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Cell-penetrating Peptides

A REEVALUATION OF THE MECHANISM OF CELLULAR UPTAKE*

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Cellular uptake of a family of cationic cell-penetrating peptides (examples include Tat peptides and penetratin) have been ascribed in the literature to a mechanism that does not involve endocytosis. In this work we re-evaluate the mechanisms of cellular uptake of Tat 48–60 and $(Arg)_9$. We demonstrate here that cell fixation, even in mild conditions, leads to the artifactual uptake of these peptides. Moreover, we show that flow cytometry analysis cannot be used validly to evaluate cellular uptake unless a step of trypsin digestion of the cell membrane-adsorbed peptide is included in the protocol. Fluorescence microscopy on live unfixed cells shows characteristic endosomal distribution of peptides. Flow cytometry analysis indicates that the kinetics of uptake are similar to the kinetics of endocytosis. Peptide uptake is inhibited by incubation at low temperature and cellular ATP pool depletion. Similar data were obtained for Tat-conjugated peptide nucleic acids. These data are consistent with the involvement of endocytosis in the cellular internalization of cell-penetrating peptides and their conjugates to peptide nucleic acids.

During the last decade, several proteins, such as HIV-1 Tat, *Drosophila* Antennapedia homeoprotein, and HSV-1 VP22 have been shown to traverse the cell membrane by a process called protein transduction and to reach the nucleus while retaining their biological activity (1–5). Short "protein-transduction domains" are responsible for the cellular uptake of these proteins (6, 7). Although the biological relevance of protein transduction remains to be understood, it has attracted much interest. Indeed, it was discovered that short peptides derived from protein-transduction domains (cell-penetrating peptides or CPPs)[1] can be internalized in most cell types and, more importantly, allow the cellular delivery of conjugated (or fused) biomolecules (8, 9). A wide range of biomolecules such as antigenic peptides (10), peptide nucleic acids (11), antisense oligonucleotides (12), full-length proteins (13–15), or even nanoparticles (16) and liposomes (17) have been delivered this way. Most peptide- and nucleic acid-based drugs are poorly taken up in cells, and this is considered a major limitation in their development as therapeutic agents (reviewed in refs. 18 and 19). Conjugation of therapeutic agents to CPPs could thus become a strategy of choice to improve their pharmacological properties.

The mechanism of internalization of CPPs and their cargo is not well understood and has recently been the subject of controversies. It has been described in the literature that internalization of these peptides is not significantly inhibited by incubation at low temperature, by depletion of the cellular ATP pool, or by inhibitors of endocytosis (7, 20–22). Moreover, structure-activity studies indicate that the internalization of CPPs do not depend on its specific primary sequence, which implies independence of receptor recognition (20, 23, 24). Based on these results, it has been commonly accepted that the internalization of CPPs do not involve endocytosis or specific protein transporters. Instead, a direct transport through the lipid bilayer of membranes has been proposed as a possible mechanism of translocation (7, 20). If correct, this mechanism would require a radical revision of current ideas on the properties of lipid bilayers, taking into account the hydrophilic nature of CPPs such as Tat or $(Arg)_9$ and the fact that there is no indication of increased membrane permeability in the presence of these peptides.

Most studies on the mechanism of CPP translocation essentially rely on two techniques, namely fluorescence microscopy on fixed cells and fluorescence-activated cell sorter (FACS) analysis. Recently it was shown that the fixation of cells with methanol induces the artificial nuclear association of VP22 and histone H1 (25). This raised concerns about the validity of histochemical detection of CPP distribution in fixed cells.

We demonstrate here that cell fixation, even under mild conditions, leads to the artifactual redistribution of CPP into the nucleus. Moreover, these peptides bind strongly to the cell plasma membrane and remain associated with cells even after repeated washings. As a consequence, FACS analysis cannot be validly used to evaluate cellular uptake unless a protease digestion step of the adsorbed CPP is included in the protocol.

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[1] The abbreviations used are: CPP, cell-penetrating peptides; FACS, fluorescence-activated cell sorter; PNA, peptide nucleic acid; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; CHO, Chinese hamster ovary; Bhoc, benzhydryloxycarbonyl; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; DIPEA, diisopropylethylamine; PyAOP, 7-azabenzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; DIPEA, diisopropylethylamine; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate.

