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A Truncated HIV-1 Tat Protein Basic Domain Rapidly Translocates through the Plasma Membrane and Accumulates in the Cell Nucleus*

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Tat is an 86-amino acid protein involved in the replication of human immunodeficiency virus type 1 (HIV-1). Several studies have shown that exogenous Tat protein was able to translocate through the plasma membrane and to reach the nucleus to transactivate the viral genome. A region of the Tat protein centered on a cluster of basic amino acids has been assigned to this translocation activity. Recent data have demonstrated that chemical coupling of a Tat-derived peptide (extending from residues 37 to 72) to several proteins allowed their functional internalization into several cell lines or tissues. A part of this same domain can be folded in an α-helix structure with amphipathic characteristics. Such helical structures have been considered as key determinants for the uptake of several enveloped viruses by fusion or endocytosis. In the present study, we have delineated the main determinants required for Tat translocation within this sequence by synthesizing several peptides covering the Tat domain from residues 37 to 60. Unexpectedly, the domain extending from amino acid 37 to 47, which corresponds to the α-helix structure, is not required for cellular uptake and for nuclear translocation. Peptide internalization was assessed by direct labeling with fluorescein or by indirect immunofluorescence using a monoclonal antibody directed against the Tat basic cluster. Both approaches established that all peptides containing the basic domain are taken up by cells within less than 5 min at concentrations as low as 100 nM. In contrast, a peptide with a full α-helix but with a truncated basic amino acid cluster is not taken up by cells. The internalization process does not involve an endocytic pathway, as no inhibition of the uptake was observed at 4 °C. Similar observations have been reported for a basic amino acid-rich peptide derived from the Antennapedia homeodomain (1). Short peptides allowing efficient translocation through the plasma membrane could be useful vectors for the intracellular delivery of various non-permeant drugs including antisense oligonucleotides and peptides of pharmacological interest.

Most "information-rich" molecules, such as oligonucleotides, genes, peptides, or proteins, are poorly taken up by cells since they do not efficiently cross the lipid bilayer of the plasma membrane or of the endocytic vesicles (Ref. 2, and references therein). This is considered to be a major limitation for their *ex vivo* or *in vivo* use in fundamental studies or in possible clinical applications. These compounds are currently delivered by various techniques including microinjection, electroporation, association with cationic lipids, liposome encapsidation, or receptor-mediated endocytosis. Various problems have been encountered in their use including low transfer efficiency, complex manipulation, cellular toxicity, or immunogenicity, which would preclude their routine use *in vivo*. As an alternative, several peptides have been successfully used to improve the intracellular delivery of nucleic acids or proteins. The fusogenic properties of influenza virus have been extensively studied in this context. They are currently assigned to a pH-dependent conformational change of the viral hemagglutinin leading to the exposure of its hydrophobic N-terminal region, and to the fusion of the viral and endosomal membranes (3). A Tat mRNA-specific antisense oligonucleotide covalently bound to this fusogenic peptide has been demonstrated to have an increased antiviral activity *in vitro*, probably as a result of increased cellular uptake (4). Peptides adopting an amphipathic conformation at acidic pH largely increased the delivery of plasmid DNA complexed with transferrin-polylysine conjugates (5). Likewise, amphipathic characteristics have been described for a peptide derived from the third domain of Antennapedia homeodomain (1), which allows the delivery of antisense oligonucleotides or biologically active peptides. Interestingly, this peptide was efficiently translocated through the plasma membrane in the absence of energy (*e.g.* via a mechanism that does not involve endocytosis). The HIV[1] Tat transactivation protein is efficiently taken up by cells (6–8), and concentrations as low as 1 nM in the culture media are sufficient to transactivate a reporter gene expressed from the HIV-1 promoter (6). The domain responsible for this translocation has been ascribed to the region centered on a basic domain of the Tat protein. A peptide extending from residues 37 to 72 allowed the internalization of conjugated proteins such as β-galactosidase or horseradish peroxidase (9). One to two Tat peptides/molecule of protein were sufficient to induce efficient translocation. Likewise, the Tat-(37–62) sequence conjugated to a Fab antibody fragment enhanced its *in vitro* cell surface association and internalization (10). Physicochemical studies involving circular dichroism and energy minimization indicated that the

