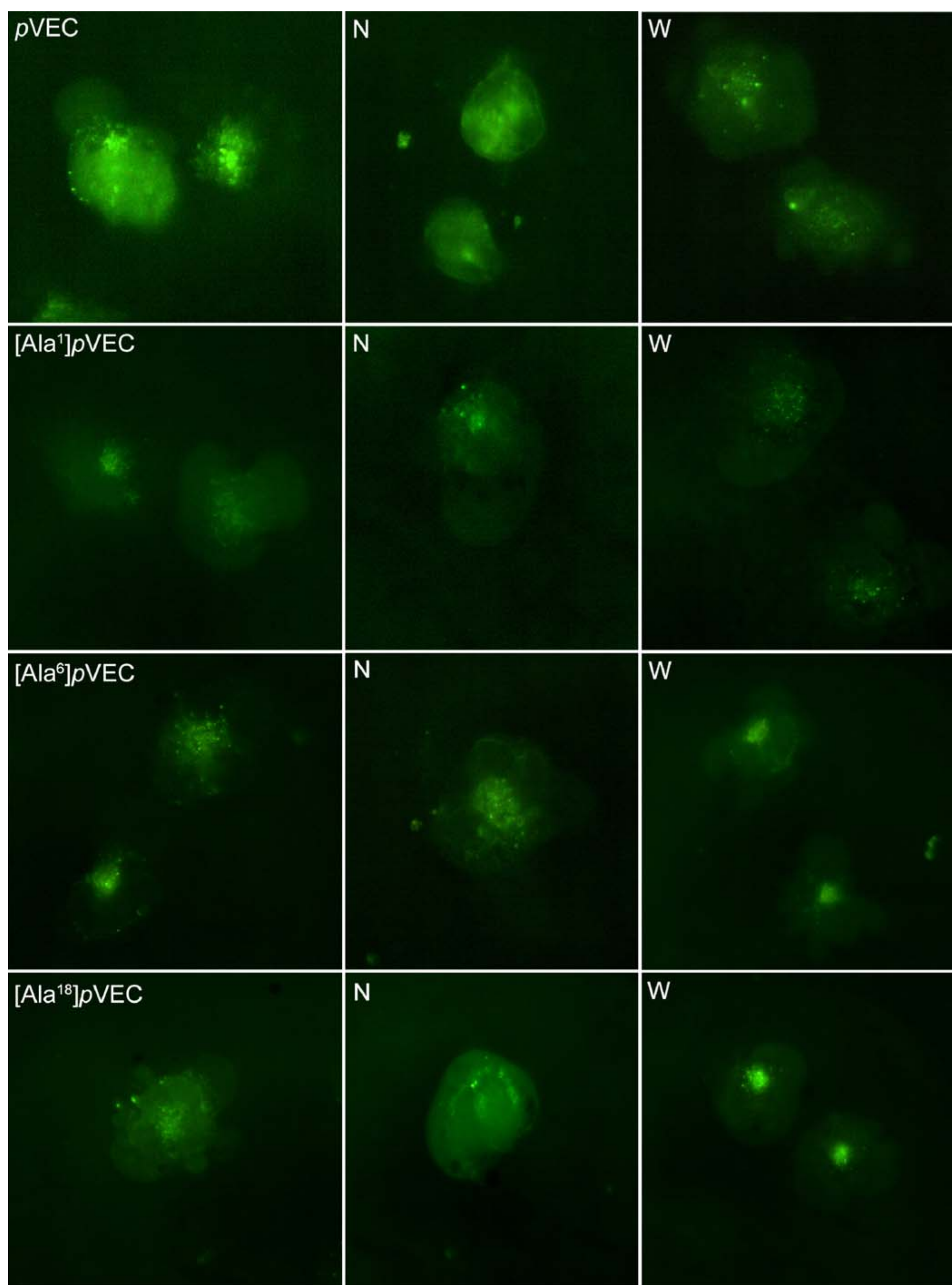

Fig. 3. Internalization of Fl-pVEC analogues into BMC. Cells, attached to the glass coverslips were treated with 10 μM of Fl-peptide in SF media for 1 h at 37 °C. After the incubation cells were treated with 0.05% trypsin for 3 min, washed twice with PBS buffer and mounted, using Vectashield mounting media. In parallel experiments nystatin (N) or wortmannin (W) were used to estimate the extent of endocytosis involved in uptake.

especially since the uptake was almost equal for both D- and L-isomer. In general, substitution made in the N-terminal hydrophobic end of the peptide tends to shorten the retention time, whereas substitution in the C-terminal part does not affect the retention time that much.