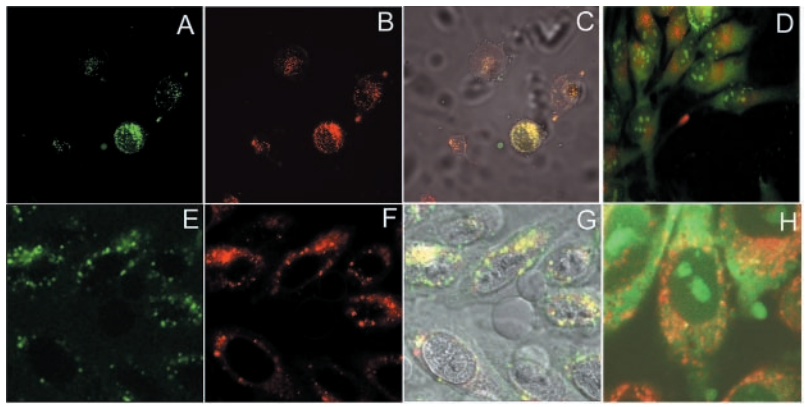

FIG. 1. **Cell fixation affects intracellular distribution of Tat peptide.** HeLa (*panels A–D*) or CHO (*panels E–H*) cells were incubated with the 1 μM or 10 μM flurochrome-tagged Tat peptide respectively, with 25 μg/ml transferrin, or with 10 μM FM 4-64 for 15 min as detailed under "Materials and Methods." Cells were either fixed with formaldehyde before fluorescence microscopy or left untreated. *A*, Alexa 488-tagged Tat peptide in unfixed HeLa cells (*green fluorescence*); *B*, Alexa 546-tagged transferrin in unfixed HeLa cells (*red fluorescence*); *C*, co-localization of Tat peptide and transferrin incubated together in unfixed cells (*yellow fluorescence*); *D*, nuclear localization of Tat peptide (*green fluorescence*) and vesicular localization of transferrin (*red fluorescence*) in fixed cells; *E*, fluorescein-tagged Tat peptide in unfixed CHO cells (*green fluorescence*); *F*, FM 4-64 in unfixed CHO cells (*red fluorescence*); *G*, co-localization of Tat peptide and FM 4-64 incubated together in unfixed CHO cells; *H*, nuclear localization of Tat peptide (*green fluorescence*) and vesicular localization of FM 4-64 (*red fluorescence*) in fixed CHO cells.

These findings have prompted us to reevaluate the mechanism of cellular uptake of two widely used CPP, namely Tat 48–60 and $(Arg)_9$, using as tools fluorescence microscopy on unfixed cells and FACS analysis on trypsin-treated cells. Both sets of data are consistent with the involvement of endocytosis in the cellular internalization of CPP and their conjugates to PNAs (peptide nucleic acids).

MATERIALS AND METHODS

Peptide Synthesis and Labeling—Synthesis of Tat peptide with sequence Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro and of $(Arg)_9$ were carried out by solid phase on a Pioneer peptide synthesizer (Applied Biosystems) following the Fmoc chemistry protocol. A cysteine was added to the C-terminal end of the peptides to provide a sulfhydryl group for ligation to a fluorochrome. Peptides were purified by preparative HPLC and characterized by analytical HPLC and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis (data not shown). Labeling with the fluorochrome was performed by conjugation with a 10 molar excess of the fluorochrome-maleimide derivatives (Molecular Probes) in 50 mM Tris-HCl buffer pH 7.2 for 4 h in the dark. Labeled peptides were purified by semi-preparative HPLC, freeze-dried, and resuspended in deionized water. Peptides were stored frozen at −20 °C until further use.

