Protein Cargo Delivery Properties of Cell-Penetrating Peptides. A Comparative Study

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Application of cell-penetrating peptides for delivering various hydrophilic macromolecules with biological function into cells has gained much attention in recent years. We compared the protein transduction efficiency of four cell-penetrating peptides: penetratin, Tat peptide, transportan, and pVEC and studied the effects of various medium parameters on the uptake. Depletion of cellular energy and lowering of temperature strongly impaired the internalization of protein complexed with cell-penetrating peptides, confirming the endocytotic mechanism of peptide-mediated protein cellular transduction. Peptide-induced protein association with HeLa cells decreased 3−6-fold in energy-depleted cells. Inhibition of clathrin-dependent endocytosis by the hyperosmolar medium decreased the uptake of peptide-avidin complexes 1.5−3-fold and the removal of cholesterol from the plasma membrane 1.2−2-fold, suggesting that both clathrin-dependent and independent endocytosis were involved in peptide-induced cellular delivery of avidin. However, even under conditions of cellular energy depletion, ceasing of cellular traffic, and partial depolarization of plasma membrane, peptide−protein complexes associated with HeLa cells, as observed by FACS analysis and spectrofluorimetry. Among the studied peptides, pTat and transportan revealed higher protein transduction efficiency than penetratin or pVEC.

INTRODUCTION

Discovery and design of various cell-penetrating peptides (CPP) (for reviews see ref 1) have opened a possibility of introducing different hydrophilic macromolecules into cells (*2*, *3*). These peptides show a capability to enter cells in culture even at low temperature and in the presence of endocytosis inhibitors. Although the primary structure of CPPs is highly variable, some general features, envisaged to be essential for efficient cell entry, can be outlined. An abundance of positive charges, especially from arginines (*4*−*6*) and the presence of amino acids with a bulky hydrophobic side-chain (*7*) in the peptide sequence have been suggested to be crucial for efficient cellular uptake. The ability of a peptide to form an amphipathic helix is in correlation with cellular uptake efficacy for some peptides (*8*), whereas for most CPPs, folding into an α-helical structure is not required.

Recently we compared the carrier properties of the four most widely used CPPs, loading them with a small model peptide as a cargo (*9*). The obtained results enabled the division of the peptides into two major classes, based on cargo delivery efficiency and membrane disturbance. Tat peptide (pTat) and penetratin had no adverse effect on plasma membrane permeability in high concentration, but possessed lower cargo delivery efficiency. The model amphipathic peptide (MAP) and transportan, on the contrary, showed higher delivery yield but induced stronger plasma membrane leakage (*9*).

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It was recently suggested that some arginine-rich peptides share a common mechanism of cell entry (*10*). On the other hand, different cellular uptake mechanisms for the arginine-rich pTat and its protein conjugates were demonstrated (*11*). However, translocation of fluoresceinyl-penetratin and -pTat into living cells was questioned lately (*12*, *13*). The uptake detected at higher concentrations was ascribed to membrane perturbation and the toxic effects of labeled peptides. Still, the conjugates of the respective CPPs with PNA oligomers internalized efficiently (*12*). The uptake of fluorescein-labeled pTat and Arg_9 was suggested to proceed mainly by the endocytic pathway (*13*). In addition, internalization of lipid-interacting peptides SynB5 and penetratin was also shown to proceed by absorptive endocytosis rather than by temperature-independent translocation (*14*, *15*). The characteristic diffuse localization of CPPs in the cytoplasm reported in numerous studies earlier was suggested to stem from the fixation and permeabilization of cells (*13*, *16*). On the other hand, the uptake of pTat and Arg_7 by living cells under endocytosis-blocking conditions was demonstrated recently (*17*), and the authors suggested the presence of an additional nonendocytic pathway. Later the observed diffuse localization of CPPs in the cytoplasm associated with the nonendocytic internalization was suggested to be caused by the escape of peptides from endosomal structures, since the inhibition of acidification of endosomes blocked the translocation of CPPs into cytoplasm (*18*, *19*). Cellular uptake of heterologous fusion proteins comprising Tat or its protein transduction domain and EGFP is shown to proceed by a cholesterol- and cellular-energy-dependent caveolae-mediated pathway (*20*, *21*). The cellular uptake of Cre-

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Table 1. Sequences and Molecular Masses of Studied Peptides

peptide (abbreviation)	sequence[a]	calculated Mw	obtained Mw	+biotin (calcd)	+biotin (obt)
Penetratin (pAntp)	R*QIKIWFQNRRMKWKK amide	2246	2249.2	2473	2473.9
pTat	G*RKKRRQRRRPPQ amide	1718	1719	1945	1945.2
pVEC	L*LIILRRRIRKQAHAHSK amide	2209	2208.7	2436	2435
Transportan (TP)	GWTLNSAGYLLGK*INLKALAA LAKKIL amide	2842	2841.1	3069	3066.7

[a] *Amino acid labeled with biotin; the peptides (except transportan) are tagged at the N-terminus of the peptide; transportan is labeled at N^{ϵ}-lysine.

recombinase fusion protein with the pTat, however, was very recently demonstrated to occur by caveolin-independent macropinocytosis of lipid rafts (*22*).