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[1] The abbreviations used are: HIV, human immunodeficiency virus; NLS, nuclear localization signal; Boc, *t*-butyloxycarbonyl; HF, hydrogen fluoride; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; FCS, fetal calf serum; NEM, *N*-ethylmaleimide; FACS, fluorescence-activated cell sorting; TAMRA-SE, tetramethylrhodamine succinimidyl ester; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide.

region covering the Tat-(38–49) domain adopted an α-helical structure with amphipathic characteristics (11). Both biological data and physicochemical studies were in keeping with a crucial role of the α-helix forming domain in Tat uptake. Most of these studies have concerned peptides extending from residues 37 to 72. These include other motifs of interest and in particular a cluster of basic amino acids extending from positions 49 to 58, which does not overlap the presumed amphipathic helical structure. This cluster of basic amino acids (Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg) appears to be unstructured due to charge repulsions (11) and contains a nuclear localization signal (NLS) sequence (12).

To delineate more precisely which determinants of the Tat-(37–60) peptide are crucial for Tat translocation and nucleolar localization, we have synthesized peptides harboring deletions in the purported α-helix domain or in the basic cluster. These peptides were assayed for their ability to translocate through the cell membrane in several cell lines. Cellular uptake and intracellular distribution were monitored by fluorescence microscopy using peptides labeled with fluorescein maleimide on their C-terminal cysteine or by indirect immunofluorescence using a monoclonal antibody directed against the Tat basic domain. Unexpectedly, the α-helix region did not appear to be required for efficient and fast cell uptake. In contrast, the whole basic domain from the Tat peptide appeared necessary for cell internalization.

MATERIALS AND METHODS

Peptide Synthesis—All peptides were chemically synthesized by solid phase method using *t*-butyloxycarbonyl (Boc)-benzyl chemistry on a 4-(oxymethyl)phenylacetamidomethyl polystyrene resin (Applied Biosystems) as described previously (8) except for the following modifications. (i) The C-terminal cysteine side chain was protected by a *p*-methylbenzyl group, and the N-terminal cysteine side chain was protected by the HF-stable protecting group acetamidomethyl. These orthogonal protecting groups allow the conjugation of various pendant groups (or drugs) at the C-terminal or N-terminal ends of the peptide after appropriate chemical treatment. (ii) The coupling reagent was benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (Castro's reagent)/*N*-hydroxybenzotriazole hydrate (5 M excess over amino group). (iii) The deprotection of the N^{α}-Boc group was performed in two steps in 100% trifluoroacetic acid with reaction times of 1 and 3 min, successively. (iv) synthesis scale was 0.2 mmol for the Tat-(43–60), Tat-(48–60), and Tat-(37–53) peptides, and 0.15 mmol for the Tat-(37–60) peptide.

A double-coupling step was performed for each amino acid. The first coupling step was monitored by a ninhydrin colorimetric test, while the second coupling step was running. Peptide cleavage and HF-labile lateral-chain deprotection were achieved by anhydrous treatment for 1 h at −5 °C (HF-*p*-cresol-ethanedithiol (85:10:5; v/v/v)). All peptides were purified by semi-preparative C18 reversed phase HPLC using a μBondapack™ column (19 × 300) (Waters, Millipore). Analytical HPLC was carried out on Hypersyl C18 5-μm column (4.6 × 250) using a Beckman System Gold 126AA solvent delivery module equipped with a System Gold 168 photodiode array detector. Homogeneous HPLC fractions were pooled and lyophilized. Peptide molecular weights were determined by electrospray ionization mass spectrometry. Peptides were quantified after hydrolysis of an aliquot for 24 h at 110 °C. Amino acid analysis were run on a Beckman amino acid analyzer. Mass values, HPLC profiles, and amino acid analyses were in excellent agreement with expected criteria. All peptides were resuspended in PBS (pH 7.3) at a concentration of 10 mg/ml and kept frozen until further use.