Substitution of leucine residues in the N-terminal hydrophobic domain shortened the retention times about 1–2 min, depending on the residue, whereas replacement of isoleucine residues has less effect on the retention times. This may indicate that leucine residues in the N-terminal hydrophobic domain are more important in forming hydrophobic interactions than isoleucine residues.

4. Discussion

We have previously reported that *p*VEC, a peptide derived from the murine VE-cadherin efficiently translocates into several cell lines [3]. To evaluate the importance of the

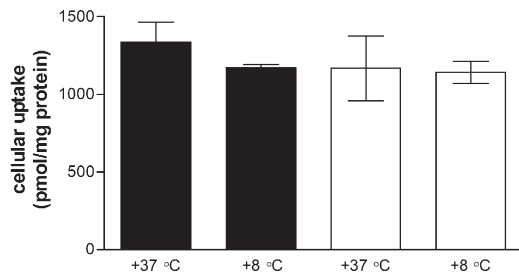

Fig. 4. Quantitative uptake of Fl-*p*VEC and Fl-D-*p*VEC in BMC at different temperatures. The cells were treated with 10 μM of Fl-pVEC (solid bars) or Fl-D-pVEC (open bars) in SF media for 1 h at either 37 °C or 8 °C. After the incubation, the cells were washed once with trypsin, twice with HKR buffer and lysed with 0.1 M NaOH. The peptide uptake was determined by measuring the fluorescence intensity of the supernatant and calculated from a standard curve of the respective peptide. The cellular uptake is expressed as picomole peptide per mg of total cellular protein. Results are expressed as mean ± S.E.M. from three independent experiments where each value was determined in duplicates.

individual amino acids in the peptide sequence, we performed an L-Ala-scan where each amino acid in *p*VEC sequence is replaced, one by one, with L-Ala, except the two original alanine residues in the sequence that were changed to D-Ala. The analogues were labeled with fluorescein at the N-terminus for monitoring the cellular internalization and for quantification of the uptake in Bowes cells. Together with the L-Ala-analogues of *p*VEC, an analogue with the inverted sequence, *retro*-*p*VEC, and the D-*p*VEC were included in this study. The scrambled *p*VEC, which was introduced recently [4], was additionally included as a negative uptake control.

Previous S.A.R studies have been published for Tat (48–60) [20] penetratin [21,22] transportan [23,24] and a model amphipathic peptide [25]. Since these peptides lack the hydrophobic domains, the studies have mostly shown the importance of positive charges in cellular uptake. However, study by Fischer et al. shows also the decreased uptake when hydrophobic residues are substituted, but this trend is less pronounced [22].

Even though the representatives of the CPP-family are largely different in their lengths, charges and structures, some general conclusions on peptide features can be drawn from the S.A.R. studies. One is the abundance of multiple positive charges, especially arginine residues, in the primary peptide sequence [26,27]. The ability of a peptide to form an amphipathic helix has also been suggested as key element for efficient translocation [25].

We used an uptake quantification method where adhered Bowes melanoma cells are treated with Fl-peptides. The cells are, after peptide treatment, rinsed thoroughly with buffer solution and trypsin, lysed with 0.1 M NaOH and the fluorescence intensity in the cell lysates determined after centrifugation. The trypsin treatment degrades extracellulary bound peptide that otherwise could be interpreted as internalized.

Generally, *p*VEC consists of a hydrophobic N-terminus, an arginine-rich middle region and a hydrophilic C-terminus. Our data show that L-Ala substitution of the five hydrophobic N-terminal residues significantly decreased the cellular uptake. Prolonging the hydrophobic stretch by changing Arg^6 to L-Ala increased the uptake. A slightly higher uptake was also recorded when substituting the Ser^{17} and the Lys^{18} at the C-terminal of *p*VEC. We also quantified the uptake of scramble *p*VEC, where the N-terminal hydrophobic domain is broken, but the overall positive charge of the peptide is not altered (Table 1). The uptake of scramble *p*VEC was significantly lower than the original peptide (Fig. 1). Moreover, when we deleted 3 amino acids from the N-terminus the uptake of the peptide was completely abolished (Fig. 5d), also the replacement of the entire 5 amino acid long hydrophobic domain by alanine decreased the uptake to undistinguishable from the background (Fig. 5g). Together these data provide evidence on the importance of the hydrophobic N-terminal amino acids for efficient peptide uptake.