The present study characterizes the ability of CPPs (see Table 1 for structures) to target a protein cargo into HeLa cells. We studied the impact of low temperature, endocytosis inhibitors, modulation of the plasma membrane composition and culture medium on the protein cellular transduction efficiency. Inhibition of relevant cellular processes affects the cargo delivery efficiency of different CPPs in a divergent manner. We assume that the observed changes in protein delivery may help elucidate the mechanism by which the four studied peptides facilitated the cellular uptake of proteins.

MATERIALS AND METHODS

Peptide Synthesis. The peptides (Table 1) were synthesized as described earlier (*23*, *24*) on a peptide synthesizer (Applied Biosystems model 431A, USA) in a stepwise manner in a 0.1-mmol scale using the *t*-Boc strategy of solid-phase peptide synthesis. *tert*-Butyloxycarbonyl amino acids were coupled as hydroxybenzotriazole (HOBt) esters to a *p*-methylbenzylhydrylamine (MBHA) resin (Neosystem, Strasbourg, France) to obtain C-terminally amidated peptides.

Biotin was coupled manually to the N-terminus (or to $N^{\epsilon 13}$-Lys in transportan) of the peptide by adding a 3-fold excess of HOBt and *o*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluorborate (TBTU) activated biotin (Chemicon, Hampshire, UK) in DMF to the peptidyl-resin. Deprotection of the dinitrophenyl group was carried out by treatment with 20% thiophenol in DMF for 1 h at room temperature. Deformylation was carried out by treatment with 20% piperidine in DMF for 40 min at room temperature. The peptides were finally cleaved from the resin with liquid HF at 0 °C for 1 h in the presence of *p*-cresol. Purification of peptides with reverse-phase 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 peptides was >98% as demonstrated by HPLC on an analytical Nucleosil 120-3 C-18 RP-HPLC column (0.4 × 10 cm). Correct molecular masses were obtained by using a MALDI-TOF mass spectrometer (Voyager-DE STR, Applied Biosystems, Foster City, CA) (Table 1).

Cell Culture. HeLa cells (ATCC CCL-2) were cultivated in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum (FCS) at 37 °C in humidified atmosphere containing 5% CO_2. Effects of CPPs on plasma membrane integrity were assayed by measuring the LDH release using the CytoTox-ONE Assay (Promega Corp. Madison, WI). In parallel, the MTT-assay was also performed.

Uptake Experiments. The cells were incubated with fluorescein-labeled avidin complexed with biotinyl-CPPs in IMDM containing 10% FCS if not stated otherwise. The CPP−avidin complexes were preformed by associating fluorescein-labeled avidin with biotinyl-CPP at room temperature in a minimal volume for 5 min and applied to the cells after dilution with the culture medium to the concentration of 0.15 μM and 0.5 μM, respectively. Incubation was carried out at 37 °C or on ice, and the incubation time was varied from 15 min to 24 h, depending on the experiment. For experiments at low temperature, the cells were preincubated on ice for 15 min. Depletion of cellular energy was achieved by inhibiting oxidative phosphorylation and glycolysis with 0.5 or 1% sodium azide and 25 or 50 mM 2-deoxyglucose (DOG), respectively, either alone or in combination (*25*) prior to the application of complexes. The hyperosmolar medium was prepared by dissolving sucrose at a concentration of 0.4 M in IMDM, and solutions containing deoxyglucose were prepared in Dulbecco's PBS. The cells were treated with a solution containing the respective agent for 30 min, and then avidin or CPP−avidin complexes in the same solution were applied and the uptake was measured after 30 min incubation. Organization of plasma membrane lipids was modified by incubating the cells in serum-free IMDM with 10 mM methyl β-cyclodextrin, which extracts cholesterol from the cells' plasma membrane (*26*), leads to the disappearance of rafts and caveolae and inhibits their re-formation (*27*).

FACS Analysis. The cells were seeded in six-well plates to a density of 250 000 cells per well in 2.5 mL medium and used for experiments 1 day after seeding at 70−90% confluence. After incubation with peptide−protein complexes, the cells were rinsed twice with PBS and detached from the plastic by incubating with trypsin solution (0.5 mg/mL in PBS containing 0.2 mg/mL EDTA) at 37 °C for 3, 15, or 30 min, respectively. Trypsinized cells were suspended, collected by centrifugation at 250*g* for 5 min at 4 °C, and resuspended in PBS. The intactness of the cells was estimated by resuspending the cells' pellet in PBS containing 1 μg/mL of propidium iodide. In the flow cytometry analysis 30 000 cells per sample were counted by the FACS Calibur, using Cell Quest software (BD Biosciences, Heidelberg, Germany). Each value represents a mean of three separate determinations.

Spectrofluorimetric Quantification of Internalized Avidin. The cells were treated with the complexes as described in uptake experiments and trypsinized for 15 min, and the washed cell pellet was dissolved in 1 mL 0.1 M NaOH. The fluorescence signal in the obtained solution was measured in a 10 × 10 mm cuvette at 520 nm using excitation at 490 nm by a F-4500 fluorescence spectrophotometer (Hitachi, Japan). Quantitative data analysis was performed by MS Excel.