Fluorescent Labeling of Peptides—Aliquots of the peptides were first reacted with dithiobisnitrobenzoic acid for SH quantification to assess the availability of the sulfhydryl group. All optical density values were in good agreement with the reduced form of the sulfhydryl group of the peptide. One milligram of Tat-(37–60), Tat-(43–60), Tat-(48–60), or Tat-(37–53) peptides dissolved in PBS was reacted for 2 h in the dark at room temperature with 2 eq of fluorescein maleimide dissolved in dimethylformamide per SH group of the peptide. Reaction was monitored by HPLC with a dual absorbance at 215 and 440 nm. Fluorescent peptides were purified by HPLC (purity > 95%). These modified peptides were lyophilized in the dark, resuspended in PBS (pH 7.3), quantified by amino acid analysis as described above, and stored at −20 °C in the dark until further use.

Cells and Cell Culture—HeLa GH cells (derived from HeLa 229) were cultured as exponentially growing subconfluent monolayers on 90-mm plates in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS) and 2 mM glutamine. HL116 cells (derived from the HT1080 human fibrosarcoma cell line) and CCL39 Chinese hamster cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FCS and 2 mM glutamine.

Fluorescence Microscopy—Exponentially growing cells were dissociated with non-enzymatic cell dissociation medium (Sigma). 3×10^4 cells/well were plated on four-well Lab-Tek coverslips (Nunc Inc.) and cultured overnight. The culture medium was discarded, and the cells were washed once with PBS (pH 7.3) The cells were preincubated in 1 ml of Opti-MEM at 37 °C for 30 min before incubation with the peptides. The peptides were dissolved in Opti-MEM and incubated at 37 °C for 15 min. Opti-MEM was discarded from the coverslips, and the cell monolayers were incubated at 37 °C with peptide solutions at the appropriate concentration for 15 min unless otherwise specified. Subsequently, cells were rinsed three times with PBS (pH 7.3) at room temperature and fixed in 3.7% (v/v) formaldehyde in PBS for 5 min at room temperature. For experiments at 4 °C, the protocol was the same except that all incubations were performed at 4 °C until the end of the fixation procedure. For direct detection of fluorescein-labeled peptides, cells were washed three times, incubated with 50 ng/ml Hoechst 33258 in PBS supplemented with 1% (v/v) FCS at room temperature, and washed again with PBS before being mounted in a PBS/glycerol mixture (2:1; v/v) containing antifading reagent. For indirect immunodetection, fixed cell monolayers were washed twice with PBS before permeabilization with cold acetone (−20 °C) for 30 s. Cells were then incubated first with a monoclonal mouse antibody directed against the Tat-(49–58) epitope (Hybridolab, Institut Pasteur) at a final dilution of 10 ng/μl in PBS supplemented with 1% (v/v) FCS for 1 h at 37 °C. Cells were then washed three times for 10 min with PBS supplemented with 1% (v/v) FCS before incubation with a fluorescein-conjugated anti-mouse IgG (Sigma) at a 1/200 dilution for 30 min. Cells were further processed as for direct detection. The distribution of the fluorescence was analyzed on a Zeiss Axiophot fluorescence microscope equipped with a 100-watt mercury lamp, plan Fluotar oil immersion objectives (40/0.70–0.40; 100/1.30–0.60) (Leica), and the following filter sets: excitation, BP340–380 nm, and emission, LP430 nm (for Hoechst staining); excitation, BP450–490 nm, and emission LP520 nm (for fluorescein). Images were captured with a slow scan charge-coupled device (CCD) (Kappa CF 8/1 DX) interfaced to a 8200 Power PC computer using the public domain NIH program (National Technical Information Service, Springfield, VA, part number PB95-500195GEI) and Adobe Photoshop version 3.0.5 software.

Okadaic Acid and N-Ethylmaleimide (NEM) Assay and FACS Analysis—2×10^5 HeLa cells were incubated for 1 h at 37 °C in Opti-MEM containing 1.5 μM okadaic acid dissolved in ethanol. Cells were then washed twice with Opti-MEM and resuspended with 5 μM fluorescein-labeled Tat-(48–60) peptide in Opti-MEM. Cells were then incubated at 37 °C for 10 min, washed four times, and resuspended in 500 μl of PBS. Fluorescence analysis were performed with a FACScan fluorescence-activated cell sorter (Becton Dickinson). The fluorescence intensity of 3000 cells was analyzed and compared with the intensity of the same amount of okadaic acid untreated cells. For NEM incubation assay, 5×10^5 cells were incubated with or without 1 mM NEM for 5 min at 37 °C and processed as described above.