Recent studies showed that the uptake of Tat (48–60), $(Arg)_9$ [16] and penetratin [17] was reduced when depleting the cellular pool of ATP and lowering the temperature, suggesting an endocytotic uptake component. Wadia and coworkers reported recently that the uptake of Tat-cre fusion protein takes place mostly by lipid-raft mediated macropinocytosis [28]. The role of macropinocytosis was also reported for oligoarginines but not for penetratin suggesting that different internalization pathways are used by different arginine-rich CPPs [29].

Wortmannin is an inhibitor of PI3K; an enzyme involved both in clathrin-dependent endocytosis [11] and macropinocytosis [30]. EIPA, another well-characterized endocytosis inhibitor specifically affects the macropinocytotic uptake pathway by inhibiting Na^+/H^+ exchange protein [15]. In our experiment 50 nM wortmannin significantly, by about 25%, lowered the uptake of *p*VEC into Bowes cells whereas 50 μM EIPA did not affect the uptake. These results indicate that clathrin-dependent endocytosis prevails over macropinocytosis in the *p*VEC uptake mechanism. Fischer and coworkers recently reported approximately 50% lower uptake for Fl-penetratin and Fl-$(Arg)_9$ after treatment with 100 nM wortmannin in MC57 cells [8]. Further experiments using chloroquine and bafilomycin A1, inhibitors of endosomal acidification and vacuolar H^+-ATPases respectively, also decreased the uptake of peptides suggesting the mechanisms of clathrin-dependent endocytosis and, further, retrograde *trans*-Golgi transport to the ER [8].

Previous report from Suzuki and coworkers shows approximately 7% decreased uptake of 1 μM Fl-Tat (48–60) and Fl-Arg_8 in HeLa cells pretreated with 50 nM wortmannin for 30 min, approximately 3% less uptake was shown when cells were pretreated with 50 μg/ml nystatin [7]. The minor effect of wortmannin can be explained with its lower concentration compared to present work and recently reported [8].

Although the decrease in uptake was significant after wortmannin treatment, remaining cellular internalization may indicate the presence of another complementary uptake mechanism. The translocation of the D-*p*VEC and the *retro*-*p*VEC shows that no specific receptor is involved in the uptake process. The significant difference in the uptake of the latter two, structurally very similar peptides can be explained