Confocal Scanning Microscopy. HeLa cells in eight-well chambered coverglasses (Nalge Nunc International, Rochester, NY) were incubated with 0.15 μM Alexa Fluor 488 labeled avidin (Molecular Probes, Leiden, Netherlands) and 0.5 μM biotinyl-CPP in 10% of FCS containing

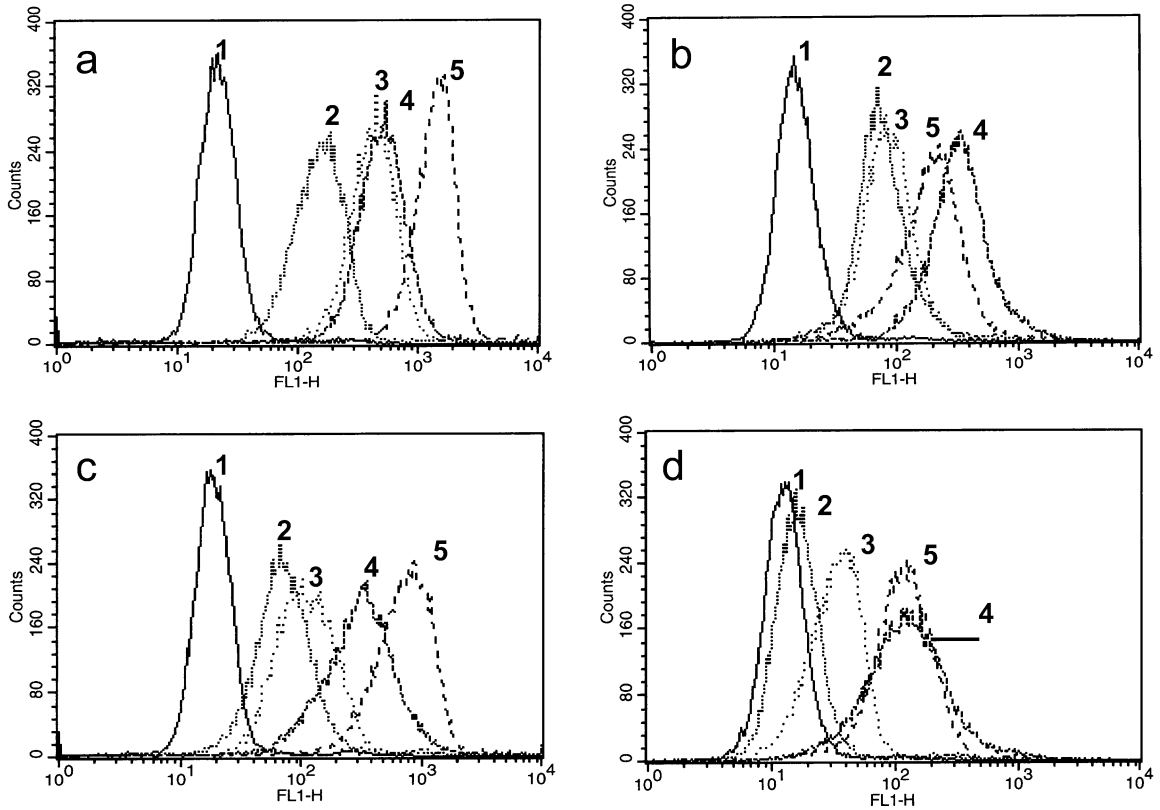

Figure 1. Cellular uptake of FITC-labeled avidin complexes with different biotinyl-CPPs. HeLa cells were incubated with complexes of 0.15 μM FITC-labeled avidin and 0.5 μM biotinyl-CPP for 30 min at 37 °C (a, c) or 4 °C (b, d) and thereafter treated with trypsin for 3 min (a, b) or 15 min (c, d). The cells were incubated with FITC-labeled avidin (1) or complexed with biotin-tagged penetratin (2), pVEC (3), pTat (4), or transportan (5). The number of cells (*y*-axis) is plotted against the fluorescence signal of cells (*x*-axis).

IMDM for 45 min at 37 °C. The medium with the complexes was removed, the cells were rinsed twice with PBS, covered with PBS (containing 1 mM of $CaCl_2$ and 0.5 mM $MgCl_2$), and the serial images were recorded using the laser confocal scanning microscope MRC-1024 (Bio-Rad Laboratories, Hercules, CA). After recording the images, an equal volume of 8% (w/v) paraformaldehyde in PBS was added. The images from the cells were obtained in the course of fixation with 4% PFA every 10 min using the identical settings of the microscope.

RESULTS

Peptides. All peptides were synthesized and biotinylated by using conventional peptide synthesis protocols. Orthogonal protection group strategy was used for the labeling of transportan at the 13th position with biotin (*23*, *28*). The studied peptides (Table 1) at the used concentrations were not toxic in the applied conditions and did not impair the viability of cells, judged by the standard methods for measuring cell viability, using a MTT-based assay and plasma membrane integrity by the LDH leakage assay (data not shown).

Protein Transduction Ability of CPPs Differs. Addition of fluorescein-labeled avidin into the culture medium and incubation for 30 min led to negligible, if any, detectable increase in the fluorescence of HeLa cells (Figure 1a). Coapplication of FITC-labeled avidin with 0.5 μM biotinyl-CPP led to a marked increase in the cells' fluorescence that ranged from about a 10-fold gain with biotinyl-penetratin to a more than a 100-fold rise with biotinyl-transportan (Figure 1a). The effect of biotinyl-derivatives of pTat and pVEC peptides lay between these limits (Figure 1a). Although the total gain of fluorescence intensity widely varied for avidin−FITC complexes with different biotinyl-CPPs, the uptake of each complex is rather uniform, i.e., all cells were labeled and only one cell population was present.