Cytotoxicity of Tat Peptides on HeLa Cells—HeLa cells (3×10^4/well) were cultured in 96-well microtiter plates in RPMI 1640 supplemented with 10% (v/v) FCS in the presence of the peptides at the indicated concentration. Cells were incubated at 37 °C for 24 h before addition of MTT (Sigma, 5 mg/ml in PBS) for 2 h. The precipitated MTT formazan was dissolved overnight in 100 μl of lysis buffer (20% (w/v) SDS in H_2O/DMF 50:50 (v/v)). The optical density at 570 nm was measured on a Dynatech multiwell plate reader. Cell viability was expressed as the ratio of A_{570} of cells treated with peptide over control sample.

RESULTS

Design, Synthesis, and Chemical Modifications of a Subset of Tat-derived Peptides—Published results have established that the chemical conjugation of a Tat-derived peptide extending from residues 37 to 72 was able to induce the cellular internalization of large proteins such as β-galactosidase or horseradish peroxidase (9). With the prospect of using similar tools for drug delivery, it would be of interest to reduce the peptide length

TAT48-60 C(Acm)GRKKRRQRRRPPQC

TAT43-60 C(Acm)LGISYGRKKRRQRRRPPQC

TAT37-60 C(Acm)FITKALGISYGRKKRRQRRRPPQC

TAT37-53 C(Acm)FITKALGISYGRKKRRC

FIG. 1. **Primary structure of the synthesized Tat peptides.** *One-letter codes* are used (*C(Acm)*, Cys-acetamidomethyl). Hydrophobic amino acids of the α-helix structure are *underlined*.

and to explore its mechanism of internalization. A peptide extending from residues 37 to 60 was used as a starting reference, since it is the shortest sequence that overlaps the two major domains supposed to be involved in membrane translocation and in nuclear targeting. Moreover, this peptide contains two consecutive proline residues at its C-terminal end between the highly basic amino acid cluster and the cysteine residue added as a linking arm (Fig. 1). These two proline residues might potentially act as a spacer between the peptide carrier and the transported drug. This could favor an interaction of the peptide with cellular structures eventually involved in cellular uptake and nuclear translocation. A peptide extending from residues 37 to 60 as well as shorter peptides carrying deletions at the C-terminal or N-terminal end were synthesized as illustrated in Fig. 1. The N-terminal part (amino acids 38–49) of the peptide has been described as a sequence adopting an amphipathic α-helical structure (11). Two peptides carrying a partial (Tat-(43–60)) or a total (Tat-(48–60)) deletion in this sequence were therefore synthesized. The C-terminal part of the peptide contains a basic amino acid-rich region including a NLS (12). A peptide (Tat-(37–53)) with a 7-amino acid deletion at the C-terminal end was therefore synthesized. All peptides also bore an additional cysteine residue at their C-terminal end. This additional sulfhydryl group allows coupling to fluorochromes or to peptides, proteins, oligonucleotides, or peptide nucleic acids to induce a biological activity. All peptides were synthesized by solid phase synthesis using Boc amino acids. The crude products were homogeneous upon analysis by HPLC after hydrogen fluoride cleavage. The synthesis of peptides carrying clusters of basic amino acids (and succession of arginine residues in particular) often leads to coupling problems, which were avoided by the use of the efficient protocol of synthesis described under "Materials and Methods." The identity and the purity of each purified peptide was assessed by HPLC chromatography, amino acid analysis, and mass spectrometry (data not shown).

