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Uptake of analogs of penetratin, Tat(48–60) and oligoarginine in live cells

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Abstract

Cell-penetrating peptides are regarded as promising vectors for intracellular delivery of large, hydrophilic molecules, but their mechanism of uptake is poorly understood. Since it has now been demonstrated that the use of cell fixation leads to artifacts in microscopy studies on the cellular uptake of such peptides, much of what has been considered as established facts must be reinvestigated using live (unfixed) cells. In this work, the uptake of analogs of penetratin, Tat(48–60), and heptaarginine in two different cell lines was studied by confocal laser scanning microscopy. For penetratin, an apparently endocytotic uptake was observed, in disagreement with previous studies on fixed cells found in the literature. Substitution of the two tryptophan residues, earlier reported to be essential for cellular uptake, did not alter the uptake characteristics. A heptaarginine peptide, with a tryptophan residue added in the C-terminus, was found to be internalized by cells via an energy-independent, non-endocytotic pathway. Finally, a crucial role for arginine residues in penetratin and Tat(48–60) was demonstrated.

Keywords: Protein transduction domain; Antennapedia homeodomain; Tat; PC-12; V79; Rotenone; Endocytosis; ATP depletion; Membrane translocation; Internalization

The delivery of large, hydrophilic molecules, such as proteins and oligonucleotides, to the cytoplasm and nucleus of cells is problematic due to their poor plasma membrane permeability. In recent years, several peptides have emerged as potent delivery vectors for such compounds, crossing the membrane barrier by a poorly understood process denoted protein transduction or cell penetration [1,2]. Most published studies of the cellular uptake of cell-penetrating peptides are based on fluorescence microscopy on fixed cells and/or flow cytometry analysis (see for example [3–12]). Based on the results of such studies, the common features of cell-penetrating peptides have been considered to be a receptor- and energy-independent uptake and the ability to transport large cargo molecules into the cytoplasm and nucleus. Now, it has been demonstrated that cell fixation, even under mild conditions, results in an artifactual uptake of peptide associated with the plasma membrane [13,14], leading to misinterpretations especially since these often highly basic peptides have a high affinity for the cell surface and are not removed even by repeated washings. Along the same lines, there is an obvious risk of causing redistribution of peptide to the cell interior when permeabilizing the membrane by the use of e.g., methanol at −20 °C or detergent, to allow entry of fluorescently labeled streptavidin for visualization of biotinylated peptide. Certainly, the strong tendency of these peptides to associate with the plasma membrane should also lead to an overestimation of cellular uptake by flow cytometry. This can only be circumvented if a distinction can be made between peptide internalized by cells and peptide associated with the membrane, as in [15].

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In the light of these findings, the question must be raised whether all of the intriguing results of cell studies on cell-penetrating peptides found in the literature are relevant for our understanding of their mechanisms of internalization in live cells. For example, structure-activity studies using fixed cells and flow cytometry are more likely to reflect differences in cell association than in cellular uptake. In fixed cells, the internalization of penetratin has been shown to be virtually unaffected by modifications such as the introduction of three helix-breaking proline residues, reversal of the amino acid sequence or substitution of all amino acids by their D-enantiomers [7], leading to the conclusions that the uptake of penetratin is receptor-independent and that peptide conformation is of minor importance.

Considering the possible artifacts in previous studies, one could in fact question the very existence of cell-penetrating peptides in the original definition. Nevertheless, there are a large number of studies where these peptides have been successfully utilized to transport oligonucleotides, peptide nucleic acids (PNAs), peptides, and proteins into living cells, resulting in significant biological effects (for a review, see for example [16,17]). Thus, there is substantial evidence that peptides such as penetratin, Tat(48–60), and oligoarginine are indeed able to somehow convey cargo molecules to the cytoplasm. Cytoplasmic and nuclear localization of fluorescently labeled peptides has also been observed in a few reports, in which fluorescence microscopy on living cells was used. For instance, Mitchell et al. [18] showed that oligoarginines are taken up by unfixed cells via a non-endocytotic pathway and localized both in the nucleus and the cytoplasm. Furthermore, although only in a single experiment, Fischer et al. [8] observed cell internalization and nuclear accumulation of penetratin in unfixed cells.