Lowering the incubation temperature from 37 to 4 °C led to a remarkable drop in fluorescence intensity, showing a decreased association/uptake of biotinyl-CPP−avidin complexes with/into cells (Figure 1b). Still, all the cells were stained and the order of potency among the four CPPs was the same as at the physiological temperature. Protein delivery with the Tat peptide was less affected upon lowering the incubation temperature as compared to complexes with the other studied CPPs.

The avidin−biotinyl-CPP complexes loosely bound at the plasma membrane may interfere with the FACS analysis (*13*). After a prolonged trypsin treatment for 15 min, cells incubated with biotinyl-CPP−avidin complexes revealed markedly lower fluorescence intensity, showing that a significant fraction of complexes is still accessible to extracellularly applied trypsin (Figure 1c). The amount of cell-associated protein complexes with biotinyl-penetratin and biotinyl-pVEC was more and complexes with biotinyl-pTat and biotinyl-transportan less affected upon a prolonged trypsin treatment, revealing lower accessibility of the latter to the enzyme. However, the changes induced by prolonged trypsin treatment did not change the principal order of delivery efficiency of the studied CPPs (Figure 1c). Analogous results were obtained with complexes of FITC-labeled streptavidin and the respective CPPs. When the biotin moiety was coupled to the CPP via an aminohexanoic acid spacer, the uptake of avidin complex was identical with the spacer-free biotinyl-CPPs (data not shown).

The avidin−CPP complexes, associated with cells at low temperature, were highly accessible and a substan-

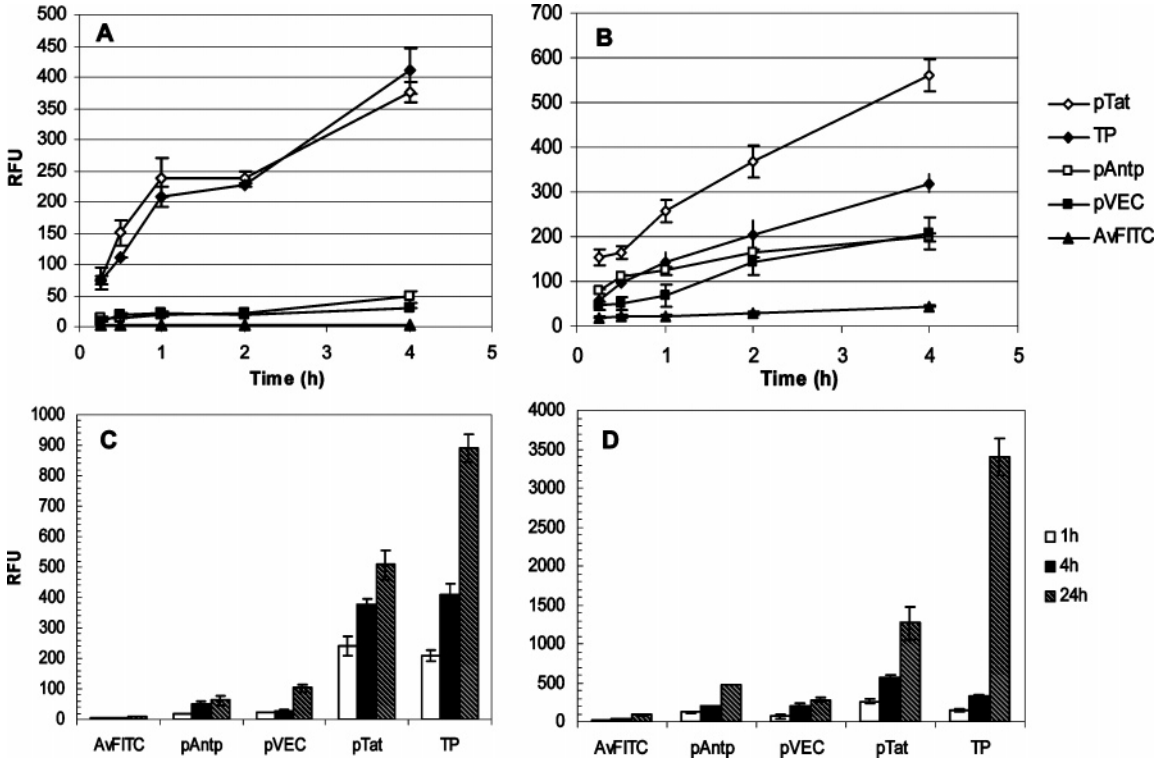

Figure 2. Cell association kinetics of CPP−avidin complexes. HeLa cells were incubated with 0.15 μM FITC-labeled avidin or its complexes with the respective 0.5 μM biotinyl-CPP and fluorescence was measured by FACS (a, c) in live cells or by spectrofluorimetry (b, d) in cell lysates. Association kinetics of complexes during the first 4 h (a, b) and from 1 to 24 h (c, d). Each value is the mean ± SD of three separate determinations. The fluorescence intensity in the cells is expressed as a ratio to the fluorescence of untreated control cells.

tial fraction of complexes was removed by trypsin. The association of avidin complexes with penetratin was more and complexes with pTat less affected in comparison with other peptides. Probably the penetratin−avidin complexes remained loosely bound to the cell surface at low temperatures. Stable association of penetratin−avidin complexes with HeLa cells seems to be more temperature-dependent than that of other studied CPPs.