Uptake and Intracellular Compartmentalization of Tat Peptides—We first tested the internalization of the full-length Tat-(37–60) peptide labeled with fluorescein maleimide on the free sulfhydryl group of its C-terminal cysteine residue. The labeled peptide was purified by HPLC before use and quantified by amino acid analysis. When added to the cell-culture medium, the peptide was mainly recovered in the nucleus with a nucleolar accumulation after a few minutes of incubation only (Fig. 2*A*). The uptake of Tat-(43–60), Tat-(48–60), and Tat-(37–53) peptides (Fig. 1) was monitored in the same experimental conditions (Fig. 2, *B–D*). Interestingly, fragments Tat-(43–60) and Tat-(48–60), which contain the complete basic domain but carry deletions in the helical domain, fully retained cell internalization and nuclear accumulation (Fig. 2, *B* and *C*). The Tat-(37–53) fragment was not taken up by cells even when used at 20 μM (Fig. 2*D*). When used at such high concentrations, the active peptides induced a saturation of the fluorescence signal. The Tat-(48–60) peptide, which contains the basic domain only, retained the full translocation activity and even appeared more efficient in terms of nuclear localization when compared

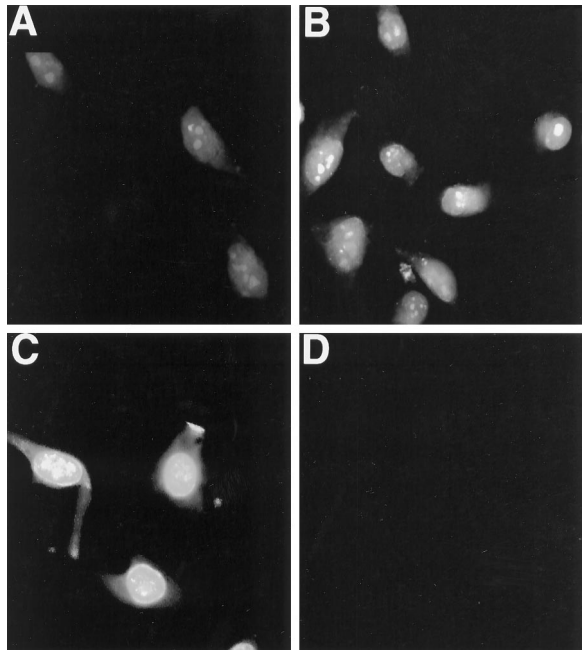

FIG. 2. **Cellular uptake of Tat peptides in HeLa cells.** 3×10^5 cells were incubated with 500 μl of peptide solution for 10 min at 37 °C. Peptides were labeled with fluorescein maleimide on their C-terminal cysteine residue as described under "Materials and Methods." *Panel A* corresponds to peptide Tat-(37–60), *panel B* to Tat-(43–60), and *panel C* to Tat-(48–60) at a concentration of 1 μM. *Panel D* corresponds to Tat-(37–53) peptide incubated at a concentration of 20 μM.

with the other active peptides at the standard dose of 1 μM (compare Fig. 2, *A*, *B*, and *C*). The α-helical structure thus appears to reduce the efficiency of internalization induced by the basic domain, and we cannot exclude that the α-helix part of the peptide slows down the diffusion of the peptide into the cells and toward the nucleus. However, it is unlikely that the helical domain causes a retention in the plasma membrane or in an endocytic compartment as no significant fluorescent signal has been observed associated with these structures, except at high concentrations. The Tat-(37–53) peptide lacked the three arginine residues and the glutamine-proline-proline C-terminal sequence (positions 58–60) (Fig. 1). An additional peptide containing the entire basic sequence (*i.e.* Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys) and lacking the Pro-Pro-Gln C terminus was synthesized to control the possible effect of these amino acids on the ability to translocate through the plasma membrane. This peptide was tested under the same conditions after direct labeling with fluorescein maleimide. No variation in the amount and localization of the internalized peptide was observed as compared with the Tat-(48–60) peptide (data not shown). This confirmed that the Pro-Pro-Gln sequence of the peptide did not directly influence the translocation event and that the full basic domain was required for Tat translocation properties.

Internalization follows a dose-dependent curve as observed by the intensity of the recorded signal (Fig. 3). The amount of internalized peptides increases linearly with its concentration in the culture medium with a saturation from around 5 μM whether quantitating the fluorescent signal or using FACS analysis on resuspended cells (data not shown). At concentrations higher than 5 μM, fluorescein-labeled peptides containing the basic domain could also be detected in the cytoplasm (data not shown). The Tat-(48–60) peptide can be unambiguously detected at a concentration as low as 100 nM (Fig. 3*C*), although the level of detection might be limited by the fluorescent marker.