In this paper, we studied the internalization of carboxyfluorescein-labeled analogs of penetratin, Tat(48–60), and heptaarginine (Table 1) in unfixed cells, using confocal laser scanning microscopy. Two different cell lines were used: rat adrenal pheochromocytoma cells (PC-12) and Chinese hamster lung fibroblasts (V79). For penetratin, an apparently endocytotic uptake was observed, in disagreement with previous studies on fixed cells found in the literature. Substitution of the two tryptophan residues, earlier reported to be essential for cellular uptake [6], did not alter the uptake characteristics. Unlike penetratin, a heptaarginine peptide, with a tryptophan residue added in the C-terminus to enable optical spectroscopic studies, was found to be internalized by cells via an energy-independent, non-endocytotic pathway. Penetratin analogs, in which the lysine residues were substituted for arginines or vice versa, exhibited strikingly different uptake characteristics. The arginine-rich variant was internalized both via endocytosis and via an energy-independent pathway. The lysine-rich penetratin analog, on the other hand, exhibited no cellular uptake and a very low affinity for the cell surface. Along the same lines, TatP59W, a Tat(48–60) analog containing a tryptophan residue, was taken up both via endocytosis and via an energy-independent pathway, while a lysine-substituted variant, Tat-LysP59W, was not internalized at all and exhibited only very weak cell surface binding.

Table 1
Names and sequences of synthesized carboxyfluorescein-labeled peptides

Peptide	Sequence
Penetratin	Fluo-RQIKIWFQNRRMKWKK-NH_2
Pen2W2F	Fluo-RQIKIFFQNRRMKFKK-NH_2
PenArg	Fluo-RQIRIWFQNRRMRWRR-NH_2
PenLys	Fluo-KQIKIWFQNKKMKWKK-NH_2
R_7W	Fluo-RRRRRRRW-NH_2
TatP59W	Fluo-GRKKRRQRRRPWQ-NH_2
TatLysP59W	Fluo-GKKKKKQKKKPWQ-NH_2

Materials and methods

Peptide synthesis. Carboxyfluorescein-labeled peptides were synthesized as described earlier [19]. For cleavage of the Tat analogs and R_7W, however, a solution of trifluoroacetic acid:water:triisopropysilane (95:2.5:2.5) and a cleavage time of 4 h was used.

Cell culture. Approximately 25,000–30,000 adherent PC-12 cells were cultivated on quadratic coverslips in Petri dishes for 3–5 days in Iscoves modification of Dulbecco's medium (IMEM) supplemented with Hepes (25 mM), penicillin/streptomycin (1%), fetal calf serum (10%), Na-pyruvate (1%), and L-glutamine in a 5% CO_2 humidified atmosphere at 37 °C.

Approximately 10,000–25,000 Chinese hamster V79 (Padua) cells were grown for 1–2 days on quadratic coverslips in Dulbecco's modified Eagle's medium, with 10% fetal calf serum and 90 U/ml penicillin in a humid atmosphere with 5% CO_2 at 37 °C. All products were from Gibco.

Cellular uptake. The culture medium was removed from cells adhered to the coverslips and the cells were incubated at 37 or 4 °C, with either 1, 5, or 10 μM of peptide and 1 μM of FM 4-64 (Molecular Probes) in Hepes-buffered saline (HBS) (10 mM Hepes, 10 mM D-glucose, 135 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.3). After incubation, the cells were washed once with HBS and the coverslips were mounted in HBS on glass slides with a Secure-Seal adhesive spacer (Molecular Probes).

In some experiments, cells were preincubated for 30 min at 37 °C with 1 μM rotenone in HBS with 10 mM 2-deoxy-D-glucose instead of D-glucose to accomplish depletion of the intracellular pool of ATP. The cells were then incubated with peptide and FM 4-64 in HBS with 2-deoxy-D-glucose and rotenone.

In another set of experiments, cells were incubated with peptide and 2 μM propidium iodide in HBS in order to assay cell viability under the various experimental conditions used in this study.

The presence of FM 4-64 did not affect the uptake of the peptides examined, since the distribution of peptide fluorescence in the cells was the same in control experiments without the membrane probe.