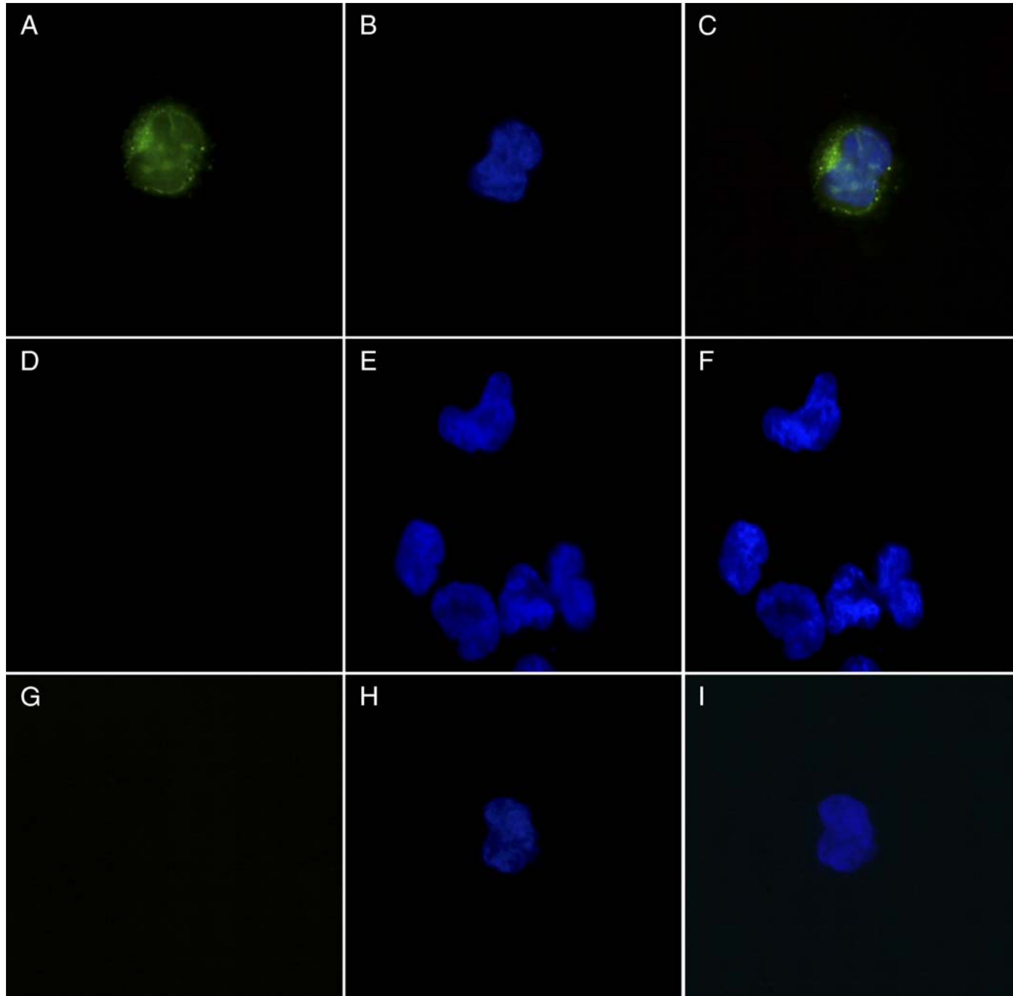

Fig. 5. N-terminal modifications abolish peptide internalization. BMC, attached to glass coverslips were treated with 10 μM Fl-pVEC (top), Fl-des-(1–3)pVEC (middle) or Fl-[Ala$^{1-5}$]pVEC (bottom row) respectively, in SF media for 1 h at 37 °C. After the incubation cells were treated with 0.05% trypsin for 3 min, washed twice with PBS buffer and nuclei were stained with fluorescent Hoechst stain. Thereafter the glass coverslips were mounted, using Vectashield mounting media. Fl-pVEC (A) is internalized into BMC. Deletion of three amino acids from N-terminus completely abolishes the uptake of Fl-labeled peptide (D), as well as replacement of entire N-terminal hydrophobic domain by alanine residues (G). B, E, H—nuclear staining with Hoechst stain. C, F, I—merged images of fluorescein and Hoechst.

with the resistance to the trypsin degradation of the D-*p*VEC prior measuring the uptake. Removal of cell-surface proteoglycans with heparinase III, which otherwise could explain the uptake of both D- and L-enantiomers as well as peptides with inversed sequences, did not alter the internalization of *p*VEC nor its D-enantiomer. Endocytotic processes are considered to be halted at temperatures below +10 °C as the flexibility of plasma membrane is drastically reduced [18,19]. However, substrate binding to the membranes still takes place at lower temperatures; therefore the trypsin treatment in order to remove membrane bound peptide is essential when investigating internalization of a CPP at low temperature. In our experiment the uptake of *p*VEC at +8 °C was not reduced, further confirming the presence of non-endocytic uptake mechanism (Fig. 6).

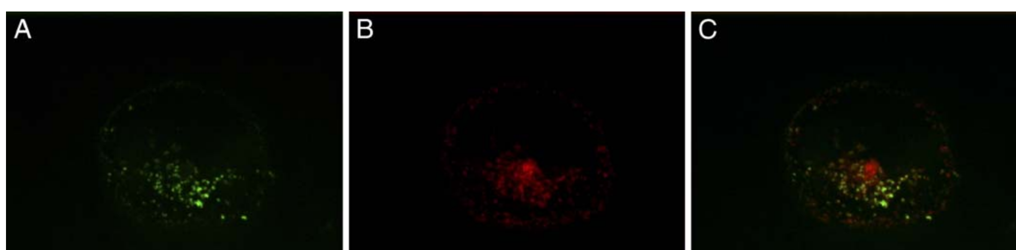

Fig. 6. Colocalization of Fl-pVEC and TMR-transferrin. BMC were coincubated with 10 μM Fl-pVEC (A) and 50 μg/ml TMR-transferrin (B) in SF media for 1 h at 37 °C. Thereafter cells were rinsed twice with PBS, covered with PBS and submitted to confocal microscopy, as described in Materials and methods. Merged image (C) shows relatively modest colocalization of pVEC and transferrin.