Synthesis of Fluorescein-Tat-PNA and Fluorescein PNA-Tat—PNA-peptide conjugates were synthesized sequentially on an ABI Pioneer peptide synthesizer (peptide part) and an ABI 380B DNA synthesizer (PNA part) using a Fmoc-PAL-polyethylene glycol polystyrene support. For the Peptide-PNA construct, the peptide and PNA parts were synthesized using the ABI 380B Synthesizer. Fmoc-PNA(Bhoc) monomers were obtained from Applied Biosystems, and Fmoc amino acids from Novabiochem with Pbf protection for Arg, Trityl for Gln, and *t*-butyloxycarbonyl (Boc) for Lys. Assembly of the Tat peptide sequence (GRKKRRQRRRP) was by the standard Fmoc procedure using PyBOP/DIPEA/*N*,*N*-dimethylformamide coupling reactions (26). The anti-TAR PNA sequence (CTCCCAGGCTCA) (27) was assembled also by the Fmoc procedure with PyAOP in *N*,*N*-dimethylformamide as coupling agent and DIPEA/2,6-lutidine (2:3) in *N*-methylpyrollidone as the base (28). Peptide and PNA parts were joined by an AEEA linker (2-aminoethoxy-2-ethoxyacetic acid, Applied Biosystems) using a standard PNA coupling cycle. After assembly of the PNA-peptide or peptide-PNA, one further coupling reaction was carried out with 6-carboxyfluorescein diacetate (Sigma, 10 eq) pre-activated with HATU in the presence of 1 equivalent of DIPEA. After 10 min, a second equivalent of DIPEA was added, and the mixture was added to the solid support. After coupling, the acetyl esters were removed by treatment with 20% piperidine/*N*,*N*-dimethylformamide, and the conjugate was cleaved from the support in trifluoroacetic acid /phenol/water/triisopropylsilane (85:10:2.5:2.5) for 4 h and precipitated with diethyl ether. After lyophilization from a 0.1% trifluoroacetic acid solution in water, the conjugates were purified by reversed-phase HPLC on a C_{18} column with acetonitrile gradient in 0.1% trifluoroacetic acid in water. Product masses determined by MALDI-TOF mass spectrometry agreed with expected values (data not shown).

Cells and Cell Cultures—HeLa cells were cultured as exponentially growing subconfluent monolayers on 90-mm plates in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum and 2 mM glutamine. CHO-K1 cells were cultured as exponentially growing subconfluent monolayers on 25-cm^2 culture flasks in F-12K medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum and 2 mM glutamine. Jurkat lymphoid cells were cultured in suspension in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum and 2 mM glutamine.

Fluorescence Microscopy—Exponentially growing cells were dissociated with a nonenzymatic cell dissociation medium (Sigma). 2.5×10^5 cells were plated and cultured overnight on 30-mm plates on a glass coverslip. The culture medium was discarded, and the cells were washed with NaCl/P_i (pH 7.3). NaCl/P_i was discarded, and the cell monolayer was incubated with the peptides dissolved in Opti-MEM at the appropriate concentration. Subsequently, cells were rinsed three times for 5 min with NaCl/P_i for the observation of the living cells. For the fixed cells, the protocol was the same, but, in addition, they were fixed in 3.7% (v/v) formaldehyde in NaCl/P_i for 5 min at room temperature. For direct detection of fluorescein-labeled peptides, cells were washed three times after fixation in NaCl/P_i at room temperature and washed again with NaCl/P_i before being processed in Vectashield mounting medium (Vector Laboratories). The distribution of fluorescence was analyzed on a Zeiss Axiophot fluorescence microscope or on a Leica DM IRBE inverted fluorescence microscope.

Flow Cytometry—To analyze the internalization of fluorochrome-labeled Tat peptides by FACS, exponentially growing HeLa cells were dissociated with nonenzymatic cell dissociation medium, centrifuged at $500 \times g$, and resuspended in Opti-MEM. 5×10^5 HeLa cells in 250 μl of Opti-MEM were then incubated with the peptides at the concentration indicated in the Figure legends. For analysis of Jurkat cells, we used a slightly different procedure. After 40 min incubation in 1 ml of serum-free medium, 2×10^6 cells were resuspended in 200 μl of serum-free medium containing the Tat peptide at 10 μM concentration. After different times of incubation at 37 °C or 4 °C in the presence of the peptide, the cell suspension was either centrifuged at $1,300 \times g$ or diluted in 2 ml of NaCl/P_i and centrifuged at $800 \times g$. The cell pellet was washed twice before incubation with trypsin (1 mg/ml) during 15 min at 37 °C. Cells were then washed once more with NaCl/Pi and finally resuspended in 500 μl of NaCl/P_i containing 0.1 mM propidium iodide (Molecular Probes).

In some experiments, cells were incubated with 10 mM sodium azide in the presence of 6 mM 2-deoxy-D-glucose for 1 h to deplete cellular ATP.

Fluorescence analysis for both cell lines was performed with a FACScan fluorescence-activated cell sorter (BD Biosciences). Cells stained with propidium iodide were excluded from further analysis. A minimum of 20,000 events per sample was analyzed.