Confocal microscopy. The PC-12 cells were imaged using a Bio-Rad MRC-1024 laser scanning confocal unit mounted on the side-port of an inverted Nikon TE-300 microscope. The confocal microscope was equipped with a fibre-coupled ArKr laser which was

used to excite the two dyes. The carboxyfluorescein was excited using the 488 nm laser line, while the FM 4-64 dye was excited using the 568 nm laser line. The two emission signals were separated using a 560DRLP dichroic mirror that reflects light in the wavelength region 500–550 nm to one photomultiplier tube and transmits wavelengths over 550 nm to a second photomultiplier tube. The carboxyfluorescein signal was sorted out using a 522DF35 filter (emission 505–540 nm), while a 585EFLP filter (emission >600 nm) was used to detect the signal from the FM 4-64 dye. Since both dyes are excited simultaneously using the 488 nm laser line, but only the FM 4-64 dye with the 568 nm laser line, the imaging was performed in sequential mode in order to avoid bleed through between the two photomultiplier tubes. Relative fluorescence was measured using LaserPix software (Bio-Rad).

For V79 cells, images were obtained with a Zeiss LSM 510 inverted confocal microscope using a planapochromate 63X/NA 1.4 oil immersion objective and an excitation wavelength of 488 nm for carboxyfluorescein. Relative fluorescence was measured using the Zeiss 3D for LSM software.

Results

The uptake of peptides in unfixed cells was studied by confocal laser scanning microscopy. V79 cells were incubated with carboxyfluorescein-labeled peptides, while PC-12 cells were incubated with both carboxyfluorescein-labeled peptides (green fluorescence) and the membrane probe FM 4-64 (red fluorescence), a commercially available marker of endocytosis.

Cellular uptake of R_7W

PC-12 cells incubated for 1 h with 5 μM R_7W and 1 μM FM 4-64 at 37 °C (Fig. 1) exhibited an uneven distribution of FM 4-64, with a stronger fluorescence in a region close to the nucleus, most probably the centrosomal area, and negligible staining of the nucleus. The peptide, on the other hand, was found to be more evenly distributed within the cells giving rise to both diffuse and punctate cytoplasmic fluorescence. Inside the nucleus, the fluorescence intensity was somewhat weaker, but clearly, there was peptide present in the nucleus as well. Furthermore, there was a strong fluorescence emanating from accumulations of peptide at the cell surface.

The uptake of R_7W was also examined at 1 and 10 μM and it was found to be proportional to the peptide concentration (data not shown). When cells were incubated with 5 μM R_7W for 5 min, only membrane-associated peptide was detected, while after 15 min, there was a diffuse cytoplasmic peptide fluorescence, weaker than after 1 h (data not shown). The presence of FM 4-64 was detectable in the centrosomal area after 5 min, albeit to a much lesser extent than after 1 h.

When PC-12 cells were incubated with 5 μM R_7W for 1 h at 4 °C (Fig. 2A), the peptide was taken up even more efficiently than at 37 °C. Unlike the situation at 37 °C, the distribution of peptide within the cells was