In the light of our current data it seems likely, that pVEC due to its partial transmembrane origin [3] has some kind of direct interaction with plasma membrane through the N-terminal hydrophobic stretch. Most likely this part inserts to the plasma membrane and this allows the cargo/marker to internalize. This would explain the equal internalization of both L- and D-isomers and the reversed sequence. This type of membrane insertion can also explain the very low toxicity of pVEC even at relatively high concentrations (10 μM).

On the other hand, scramble pVEC does not have hydrophobic domain in its N-terminus (Table 1), hence it is logical to see the decreased uptake. Additionally, the removal of 3 hydrophobic amino acids abolished the uptake.

The potential limitations of using high concentrations of peptides are their possible effects on the composition of plasma membrane. Massive binding of hydrophobic peptide to the membrane may potentially lead to the disturbance in the coordination of membrane lipids, which in turn may lead to the increase in peptide internalization. However, this kind of disturbance would probably also lead to the increased cellular toxicity and could be detected as leakage of LDH. Since in present study, 10 μM peptide concentration showed no leakage of LDH after 1 h, we assumed that composition of plasma membrane was not disturbed.

Previously we have reported that *p*VEC–avidin complex is mostly internalized in an ATP-dependent manner and inhibition of clathrin-dependent endocytosis only slightly decreased the uptake. The internalization of *p*VEC–avidin complex was also remarkably decreased when cholesterol was depleted from cellular membranes [5]. This indicates, together with present data, that different uptake pathways may be used for different CPPs. Also, different pathways may exist, depending on whether the cargo is attached to the peptide or not.

In conclusion, the N-terminal hydrophobic stretch is crucial for efficient cellular uptake of *p*VEC. Substitution of the hydrophobic N-terminal amino acids to Ala significantly decreased the cellular uptake. Our results further revealed the presence of an endocytotic component in the uptake of *p*VEC.

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References

[1] C. Rousselle, P. Clair, J.M. Lefauconnier, M. Kaczorek, J.M. Scherrmann, J. Temsamani, New advances in the transport of doxorubicin through the blood–brain barrier by a peptide vector-mediated strategy, Mol. Pharmacol. 57 (2000) 679–686.

[2] M. Pooga, U. Soomets, M. Hällbrink, A. Valkna, K. Saar, K. Rezaei, U. Kahl, J.X. Hao, X.J. Xu, Z. Wiesenfeld-Hallin, T. Hökfelt, T. Bartfai, Ü. Langel, Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission in vivo, Nat. Biotechnol. 16 (1998) 857–861.

[3] A. Elmquist, M. Lindgren, T. Bartfai, Ü. Langel, VE-cadherin-derived cell-penetrating peptide, pVEC, with carrier functions, Exp. Cell. Res 269 (2001) 237–244.

[4] A. Elmquist, Ü. Langel, In vitro uptake and stability study of pVEC and its all-D analog, Biol. Chem. 384 (2003) 387–393.

[5] P. Säälik, A. Elmquist, M. Hansen, K. Padari, K. Saar, K. Viht, Ü. Langel, M. Pooga, Protein cargo delivery properties of cell-penetrating peptides. A comparative study, Bioconjug. Chem. 15 (2004) 1246–1253.

[6] N. Nekhotiaeva, A. Elmquist, G.K. Rajarao, M. Hällbrink, Ü. Langel, L. Good, Cell entry and antimicrobial properties of eukaryotic cell-penetrating peptides, FASEB J. 18 (2004) 394–396.

[7] T. Suzuki, S. Futaki, M. Niwa, S. Tanaka, K. Ueda, Y. Sugiura, Possible existence of common internalization mechanisms among arginine-rich peptides, J. Biol. Chem. 277 (2002) 2437–2443.

[8] R. Fischer, K. Kohler, M. Fotin-Mleczek, R. Brock, A stepwise dissection of the intracellular fate of cationic cell-penetrating peptides, J. Biol. Chem. 279 (2004) 12625–12635.

[9] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.

[10] C. Korzeniewski, D.M. Callewaert, An enzyme-release assay for natural cytotoxicity, J. Immunol. Methods 64 (1983) 313–320.

[11] A. Simonsen, R. Lippe, S. Christoforidis, J.M. Gaullier, A. Brech, J. Callaghan, B.H. Toh, C. Murphy, M. Zerial, H. Stenmark, EEA1 links PI (3)K function to Rab5 regulation of endosome fusion, Nature 394 (1998) 494–498.

[12] K.G. Rothberg, Y.S. Ying, B.A. Kamen, R.G. Anderson, Cholesterol controls the clustering of the glycophospholipid-anchored membrane receptor for 5-methyltetrahydrofolate, J. Cell Biol. 111 (1990) 2931–2938.