Lowering of the incubation temperature or the prolongation of trypsin treatment did not cause any detectable increase in the number of cells taking up propidium iodide as compared to untreated control cells (data not shown). Therefore, we believe that the barrier function of the cells plasma membrane was not compromised and the integrity of the cells was not impaired by these treatments.

Uptake Kinetics of CPP−Avidin Complexes. Marked reduction in the fluorescence signal under an extended trypsin treatment implies that after 30 min a substantial fraction of biotinyl-CPP complexes with FITC-labeled avidin are still located on the cell surface. The uptake of complexes was measured in a serum-containing culture medium in order to maintain favorable conditions for cells. Prolongation of incubation time with CPP−avidin complexes from 15 min to 4 h led to a gradual increase in cell-associated fluorescence (Figure 2a).

Analogous time-dependency of internalization was observed for all the biotinyl-CPP complexes studied. However, in the serum-supplemented culture medium the uptake of complexes with transportan was reduced and slightly less efficient than that of Tat complexes.

Although the protein transduction efficiency of the studied CPPs is different, the initial stable association is very fast and already in 15 min a substantial amount of complexes is stably associated with the cells. Cellular fluorescence increases linearly during the first hour. After 2-h incubation a deceleration in the uptake was observed, followed by a new fast increase (Figure 2a), and the internalization of CPP−avidin complexes continued for at least 24 h at this peptide concentration (Figure 2c). Uptake of fluorescein-labeled avidin shows a rather similar time-dependence, though at a markedly lower level. Spectrofluorimetry did not reveal any deceleration in the uptake of fluorescent CPP−avidin complexes after 1 h, but a continuous increase during the first 4 h. While the FACS analysis shows an approximately 10-fold difference in transduction efficiency between the Tat and penetratin peptides, comparison by spectrofluorimetry reveals less than a 3-fold difference after 4 h incubation (Figure 2b). Prolonged incubation of cells with complexes from 4 to 24 h approximately doubles the intracellular concentration of the labeled complex (Figure 2c). Surprisingly, the cellular uptake of transportan−avidin complexes did not slow after 4 h incubation but continued increasing at high speed. Growing accumulation of transportan−avidin complexes in HeLa cells from 4 to 24 h was detected by both, FACS and spectrofluorimetry (Figure 2c, d). Perforation of the plasma membrane and free entrance into cells is not the reason for the observed accumulation since the integrity of cells is not compromised as confirmed by the exclusion of propidium iodide.

Constituents of Serum and Tissue Culture Medium Slightly Modulate the Uptake of CPP−Avidin Complexes. It is usually considered that the cellular uptake of CPPs and their cargoes is reduced in the presence of serum. However, the uptake of CPP−avidin complexes by HeLa cells was more efficient in a serum containing medium and less efficient in PBS (Figure 3). Transportan was an exception, inducing the internalization of a higher amount of avidin in a serum-free than serum containing medium (Figure 3). On the other hand, a less efficient uptake of transportan−avidin complexes

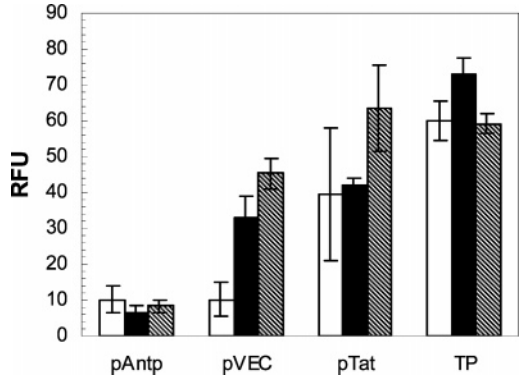

Figure 3. Effect of culture medium on protein cellular uptake. HeLa cells were incubated with complexes of 0.15 μM FITC-labeled avidin with the respective 0.5 μM biotinyl-CPP in PBS (empty bars), serum-free IMDM (black bars), or serum-containing IMDM (striped bars) for 30 min at 37 °C. Fluorescence intensity in the cells is expressed as a ratio to the fluorescence of control cells that were incubated with FITC-labeled avidin.

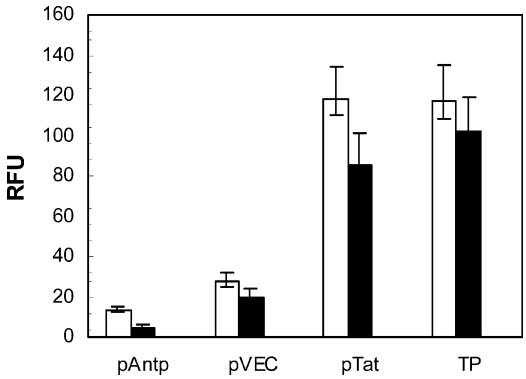

Figure 4. Inhibition of the cellular uptake of CPP−avidin complexes in hyperosmolar cell culture medium. HeLa cells were incubated with 0.15 μM FITC-labeled avidin with the respective 0.5 μM biotinyl-CPPs in isotonic IMDM (empty bars) or hyperosmolar culture medium containing 0.45 M sucrose (black bars).

in the presence of serum was observed only with short incubation times of 15 to 30 min. In less than 1 h the uptake of complexes with transportan was equal both in serum-containing and serum-free media (data not shown). After longer incubations, the internalization of transportan−avidin complexes followed the rule: a higher uptake took place in the presence of serum.