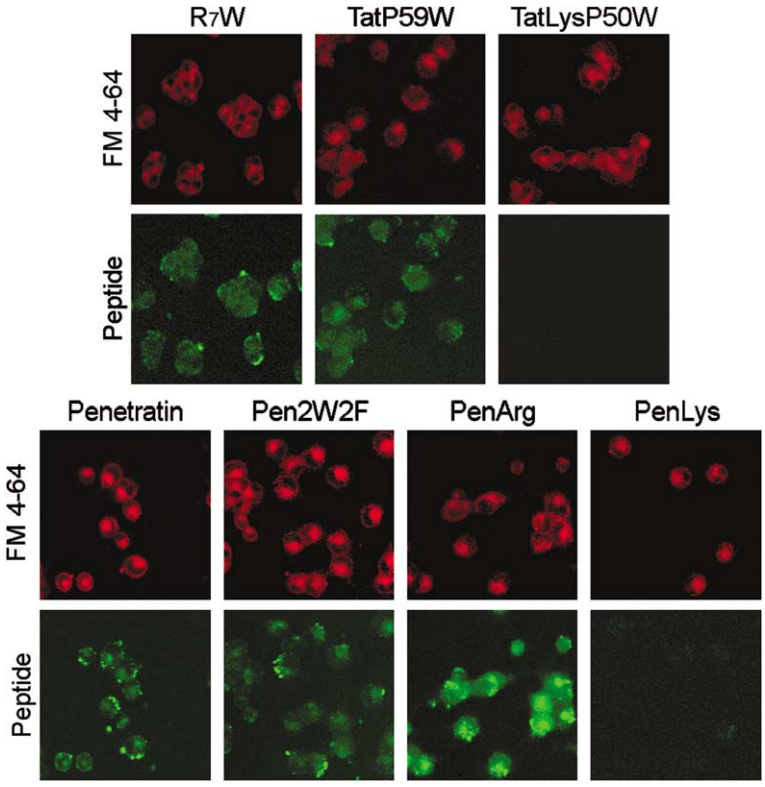

Fig. 1. Confocal laser scanning microscopy images of unfixed PC-12 cells incubated for 1 h at 37 °C with 1 μM FM 4-64 (red fluorescence) and 5 μM of carboxyfluorescein-labeled peptide (green fluorescence).

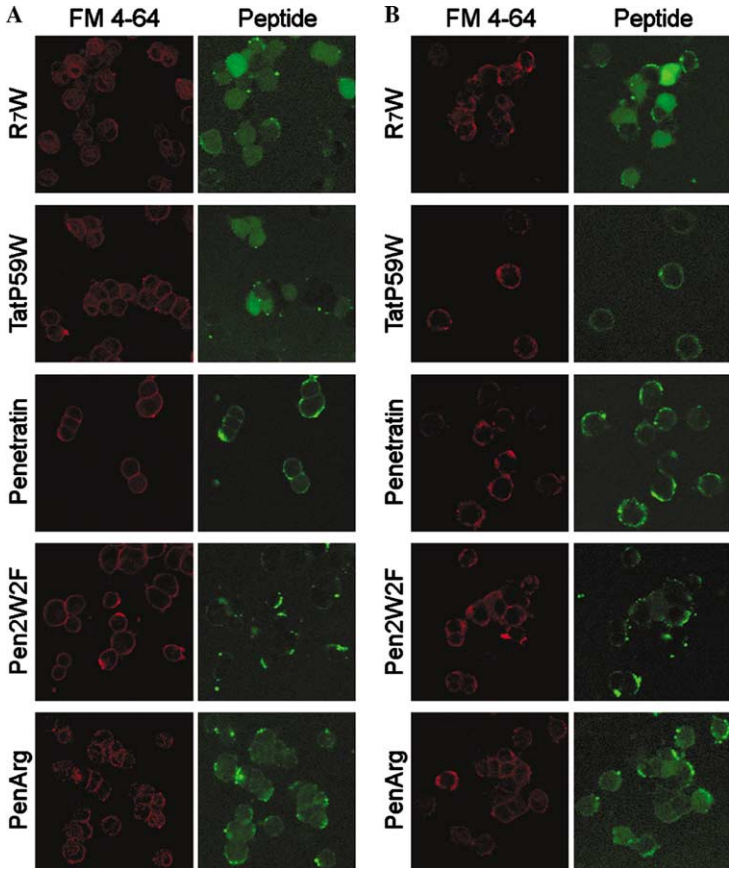

Fig. 2. Confocal laser scanning microscopy images of unfixed PC-12 cells incubated for 1 h with 1 μM FM 4-64 (red fluorescence) and 5 μM of carboxyfluorescein-labeled peptide (green fluorescence) (A) at 4 °C or (B) with depletion of intracellular ATP, as detailed in Materials and methods.