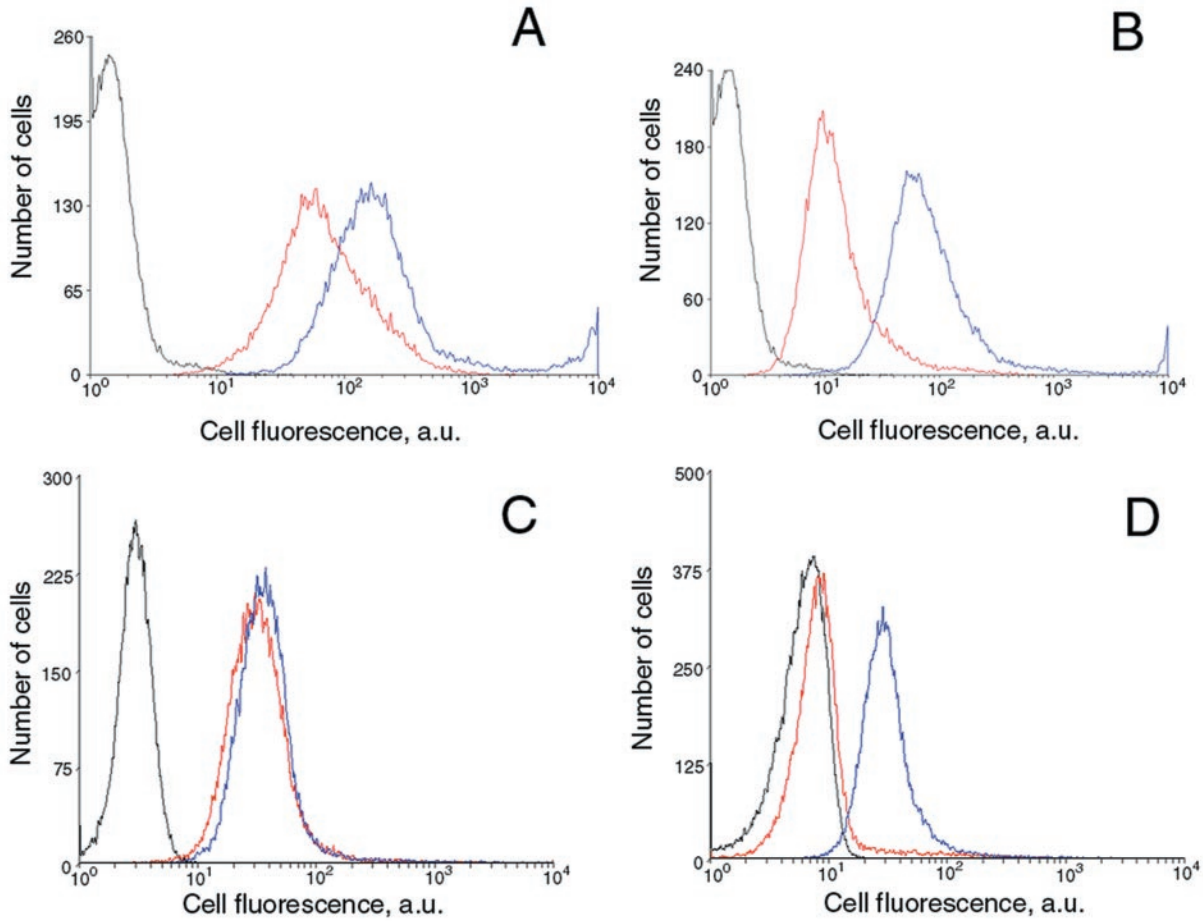

FIG. 2. **Cell-associated Tat and $(Arg)_9$ with and without trypsin treatment.** HeLa cells were incubated for 15 min in the presence of 1 μM fluorescein-tagged $(Arg)_9$ at 37 °C (*A*) or 4 °C (*B*) with (*red curves*) or without (*blue curves*) trypsin treatment before FACS analysis. Jurkat cells were incubated for 15 min in the presence of 10 μM fluorescein-tagged Tat at 37 °C (*C*) or 4 °C (*D*) with (*red curves*) or without (*blue curves*) trypsin treatment before FACS analysis. The *black curves* correspond to cells incubated in the absence of peptides.