Role of Clathrin-Mediated Endocytosis in the Uptake of CPP−Avidin Complexes. The cellular uptake of FITC-labeled avidin complexed with biotinylated CPPs decreased under hyperosmolar conditions, which inhibits clathrin-dependent endocytosis as compared to a normal isotonic medium. The uptake of avidin complexes formed with penetratin decreased about 3-fold under hyperosmolar conditions, but the uptake could not be completely blocked by inhibiting clathrin-dependent endocytosis (Figure 4). The uptake of pTat-avidin, transportan−avidin, and pVEC−avidin complexes by HeLa cells decreased in the hyperosmolar medium only slightly, about 1.5-fold, pointing at a possible different mode of interaction at the plasma membrane and the internalization pathway for penetratin.

Depletion of Cellular Energy Minimizes the Association of Peptide−Protein Complexes with HeLa Cells. Deprivation of cellular energy strongly impaired the uptake of avidin complexed with any of the four CPPs (Figure 5). Sodium azide inhibited the uptake of CPP−avidin complexes more, reducing the fluorescence signal about 2−5-fold, while the change from glucose to DOG

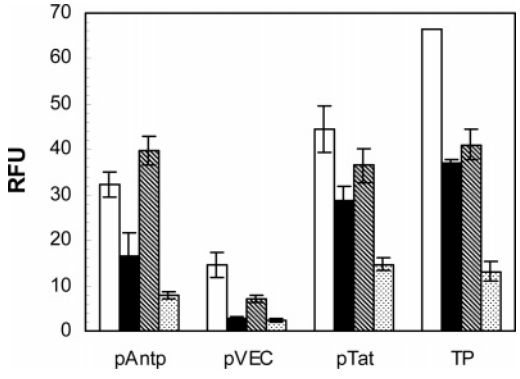

Figure 5. Inhibition of uptake of avidin−CPP complexes by depletion of cellular energy. Uptake in the presence of 0.5% NaN_3 in IMDM (black bars), 25 mM 2-deoxyglucose in PBS (striped bars), or both inhibitors in PBS (dotted bars). Control cells were incubated in IMDM (empty bars). HeLa cells were treated with the respective inhibitors for 30 min at 37 °C, and then the complexes of 0.15 μM FITC-labeled avidin with the respective 0.5 μM biotinyl-CPPs in the same solution were applied and incubated for additional 30 min at 37 °C.

had almost no effect as with penetratin−avidin complexes, or a small inhibitory effect of 1.2−2-fold. Coapplication of inhibitors had an additive effect, and the fluorescence signal associated with the cells was reduced about 3−6-fold, depending on the CPP examined. Identical reduction in the uptake was obtained with 1% sodium azide (i.e., by doubling the concentration) alone. Increase in the DOG concentration from 25 to 50 mM slightly (about 1.5-fold) enhanced its inhibitory effect (not shown). Still, a clearly detectable fraction of CPP−avidin complexes stably associated with cells in the presence of both inhibitors with all the tested peptides. This residual fluorescence could not be eliminated by extensive treatment with trypsin or pronase (not shown).

In the absence of cellular energy, about 2- to 5-fold more FITC-labeled avidin associated with cells when complexed to a biotinyl-CPP than without a carrier peptide. This is in the line with the results obtained at low temperature, suggesting that by energy depletion the stable association of CPPs with the cells was not completely blocked.

Organization of Membrane Lipids is Essential for CPP-Mediated Protein Delivery. Extraction of cholesterol from cells leads to the disappearance of caveolae, flattening of the plasma membrane, and the loss of rafts and abolishes staining with filipin as observed in electron microscopy (*27*). Treatment of HeLa cells with methyl-β-cyclodextrin removed more than 2/3 of cholesterol from the plasma membrane as indicated the staining of cells with filipin. Cholesterol-depleted HeLa cells took CPP−avidin complexes up less avidly than the untreated cells. However, a drop in the uptake of complexes was not uniform for all the four CPPs examined (Figure 6). Uptake of avidin, complexed with penetratin or pVEC decreased about 2-fold, while that of transportan and pTat complexes by only about 20%.

Intracellular Localization of CPP−Avidin Complexes. Incubation of cells with complexes of any used biotinyl-CPP and fluorescently labeled avidin for more than 15 min led to an intense labeling of all cells in the culture as revealed by fluorescence microscopy. The complexes appeared first as punctuate structures on the plasma membrane and of spherical or tubular shape in cortical cytoplasm. Later the complexes shifted more centrally and were detectable also in the perinuclear region of HeLa cells (Figure 7). The punctuate pattern