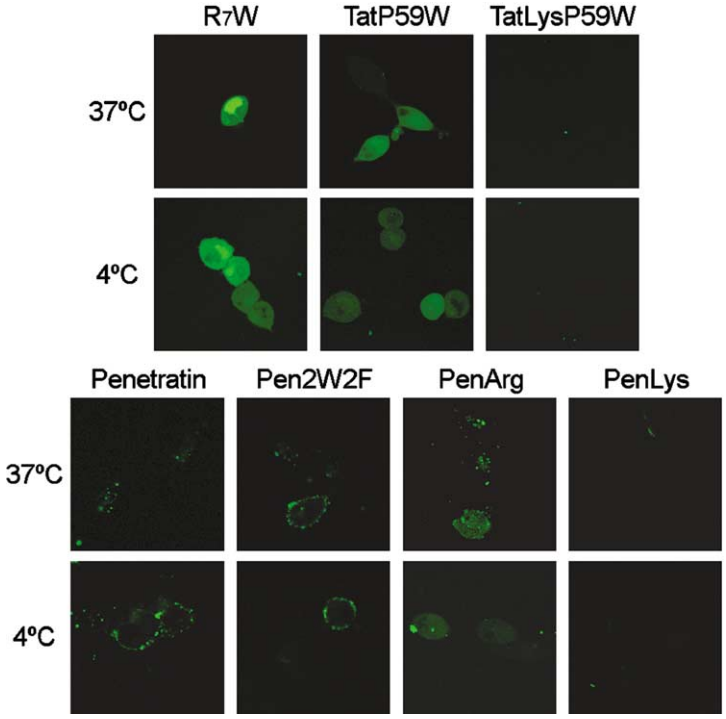

Fig. 3. Confocal laser scanning microscopy images of unfixed V79 cells incubated for 1 h with 5 μM of carboxyfluorescein-labeled peptide at 37 or 4 °C, as indicated in the figure.

uniform, with no difference between nucleus and cytoplasm and no distinct membrane fluorescence. There were, however, large differences between cells in the amount of internalized peptide. While most cells exhibited uniform peptide fluorescence of varying intensity, some 5–10% of the cells had not taken up any peptide at all. In the corresponding experiments using peptide coincubated with propidium iodide, a non-permeant dye that is taken up by dead/dying cells, neither uptake of R_7W nor lack of uptake was correlated with cell staining with propidium iodide (data not shown). The internalization of FM 4-64 was almost completely abolished at 4 °C, indicating that endocytosis was efficiently inhibited. The uptake of R_7W was found to be relatively slow also at 4 °C. After 15 min incubation, the intracellular fluorescence was weaker than after 1 h, but instead there was a much more accentuated membrane fluorescence, also in the fraction of cells devoid of internalized peptide (data not shown).

Depletion of the cellular pool of ATP by preincubation of PC-12 cells with 1 μM rotenone in the presence of 2-deoxy-D-glucose prior to addition of peptide and membrane probe had an effect similar to that of incubation at low temperature. The internalization of FM 4-64 was inhibited, but not the uptake of R_7W (Fig. 2B). As in the case of incubation at 4 °C, there was a wide variation between the cells in intracellular peptide fluorescence.

The uptake of R_7W in V79 cells was also examined at 37 and 4 °C (Fig. 3) at a concentration of 5 μM with an incubation time of 1 h. R_7W was efficiently internalized at both temperatures. Here, no punctate fluorescence in the cytoplasm was found. Instead, there was diffuse intracellular fluorescence, particularly strong in the nucleus. As in the case of PC-12 cells, the uptake was much more pronounced at 4 °C, but a fraction of the cells did not exhibit any peptide fluorescence. No distinct membrane fluorescence was observed.

Cellular uptake of penetratin and Pen2W2F

When PC-12 cells were incubated with 5 μM penetratin for 1 h at 37 °C, an uneven distribution of the peptide was obtained, with marked accumulation of fluorescence in the centrosomal area, as was the case with FM 4-64 (Fig. 1). A fraction of the cells (~1/3) also exhibited weak peptide fluorescence inside the nucleus. In the plasma membranes, the peptide fluorescence was distinctly punctate. When examined at 1 and 10 μM, the uptake was found to be proportional to the peptide concentration (data not shown). Uptake of penetratin was not observed after 5 min incubation; instead the peptide was localized in the plasma membrane (data not shown). After 15 min, peptide internalization was detectable, with some accumulation at the centrosome. The intracellular fluorescence was, however, much weaker than after 1 h (data not shown). When cells were incubated with penetratin at 4 °C or with depletion of intracellular ATP (Figs. 2A and B), uptake of peptide as well as FM 4-64 was abolished. The peptide was instead localized at the cell surface, staining the entire plasma membrane.

V79 cells incubated at 37 °C with 5 μM penetratin (Fig. 3) exhibited punctate peptide fluorescence mainly on the cell surface and, in contrast to PC-12 cells, only sparse fluorescent dots were observed inside the cells. At 4 °C, the peptide remained at the plasma membrane.