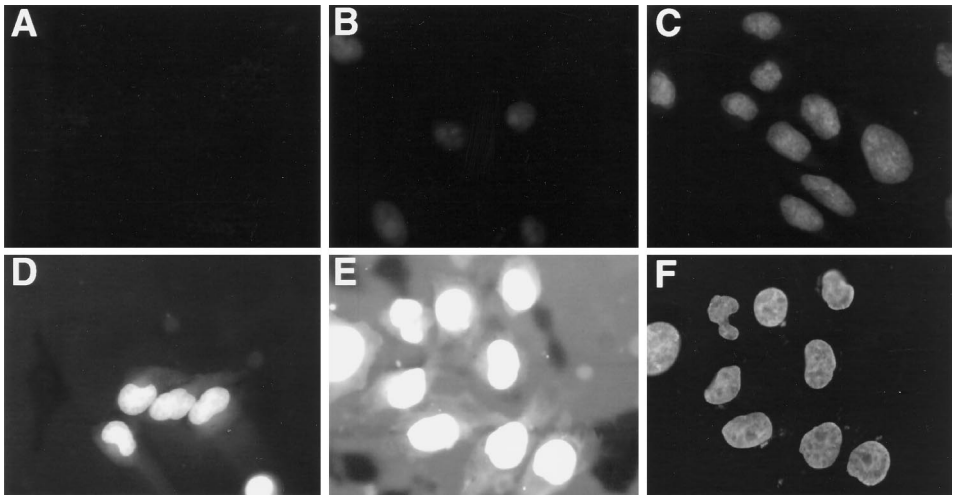

FIG. 3. **Dose-response study of fluorescein-labeled Tat-(48–60) peptide internalization.** 3×10^5 cells/well were incubated with 500 μl of peptide solution for 10 min at 37 °C at the indicated concentration. *Panel A*, no peptide; *panel B*, 50 nM; *panel C*, 100 nM; *panel D*, 250 nM; *panel E*, 500 nM. *Panel F* shows the Hoechst's reagent labeling of cell nuclei corresponding to *panel E*. All views were recorded with the same camera acquisition parameters.

The fluorescence signal could have been due to the internalization of peptide degradation products or of the fluorochrome itself. To exclude these possibilities, the peptide was extensively digested with trypsin (60 min at 37 °C) immediately before its incubation with cells. The digestion of the peptide was monitored by HPLC at dual absorbance values (215 and 440 nm). Absorbance profile recorded at 440 nm indicated that several subfragments containing the fluorescent moiety were generated upon enzymatic digestion (data not shown). When the digested peptide was used in the same *in vitro* assay conditions, no cellular fluorescence was observed at concentrations up to 5 μM (Fig. 4*B*). The same concentration of the free fluorescein fluorochrome induced a cytoplasmic and a nuclear distribution in saponin-permeabilized cells (data not shown). The conjugation of the fluorescein moiety to the cysteine residue of the peptides could potentially alter their cellular uptake or their transport properties. To avoid possible artifacts caused by the attachment of the fluorescent pendant group, cell uptake and intracellular compartmentalisation were monitored by indirect immunofluorescence using monoclonal antibodies directed against the basic region of the Tat protein as described under "Materials and Methods." Most of the experiments described above were repeated using this latter detection procedure. The data obtained by indirect immunofluorescence confirmed previous observations in terms of nuclear localization and relative efficiency of translocation through the plasma membrane for the different peptides (Fig. 5). The Tat-(37–53) peptide was poorly detected, as it partially lacks the antigenic domain (data not shown). The three other peptides (Tat-(43–60), Tat-(48–60) and Tat-(37–60)), which contain the full length basic domain, were detected, although the sensitivity of this technique was lower than for direct labeling. However, the most active peptide (*i.e.* Tat-(48–60)) could be detected at doses as low as 750 nM. Detection at lower peptide concentration by indirect immunofluorescence was limited by cellular autofluorescence and by nonspecific binding of antibodies.