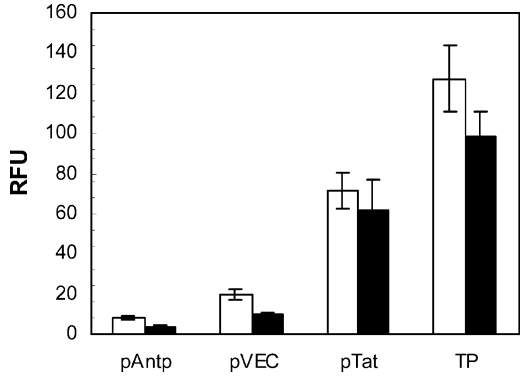

Figure 6. Effect of cholesterol removal from the plasma membrane on the uptake of avidin−CPP complexes. Cholesterol was extracted from the HeLa plasma membranes by incubating cells for 30 min in the medium with 10 mM methyl-β-cyclodextrin. The complexes of 0.15 μM FITC-labeled avidin with the respective 0.5 μM biotinyl-CPPs were applied in the same solution and incubated for additional 30 min at 37 °C. Uptake by cholesterol-depleted HeLa cells (black bars) and control cells (empty bars).

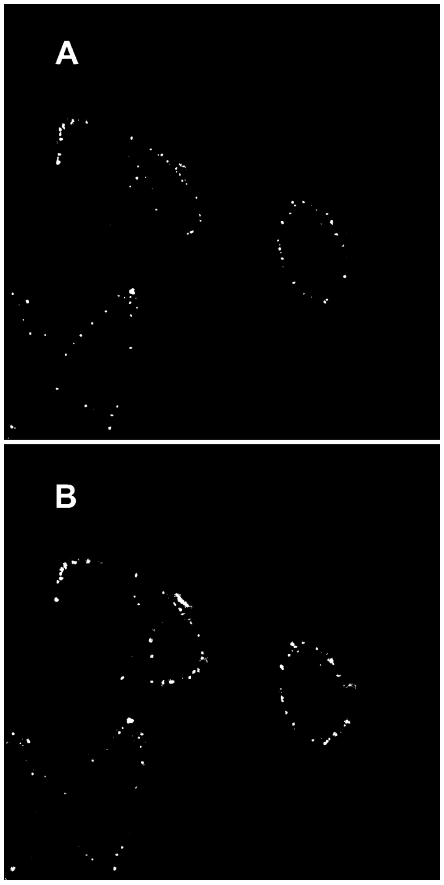

Figure 7. Localization of avidin−FITC complexes with biotinylated transportan in living and fixed HeLa cells. Confocal fluorescence microscopy images of HeLa cells (equatorial view) incubated with 0.5 μM biotinyl-transportan and 0.15 μM avidin−Alexa Fluor 488 for 45 min before (A) and after fixation (B) in 4% paraformaldehyde for 15 min.

of staining of HeLa cells prevailed also after 4 h incubation, suggesting the localization of avidin−CPP complexes mainly in vesicular structures. However, some uniform diffuse staining of cytoplasm was also detectable after at least 30 min incubation.

Distribution of fluorescein-labeled CPPs from the plasma membrane into intracellular structures upon fixation leading to an artifactual staining pattern was demonstrated recently (*12*, *13*, *16*). To study the redistribution, we assessed the localization of fluorescent CPP−avidin complexes in the course of fixation with 4% paraformaldehyde by confocal scanning microscopy during 40 min. No marked redistribution of avidin−CPP complexes upon fixation was observed within this time period (Figure 7a,b).

DISCUSSION

We estimated the ability of different CPPs to target/deliver proteins into cells by following the cellular uptake of fluorescein-labeled avidin complexed with biotinylated peptides by fluorescence microscopy, flow cytometry and spectrofluorimetry. Transduction of fluorescently labeled avidin and/or streptavidin into cells after complexing with biotinylated transportan (*29*), pVEC (*24*), pTat, penetratin (*15*), and proline-rich peptides (*30*) was demonstrated recently. Therefore, instead of synthesizing a multitude of CPP−protein covalent conjugates, we used the complexes of a respective biotinyl-CPP with fluorescein-labeled avidin. Tagging of a peptide with biotin could introduce conformational constraints and interfere with association with avidin or influence the translocation activity. We assumed that biotinylation restrains peptides analogously and all the studied CPPs complexed with fluorescein-labeled avidin and induced its cellular uptake under physiological conditions. The biotin moiety associating a peptide with avidin, on the other hand, may be considered as a spacer reducing constraints posed by a cargo to CPP activity.

Penetratin, pTat, transportan, and pVEC were chosen as representatives of the respective CPP families. The motifs that are considered to be essential for cell penetration are, in general, arginines for pTat (*5*, *31*), positively charged amino acids (*32*) and tryptophan for penetratin (*33*), hydrophobic N-terminal region for pVEC (A. Elmquist personal communication), and the amphipathic carboxyterminal part for transportan (*34*). A multitude of molecular determinants necessary for the cellular uptake of particular peptides suggests that different CPPs may vary in cellular penetration ability (*35*).