In all experiments, in both cell lines examined, the Pen2W2F peptide gave results very similar to those of penetratin (Figs. 1–3).

Cellular uptake of PenArg and PenLys

PC-12 cells incubated with 5 μM PenArg for 1 h at 37 °C exhibited a non-uniform distribution of the peptide, with punctate peptide fluorescence in the plasma membrane and distinct accumulation of fluorescence in the centrosomal area, as was the case for penetratin (Fig. 1). However, the intracellular fluorescence was stronger for PenArg than for penetratin and specifically, the nucleus exhibited a much more intense fluorescence. As for penetratin, the uptake was found to be proportional to the peptide concentration. When cells were incubated with peptide for 5 min, most of the peptide was localized on the cell surface. However, weak diffuse cytoplasmic fluorescence was also observed (data not shown). After 15 min incubation, both punctate and diffuse fluorescence were observed in the cytoplasm, the peptide starting to accumulate at the centrosome (data not shown). No peptide was observed in the nucleus at short incubation times.

In contrast to the results obtained for penetratin, the uptake of PenArg was not completely abolished in PC-12 cells incubated at 4 °C or with depletion of intracellular ATP (Figs. 2A and B). After 1 h, most of the cell-associated peptide fluorescence clearly emanated from the plasma membranes, but a weak, uniform intracellular fluorescence was also obtained. At 4 °C, no uptake was observed after 15 min incubation (data not shown). With depletion of ATP, on the other hand, the amount of peptide internalized after 15 min was comparable to that after 1 h (data not shown).

In V79 cells, punctate fluorescence from PenArg was found at the plasma membrane at 37 °C. Furthermore, sparse, punctate fluorescence as well as weak, diffuse fluorescence in the cytoplasm was observed (Fig. 3). At 4 °C, weak, diffuse cytoplasmic peptide fluorescence was obtained, along with punctate membrane fluorescence.

Surprisingly, the interaction of PenLys with both PC-12 and V79 cells was completely different from that of

the other penetratin peptides examined here (Figs. 1 and 3). No uptake of PenLys was obtained in any experiment. In fact, the peptide had a very low affinity for the cells, and most cells lacked peptide fluorescence. Only very sparse and weak, punctate fluorescence could be observed in the membranes of a small fraction of the cells.

Cellular uptake of TatP59W and TatLysP59W

The uptake of TatP59W in PC-12 cells at 37 °C was comparable to that of penetratin, with a similar distribution of peptide fluorescence inside the cells but less peptide associated with the cell surface. Also, the amount of internalized peptide was proportional to the peptide concentration. Depletion of intracellular ATP abolished the uptake of TatP59W (Fig. 2B), as for penetratin. Interestingly, however, the amount of TatP59W internalized was not reduced by incubation at low temperature (Fig. 2A). As for R_7W, the intracellular distribution of peptide was uniform, and no distinct membrane fluorescence was observed. Furthermore, large differences between cells in the amount of internalized peptide were found, with a fraction of the cells lacking uptake.

In V79 cells, the uptake of TatP59W was quite different from that of penetratin (Fig. 3). At both 37 and 4 °C, the peptide gave rise to a diffuse intracellular fluorescence of varying intensity and no distinct membrane fluorescence. The nucleus had more intense fluorescence than the cytoplasm. A fraction of the cells lacked uptake.

As for the lysine-substituted penetratin peptide, TatLysP59W was found to have a very low affinity for both PC-12 and V79 cells (Figs. 1 and 3) and therefore, no uptake was observed.

Discussion

Most published reports on the cellular uptake of cell-penetrating peptides rely on fluorescence microscopy studies using fixed cells and/or flow cytometry analysis. In the search for an understanding of the mechanism of uptake, extensive structure–activity studies have been performed, especially for penetratin and Tat. However, since it has recently been demonstrated that the use of these methods can lead to overestimations of the cellular uptake and misinterpretations regarding the mechanism of cell internalization [13,14], much of the knowledge acquired to date must now be reevaluated. In the present study, live (unfixed) cells were used to examine the uptake of analogs of penetratin, Tat(48–60), and hepta-arginine. To assay the involvement of endocytosis in the uptake, the endocytotic marker FM 4-64 was added together with the carboxyfluorescein-labeled peptides. By use of a proper experimental setup, these two fluorophores can easily be distinguished.