RESULTS

Cellular Uptake of Tat and $(Arg)_9$ Peptides—The distribution of fluorescein- or Alexa 488-tagged Tat peptide (green fluorescence) and that of Alexa 546-conjugated transferrin or FM 4-64 (red fluorescence) were followed in living unfixed HeLa and CHO cells (Fig. 1). On both cell lines, a punctate cytoplasmic distribution of the peptide was observed (Fig. 1, *A* and *E*) similar to the localization of the endocytic markers used in this study (Fig. 1, *B* and *F*). On both cell lines, the Tat peptide predominantly co-localized with the marker of endocytosis (transferrin or FM 4-64) (Molecular Probes) in living cells as indicated by the resulting yellow coloration (Fig. 1, *C* and *G*). However, a mild fixation with formaldehyde drastically changed the distribution of the Tat peptide (giving rise to the characteristic nuclear localization reported in most published data), whereas the distribution of transferrin or FM 4-64 remained unchanged (Fig. 1, *D* and *H*). These data indicate that even mild fixation led to artifactual redistribution of Tat peptide, resulting in its apparent nuclear localization. Likewise, fluorescein-tagged $(Arg)_9$ appeared to be concentrated in nuclei in formaldehyde-fixed cells, whereas they were located in cytoplasmic vesicles in unfixed cells (data not shown).

These images of Tat localization in living cells suggest that endocytosis plays an important role in the uptake of the CPPs at variance with the prevailing concept and prompted us to re-evaluate their uptake mechanism.

Flow Cytometry Analysis of Tat and $(Arg)_9$ Peptide Uptake—FACS analysis is a conventional tool to quantify cellular association of fluorochrome-tagged peptides. However, because flow cytometry does not discriminate between membrane-bound and internalized fluorochrome, special care should be taken to minimize any contribution of surface-bound peptide in measuring peptide uptake. A simple and versatile protocol involving trypsin treatment of the cells before FACS analysis has been used here to assess cellular uptake of fluorochrome-labeled Tat and $(Arg)_9$ peptides. We incubated cells with fluorescein-labeled peptides at 37 °C or 4 °C for various periods of time. The trypsinization step was either included or omitted in the washing protocol, and cell fluorescence was measured by flow cytometry. As shown in Fig. 2 for HeLa and Jurkat cells incubated with Tat or $(Arg)_9$, FACS analysis in the absence of trypsin treatment generally overestimated the cell uptake. At 4 °C, most of the signal was trypsin-sensitive (Fig. 2*A* *versus* 2*B* and Fig. 2*C* *versus* 2*D*). For a given temperature, the extent of the overestimation varied according to cell line and peptide. These data suggest that trypsin treatment removed surface-bound peptide by digestion of peptide and/or membrane proteins, whereas simple washing with NaCl/P_i did not remove all bound peptide. This is not very surprising, considering the highly cationic nature of these peptides and the overall negative charge of the cell surface. A standard protocol including a trypsin treatment before FACS analysis was therefore used in all subsequent experiments.

An intriguing feature of CPP behavior was their reported fast internalization. We measured the kinetics of CPP uptake using flow cytometry as cell-associated fluorescence after the

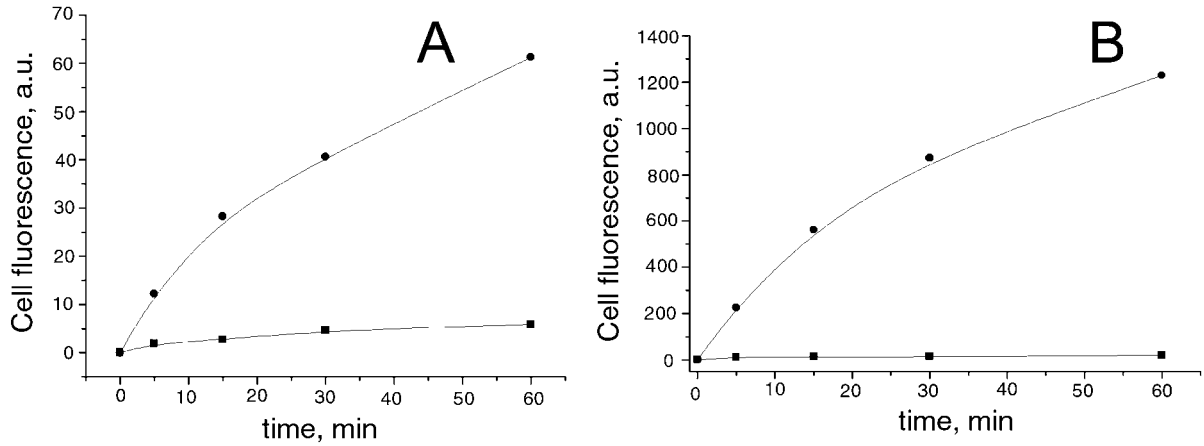

FIG. 3. **Kinetics of cell uptake of Tat at 37 °C and 4 °C.** Jurkat cells were incubated in the presence of 10 μM fluorescein-tagged Tat (*A*) or 10 μM FM 4-64 (*B*) at 37 °C (*closed circles*) or at 4 °C (*squares*) for the indicated periods of time. Samples were treated with trypsin before FACS analysis.