The four CPPs studied here were capable of targeting the complexed FITC-labeled avidin to the HeLa cell plasma membrane and inducing cellular uptake. Transportan and pTat were more efficient in the transduction of complexed avidin than penetratin and pVEC. A substantial fraction of CPP−avidin complexes was not internalized but tightly bound extracellularly and removed only by extending trypsin treatment to 15 min. Extensive trypsin treatment affected the amount of cell-bound penetratin−avidin complexes more, implying a slower passage across the plasma membrane into cytoplasm or better accessibility to the enzyme in the plasma membrane. Lower avidin/streptavidin delivery efficiency with biotinyl-penetratin compared to biotinylated Tat peptide has been observed also earlier (*15*). Avidin cargo may introduce conformational constraints on a penetratin and pVEC molecule, leading to a reduced uptake as suggested for a human calcitonin-derived peptide (*36*). On the other hand, the interaction of penetratin and pVEC with the plasma membrane of cells might be more dependent on the concentration of peptide, temperature, and cellular energy. Tryptophan in the sixth position of pAntp has been shown to serve as a membrane anchor and induce cellular transduction (*37*); therefore, higher stiffness of plasma membrane at lower temperatures might impede stable association of penetratin with HeLa cells and subsequent translocation.

The amount of internalized CPP−avidin complexes increased rapidly in physiological conditions during the first 4 h. Spectrofluorimetry showed an about 2−3-fold difference in the transduction potential between pTat and penetratin, whereas the FACS analysis suggested an almost 10-fold difference. Discrepancy between the obtained results could be explained by the sensitivity of fluorescein emission to the microenvironment and partial quenching in the endosomal structures of living cells used for the FACS analysis (*19*). Transportan or pTat might disrupt the acidic vesicular structures and escape into cytoplasm more efficiently than penetratin or pVEC. By analogy, a relative deceleration in the cellular uptake of complexes observed by FACS may also be caused by the translocation of complexes into acidic vesicular structures. The uptake of complexes did not cease after 4 h and continued slowly for three studied peptides for 24 h. Transportan−avidin complexes continued to concentrate intracellularly after 4 h incubation at high speed, suggesting that transportan could stimulate the uptake by modulating intracellular processes without compromising the intactness of cells (*38*).

The lowering of temperature decreases plasma membrane permeability and partially depolarizes the cells leading to a dramatically reduced internalization of CPPs (*14*). CPP-mediated protein cellular delivery decreased even more, suggesting that the uptake process was energy-dependent and the stiffening of the plasma membrane minimized the uptake. About 80−90% of FITC-labeled avidin associated with HeLa cells by biotinyl-CPPs was removed by an extensive trypsin treatment, demonstrating that at low temperature the majority of complexes remained in the plasma membrane and were not internalized. Cellular energy deficiency reduced the uptake of CPP−avidin complexes even more strongly. Still, a small fraction of CPP−protein complexes associated with energy-deficient cells and association took place also at low temperature, suggesting a stable association or uptake. This might imply that the initial interaction of CPPs with the plasma membrane was not dependent on cellular metabolic energy and activity.

All endocytic processes, clathrin-dependent and -independent endocytosis, micro- and macropinocytosis, are blocked at low temperature and in energy-deficient cells (*39*). Hyperosmolar medium, which inhibits clathrin-mediated endocytosis, led to a minor inhibition of the internalization of avidin−CPP complexes, showing a small role of this mechanism in the uptake and a prevalence of other, clathrin-independent endocytic processes. Detergent resistant membrane fractions (DRM) that are rich in cholesterol and sfingolipids form "rafts" and caveolae in the plasma membrane are necessary for clathrin-independent endocytosis to occur (*40*). Caveolae are suggested to mediate Tat-phage internalization into COS-1 cells (*41*) and EGFP fusion proteins with the Tat protein or peptide into HeLa cells (*20*, *21*). On the other hand, it has been recently shown that caveolae are highly immobile plasma membrane microdomains that are not involved in constitutive endocytosis (*42*), and the raft regions might mediate the uptake. Uptake of avidin complexed with the tested CPPs by HeLa cells was strongly impaired after removing cholesterol from the plasma membrane by methyl-β-cyclodextrin. Cell transducible fusion proteins with Tat are suggested to enter HeLa cells by caveolar endocytosis (*20*, *21*) and mouse lymphocytes by macropinocytosis (*22*). Both these processes are cholesterol-dependent and start from lipid rafts. Our data do not enable us to distinguish between these two pathways of cellular uptake. However, in our earlier studies with fixed cells we detected transportan−protein conjugates and complexes at the plasma membrane in structures of various size and volume but not in highly uniform and morphologically defined caveolae.

The potential application of CPP-mediated transport in vivo necessitates knowledge about the influence of serum and other medium constituents on the cellular uptake of particular CPPs. Serum is usually considered to reduce the cellular uptake of CPPs and their cargoes but the protein delivery efficiency of the studied CPPs was slightly enhanced in the presence of serum. Potentiation of cargo delivery by the presence of serum could give CPP-mediated cell transduction an advantage over lipofection, for example, for in vivo use.

In conclusion, all four studied peptides are capable of promoting the cellular delivery of avidin. Although these peptides are classified as cell-penetrating, their protein delivery potential differs substantially. Changes in experimental conditions such as temperature, medium composition and osmolarity, cellular energy, and plasma membrane composition affect protein transduction by these peptides in a rather similar manner. This suggests that the four studied transport peptides could share a common mechanism to promote the cellular uptake by endocytosis. The peptide-mediated cellular transduction of proteins comprises several subsequent steps of binding to the cellular surface, interaction at the plasma membrane, and translocation into and traffic inside the cell. Probably, various interaction modes at the plasma membrane cause most of the observed differences in the internalization efficiency of CPP−protein complexes (*43*, *44*).

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