The results obtained for penetratin are indicative of an endocytotic uptake mechanism. At 37 °C, the internalized peptide is mainly located in the centrosomal region of the cells, where also the endocytotic marker FM 4-64 is accumulated. Certainly, one could argue that peptide taken up non-endocytotically might associate with endosomes after internalization, considering that penetratin has been shown to have a high affinity for lipid bilayers [20]. However, taken together with the findings that penetratin remains on the cell surface and is not internalized when cells are incubated at 4 °C or with depletion of intracellular ATP, it is reasonable to conclude that endocytosis is the predominant uptake route. The arrest of endocytosis in energy-deprived cells and in cells incubated at low temperature is manifested by the inhibition of uptake of FM 4-64. The very weak FM 4-64 fluorescence found inside these cells most probably emanates from probe molecules that have diffused into the cell interior from the plasma membrane. The FM 4-64 fluorescence in the plasma membrane varies somewhat in intensity but is always weak in all of our experiments, since most of the water-soluble probe bound to the cell surface is removed during the washing step after incubation.

Interestingly, we find no significant differences in cell association and uptake characteristics between penetratin and the Pen2W2F analog, in which the two tryptophan residues have been substituted for phenylalanines. In fixed cells, this substitution has earlier been reported to result in virtually complete abolishment of internalization [6]. This discrepancy is surprising, since the similar distribution of these two peptides in unfixed cells is apparently inconsistent with a large difference in cellular uptake in fixed cells. Possibly, variations in DNA-binding properties are decisive for the accumulation of peptide in fixed cells. Penetratin is derived from the third helix of the homeodomain of Antennapedia, a *Drosophila* transcription factor. This helix plays a key role in DNA recognition [21].

Unlike penetratin, R_7W is internalized both at 4 °C and in ATP-depleted cells, exhibiting diffuse fluorescence uniformly distributed in the nucleus and cytoplasm. These results point to the existence of an energy-independent, non-endocytotic uptake pathway, yet to be identified. In our study on PC-12 cells, the observation of some punctate cytoplasmic peptide fluorescence at 37 °C suggests that in those experiments, some peptide is also internalized via endocytosis. Intriguingly, the uptake is even more pronounced at low temperature and in cells deprived of ATP. This would be consistent with the arrest of an active, energy-dependent transport of R_7W out of the cells under these conditions, which should be examined in detail in future studies. The large differences between cells in the amount of internalized peptide

cannot be explained at this point. It is observed in all experiments on both cell lines with the exception of PC-12 cells incubated at 37 °C. We note, however, that the same phenomenon was observed for fluorescein-labeled R_9 by Mitchell et al. [18] in a large number of cell lines. One could speculate that for some unknown reason, cells are unable to internalize peptide at certain stages of the cell cycle. Our results disagree with those of Mitchell et al. in one important respect. We do not observe inhibition of R_7W uptake when cells are depleted of ATP by use of a metabolic inhibitor, while Mitchell et al. found for oligoarginines that ATP depletion abolished uptake. We do not know the reason for the different results. However, besides the fact that slightly different peptides were used, it is noteworthy that in our experiments, rotenone in the presence of 2-deoxy-D-glucose was used, while sodium azide was used in the other study.

PenArg appears to share properties with both penetratin and R_7W. As for penetratin, there is a substantial endosomal uptake at 37 °C, manifested by a distinct accumulation of peptide in the centrosomal area. On the other hand, the internalization of PenArg is not completely eliminated in cells incubated at 4 °C or with ATP-depletion. Under these conditions, peptide fluorescence uniformly distributed in the intracellular space is obtained, albeit with much weaker intensity than for R_7W. Furthermore, the nuclear peptide fluorescence at 37 °C is clearly stronger for PenArg than for penetratin. Taken together, these findings suggest that PenArg is taken up mainly via endocytosis, but also to a small extent by an energy-independent mechanism.

For TatP59W, the situation is more complex. In PC-12 cells, there appears to be a significant endocytotic contribution to the cellular uptake, since peptide accumulation at the centrosome is observed at 37 °C and ATP-depletion inhibits uptake. On the other hand, the peptide is internalized at 4 °C, which would not be expected for a solely endocytotic uptake. In V79 cells, nuclear accumulation and uniform cytoplasmic fluorescence without punctate distribution is obtained at both 37 and at 4 °C, suggesting a non-endocytotic internalization. There is thus apparently more than one uptake route for TatP59W, their relative contributions possibly being cell-type dependent.