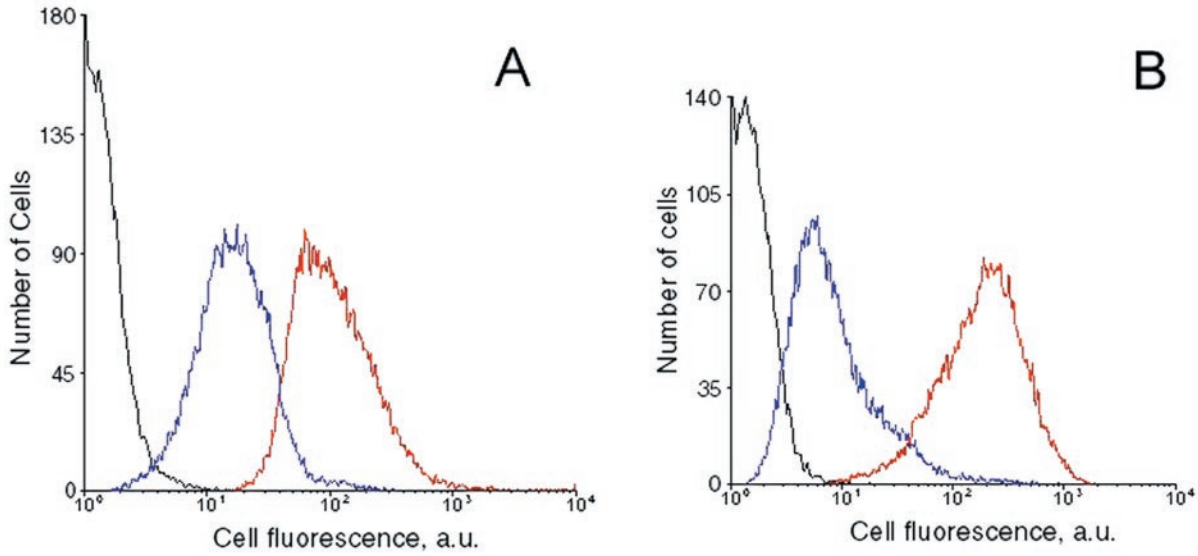

FIG. 4. **Depletion of cellular ATP inhibits uptake of Tat and $(Arg)_9$.** HeLa cells were incubated for 15 min in the presence of 5 μM fluorescein-tagged Tat (*A*) or 2 μM fluorescein-tagged $(Arg)_9$ (*B*). Intracellular ATP pool has been depleted (*blue curves*) or not (*red curves*) by preincubation with sodium azide and deoxyglucose. Samples were treated with trypsin before FACS analysis.

trypsin digestion step. Accumulation of Tat in Jurkat cells (Fig. 3*A*) was slow, with kinetics comparable to that of the classical marker of endocytosis FM 4-64 (Fig. 3*B*). Data in Fig. 3 also show that FM 4-64 endocytosis (Fig. 3*B*) and Tat uptake (Fig. 3*A*) were both severely impaired by incubation of cells at 4 °C. This is in agreement with an energy-dependent mechanism of internalization and at variance with early data in the field. Again, similar data have been obtained in HeLa and in CHO cells (data not shown).

An energy-dependent uptake mechanism was also confirmed in the experiments in which the cellular ATP pool was depleted by preincubation of the cells with sodium azide and deoxyglucose. ATP depletion significantly reduced the uptake of Tat (Fig. 4*A*) and of $(Arg)_9$ (Fig. 4*B*) in HeLa cells. The extent of the inhibition was close to that observed for transferrin, a classical marker of receptor-mediated endocytosis (data not shown). Similar results were obtained for Jurkat cells (data not shown).

Cell Uptake and Intracellular Distribution of Tat-PNA Conjugates—CPPs are being considered as promising new tools for the delivery of non-permeant or poorly permeant drugs. In this work, we tested Tat peptide conjugated to peptide nucleic acids. PNAs are nuclease-resistant DNA mimics, which hybridize specifically (by Watson-Crick base pairing) and with unusually high affinity to complementary RNA. However, the cellular uptake of these uncharged compounds is poor and, unlike DNA, cannot be increased by complexation with cationic lipids, thus limiting their use in antisense strategies (see Ref. 29 for a review). Tat-PNA conjugates carrying a fluorochrome on the N terminus of the Tat (data not shown) or PNA-Tat conjugates carrying a fluorochrome on the N terminus of the PNA (Fig. 5) moiety were taken up in HeLa cells and accumulated in vesicles. As for Tat peptide, cellular uptake of these Tat-PNA conjugates measured by flow cytometry was greatly reduced by incubation at low temperature and by ATP depletion (Fig. 6).