[13] K.G. Rothberg, J.E. Heuser, W.C. Donzell, Y.S. Ying, J.R. Glenney, R.G. Anderson, Caveolin, a protein component of caveolae membrane coats, Cell 68 (1992) 673–682.

[14] J. Yahalom, A. Eldor, Z. Fuks, I. Vlodavsky, Degradation of sulfated proteoglycans in the subendothelial extracellular matrix by human platelet heparitinase, J. Clin. Invest. 74 (1984) 1842–1849.

[15] M.A. West, M.S. Bretscher, C. Watts, Distinct endocytotic pathways in epidermal growth factor-stimulated human carcinoma A431 cells, J. Cell Biol. 109 (1989) 2731–2739.

[16] J.P. Richard, K. Melikov, E. Vivés, C. Ramos, B. Verbeure, M.J. Gait, L.V. Chernomordik, B. Lebleu, Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake, J. Biol. Chem. 278 (2003) 585–590.

[17] P.E. Thorén, D. Persson, P. Isakson, M. Goksör, A. Önfelt, B. Nordén, Uptake of analogs of penetratin, Tat(48–60) and oligoarginine in live cells, Biochem. Biophys. Res. Commun. 307 (2003) 100–107.

[18] P.H. Weigel, J.A. Oka, Temperature dependence of endocytosis mediated by the asialoglycoprotein receptor in isolated rat hepatocytes. Evidence for two potentially rate-limiting steps, J. Biol. Chem. 256 (1981) 2615–2617.

[19] S.C. Silverstein, R.M. Steinman, Z.A. Cohn, Endocytosis, Annu. Rev. Biochem. 46 (1977) 669–722.

[20] P.A. Wender, D.J. Mitchell, K. Pattabiraman, E.T. Pelkey, L. Steinman, J.B. Rothbard, The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 13003–13008.

[21] G. Drin, M. Mazel, P. Clair, D. Mathieu, M. Kaczorek, J. Temsamani, Physico-chemical requirements for cellular uptake of pAntp peptide. Role of lipid-binding affinity, Eur. J. Biochem. 268 (2001) 1304–1314.

[22] P.M. Fischer, N.Z. Zhelev, S. Wang, J.E. Melville, R. Fahraeus, D.P. Lane, Structure–activity relationship of truncated and substituted analogues of the intracellular delivery vector Penetratin, J. Pept. Res. 55 (2000) 163–172.

[23] U. Soomets, M. Lindgren, X. Gallet, M. Hällbrink, A. Elmquist, L. Balaspiri, M. Zorko, M. Pooga, R. Brasseur, Ü. Langel, Deletion analogues of transportan, Biochim. Biophys. Acta 1467 (2000) 165–176.

[24] M. Lindgren, X. Gallet, U. Soomets, M. Hällbrink, E. Bråkenhielm, M. Pooga, R. Brasseur, Ü. Langel, Translocation properties of novel cell penetrating transportan and penetratin analogues, Bioconjug. Chem. 11 (2000) 619–626.

[25] A. Scheller, J. Oehlke, B. Wiesner, M. Dathe, E. Krause, M. Beyermann, M. Melzig, M. Bienert, Structural requirements for cellular uptake of alpha-helical amphipathic peptides, J. Pept. Sci. 5 (1999) 185–194.
[26] S. Futaki, T. Suzuki, W. Ohashi, T. Yagami, S. Tanaka, K. Ueda, Y. Sugiura, Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery, J. Biol. Chem. 276 (2001) 5836–5840.
[27] D.J. Mitchell, D.T. Kim, L. Steinman, C.G. Fathman, J.B. Rothbard, Polyarginine enters cells more efficiently than other polycationic homopolymers, J. Pept. Res. 56 (2000) 318–325.
[28] J.S. Wadia, R.V. Stan, S.F. Dowdy, Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis, Nat. Med. 10 (2004) 310–315.
[29] I. Nakase, M. Niwa, T. Takeuchi, K. Sonomura, N. Kawabata, Y. Koike, M. Takehashi, S. Tanaka, K. Ueda, J.C. Simpson, A.T. Jones, Y. Sugiura, S. Futaki, Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement, Mol. Ther. 10 (2004) 1011–1022.
[30] N. Araki, M.T. Johnson, J.A. Swanson, A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages, J. Cell Biol. 135 (1996) 1249–1260.