The lack of cell association of the lysine-rich peptides (PenLys and TatLysP59W) is indeed remarkable but consistent with the finding that oligolysines associate to a much lesser extent with Jurkat cells than do oligoarginines [18]. Arginine thus appears to be crucial for the cell surface binding and internalization of cell-penetrating peptides. Since substitution of lysines for arginines does not alter the peptide charge, it is reasonable to believe that there exists a specific interaction between the guanidinium group of arginine and a hydrogen-bond acceptor moiety in the plasma membrane. Strong, bidentate hydrogen bonds can be formed with anions such as phosphate, sulfate, and carboxylate (for a review, see [22]), all of which are abundant in the plasma membrane in e.g., phospholipids and various polysaccharides. In agreement with this, computer simulations indicate an exceptionally strong and specific interaction between arginine residues and the phosphate group of membrane lipids [23]. Furthermore, it is noteworthy that polysialic acid has been suggested to be essential for the internalization of the Antennapedia homeodomain by providing surface binding sites [24], while translocation of the Tat protein was reported to rely on binding to cell surface heparan sulfate proteoglycans [25,26]. In our studies, large differences in the extent of uptake between PC-12 cells and V79 cells were observed for the penetratin peptides and for TatP59W. One could speculate that the two cell lines differ in the expression of membrane components important for the cellular association and internalization of these peptides.

The uptake of the peptides in this study was found to be proportional to the peptide concentration up to 10 μM, suggesting a non-saturable uptake in this concentration range. There were no indications of peptide toxicity in any of our experiments, since the fraction of cells stained with propidium iodide was very small and unaffected by incubation with peptide. Neither incubation at low temperature nor ATP depletion affected cell viability during these experiments.

The uptake kinetics was not examined in detail in this study. However, a few interesting observations were made. For penetratin and Pen2W2F, the accumulation of peptide inside the cells was found to be slow and correlated with the uptake of the endocytotic marker FM 4-64. On the other hand, cells incubated with PenArg exhibited diffuse fluorescence already after 5 min, suggesting a rapid, non-endocytotic uptake. Likewise, there was clearly a diffuse intracellular staining of R_7W after 15 min.

The study of Richard et al. [14] pointed to a central role of endocytosis in the internalization of cell-penetrating peptides. Although our results are not fully consistent with their findings, considering the largely non-endocytotic character of the uptake of R_7rW in particular, we also find a significant endocytotic contribution to the uptake of cell-penetrating peptides. This is most obvious in the case of penetratin. Certainly, this casts doubts on the use of these peptides as delivery vectors, since the therapeutic potency of e.g., antisense oligonucleotides would be severely impaired by confinement in endosomal vesicles. There are, however, a large number of studies in which various membrane-impermeable entities when conjugated to e.g., peptides have been shown to have the desired biological activity [16,17], implying that the conjugates must, to some extent, be able to escape the endosomes without extensive degradation. This will have to be investigated in future studies.

Our results suggest that a non-endocytotic uptake pathway exists for arginine-rich peptides. The nature of

this mechanism remains a puzzle. However, investigation of peptide–lipid interactions in model systems can provide valuable clues in mechanistic studies of translocation across cell membranes. Since the fluorescence of a tryptophan residue provides a very useful spectroscopic tool for such studies, we synthesized here variants of heptaarginine and Tat(48–60) having a tryptophan residue in the amino acid sequence. These peptides will be used in future studies using membrane model systems. Whether the inclusion of a tryptophan residue had any influence on the cellular uptake of these peptides per se was not investigated in this study, but in the light of the similarities found for penetratin and its tryptophan-free analog, we do not expect the tryptophan-modified peptides to exhibit internalization properties significantly different from their parent peptides.

In conclusion, this investigation of the cellular uptake of analogs of penetratin, Tat(48–60), and heptaarginine in unfixed cells has shown that both non-endocytotic and endocytotic uptake pathways are involved in their cellular internalization. We have also demonstrated that lysine and arginine residues have a remarkably different influence on the cellular association and internalization of these peptides.

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