DISCUSSION

It has been previously reported that cell incubation with various CPPs results in their rapid uptake with a predominantly nuclear localization and the lack of the punctate cytoplasmic labeling characteristic of endocytic uptake (7, 20–22, 30). Intriguingly, the same picture was observed at 37 °C and at 4 °C or in the presence of a number of inhibitors of endocytosis and cell metabolism (see ref. 31 for a comprehensive overview). In the present study we found that, at least for Tat

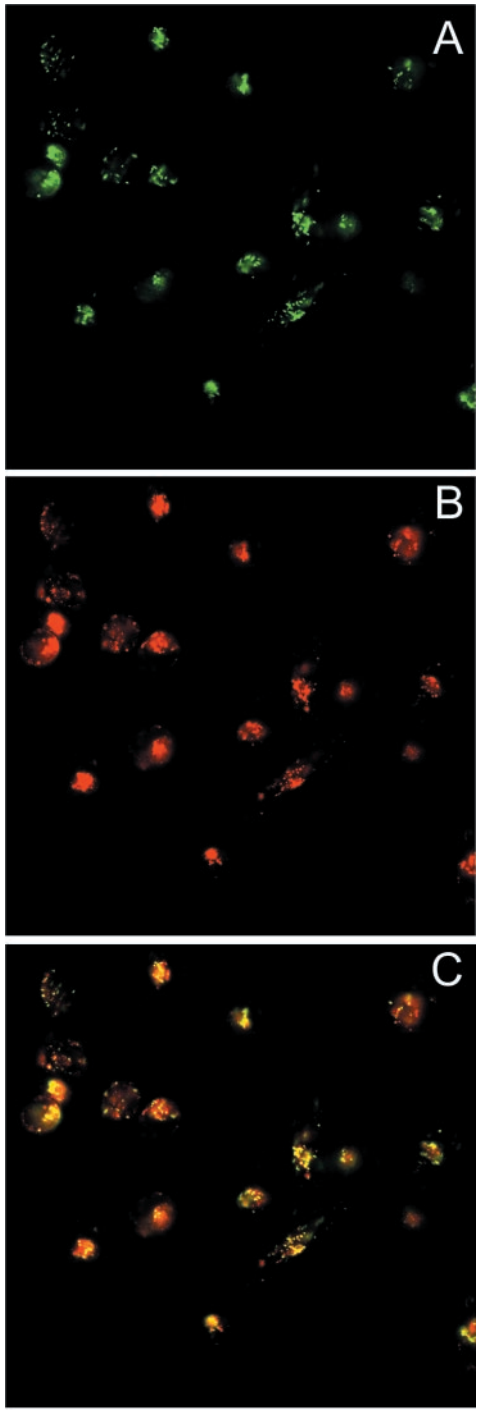

FIG. 5. **Intracellular distribution of Tat-PNA conjugates.** HeLa cells were co-incubated for 15 min with 1 μM fluorescein-tagged Tat-PNA (*green fluorescence*) (*A*) and 25 μg/ml Alexa 546-tagged transferrin (*red fluorescence*) (*B*); *C*, image merging of Tat-PNA peptide and transferrin (*yellow fluorescence*). Cells were not fixed before observation by fluorescence microscopy.

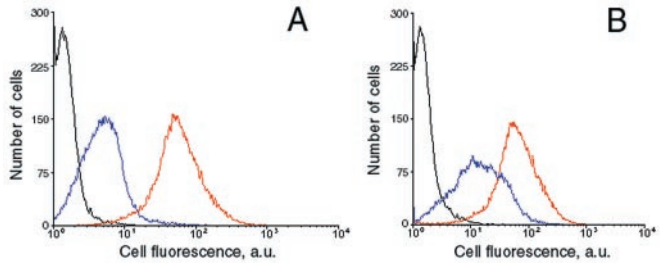

FIG. 6. **Cellular uptake of Tat-PNA is inhibited at 4 °C and by depletion of cellular ATP.** HeLa cells were incubated for 15 min in the presence of 1 μM fluorescein-tagged Tat-PNA. *A*, cells were incubated at 37 °C (*red curve*) or at 4 °C (*blue curve*). *B*, cells were preincubated (*blue curve*) or not (*red curve*) with sodium azide and deoxyglucose to deplete cellular ATP. Samples were treated with trypsin before FACS analysis.

and $(Arg)_9$, even the reportedly mild cell-fixation protocols commonly used to evaluate uptake by fluorescence microscopy (32) caused an artifactual redistribution of these peptides into the nucleus and gave rise to altered images as compared with the experiments made on unfixed cells. This was not observed for classical endocytic markers such as transferrin and FM 4-64. This artifact could be related to the highly cationic nature of the peptides, which is a common and even necessary characteristic for CPP (21, 23, 24, 33). A high density of positive charges leads to rather strong binding of the peptides to the overall negatively charged plasma membrane as was evidenced by our flow cytometry analysis data. Even mild fixation caused a disruption of membrane barrier function and increased transfer of hydrophilic probes such as propidium iodide into the cytoplasm and nucleus, as detected by the labeling of the cell nucleus under these conditions (data not shown). High positive charge of the peptides most probably causes their strong binding to nucleic acids and ultimately leads to concentration of CPP into the nucleus, which is an abundant reservoir of cellular nucleic acids. Cell fixation should therefore be avoided for further studies on the intracellular distribution of CPP and of their conjugates to various cargoes. The cargo itself might influence the final distribution of the conjugate, as attested to by two recent papers. PNA-Tat (or Antennapedia) conjugates were found predominantly segregated in endocytic vesicles (29), in keeping with our own data, whereas phosphorothioate antisense oligonucleotides conjugated to these CPP were at least partly found in nuclei (12).

Strong binding of CPP to the cell surface also leads to the overestimation of uptake by flow cytometry, and special care should be taken to remove membrane-bound peptide for the evaluation of cell uptake. For flow cytometry experiments, we used a simple protocol based on the treatment of cells with trypsin before FACS analysis. Although only trypsin treatment was used in this study, the contribution of the bound peptide could possibly be eliminated by other means, including treatment with other proteases. Most probably, trypsin digested the peptide and decreased binding of the remaining degradation products to the cell surface. Alternative approaches have recently been proposed that rely on the use of fluorescence quenching groups. A 3-nitrotyrosine quenching group has been introduced in Transportan (and in other CPPs), and these modified peptide carriers were conjugated through a disulfide bridge to a small peptide cargo carrying the fluorochrome. This elegant approach allows measurement only of cellular uptake of the peptide-conjugated cargo, because release of the fluorescent cargo strictly requires the reduction of the disulfide bridge, which is supposed to occur only in the intracellular reductive environment (34). Along the same lines, Drin *et al.* (35) conjugated the *Antennapedia* peptide to 7-nitrobenz-2-oxo-1,3-diazol-4-yl (NBD), whose fluorescence could be quenched by dithionite, a membrane-impermeant compound. Cell association could therefore be discriminated from cell uptake. It is worth noting that both studies led to the conclusion of a relatively slow rate of uptake of these CPPs (see below). In contrast to the trypsin treatment, these interesting strategies require the synthesis of the appropriate peptide analogs.

Reevaluation of the mechanism of the internalization of Tat and $(Arg)_9$ peptides demonstrated that, at variance with most published data, internalization of these peptides was strongly

inhibited by low temperature or by depletion of the cellular pool of ATP. Moreover, the kinetics of peptide uptake was as slow as that characteristic of endocytosis. The observation by fluorescence microscopy of living cells incubated with these peptides or their PNA conjugates demonstrated a punctate distribution characteristic of endocytosis and overlapping with common endocytic markers. These results strongly support the involvement of endocytosis as the major route for the internalization of Tat and $(Arg)_9$. The applicability of this conclusion to other CPP with different cargoes remains to be verified. Also, we cannot formally exclude the possibility that a small fraction of CPP enters cells by an endocytosis-independent but biologically relevant pathway.

One can hypothesize that the high positive charge of the peptides used in the present study promote binding of the peptide and of the conjugated cargo to the cell surface. Interestingly, the internalization of the full-length HIV-1 Tat protein required an interaction of the basic domain of full-length Tat with cell surface heparan sulfate proteoglycans (36, 37). Whether these receptors or other cell surface determinants are involved in the endocytosis of CPP will have to be reinvestigated using the tools described here.

In conclusion, we present here data unveiling artifacts associated with the experimental techniques most commonly used to study the internalization of CPP. Using simple and versatile improved methodologies, we reestablish the role of endocytosis in the internalization of cationic CPP.

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