When using indirect immunofluorescence, the C-terminal cysteine residues of these peptides carried a free sulfhydryl group. To assess whether a free sulfhydryl group did not affect the internalization process or the subcellular localization, the peptides were carboxymethylated on their sulfhydryl group. No difference in the behavior between peptides carrying a free or a protected sulfhydryl group was observed (data not shown).

Whatever the method used for their detection, these Tat-derived peptides appear mainly localized in the nucleus with a nucleolar accumulation. It is noteworthy that the Tat basic domain contains a GRKKR NLS within its sequence (12). The presence of this NLS sequence is not sufficient to induce the intracellular translocation of the peptide as the Tat-(37–53) peptide, which contains the NLS sequence but lacks three basic charges on its C-terminal region (Fig. 1), is not taken up by cells (Fig. 2*D*). Further studies are in progress to define more precisely which of the deleted amino acid residues from Tat-

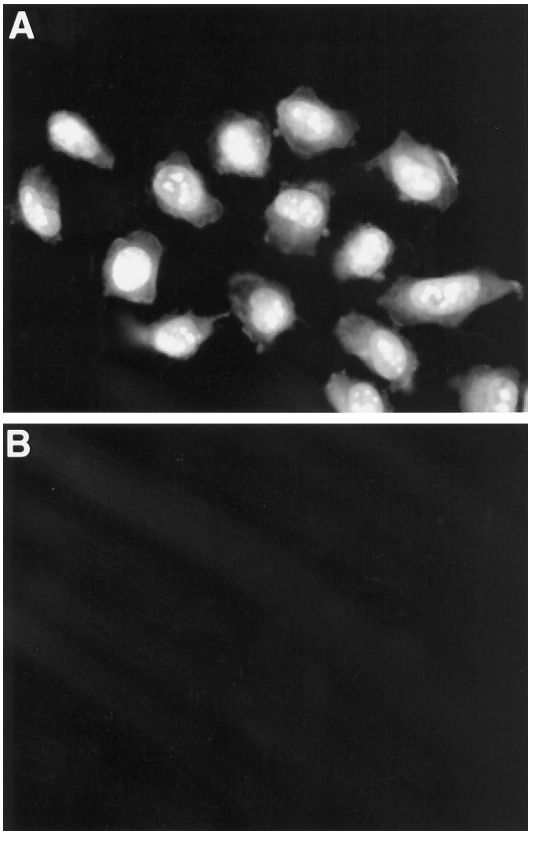

FIG. 4. **Effect of trypsinization on peptide uptake.** 3×10^5 HeLa cells were incubated with 5 μM fluorescein-labeled Tat-(48–60) peptide for 15 min at 37 °C (*panel A*) or with the same amount of peptide digested with trypsin for 1 h at 37 °C before incubation with cells (*panel B*).

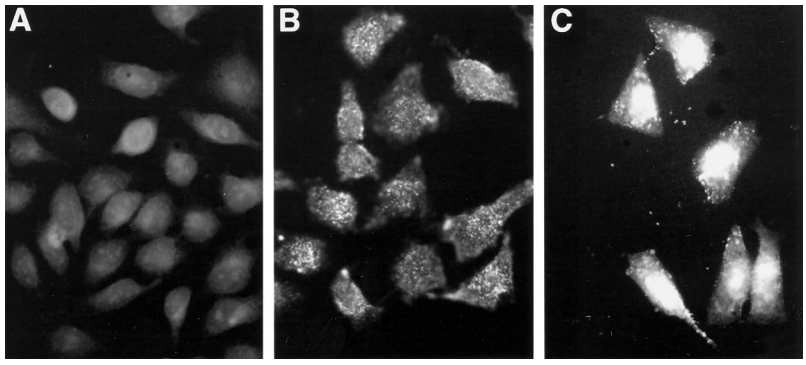

FIG. 5. **Peptide detection by indirect immunofluorescence.** Peptides Tat-(37–60) (*panel A*), Tat-(43–60) (*panel B*), and Tat-(48–60) (*panel C*) were incubated at a final concentration of 10 μM with HeLa cells for 15 min at 37 °C before indirect immunofluorescence detection with a primary monoclonal mouse antibody directed against the Tat-(49–58) epitope and a secondary fluorescein-conjugated anti-mouse IgG.

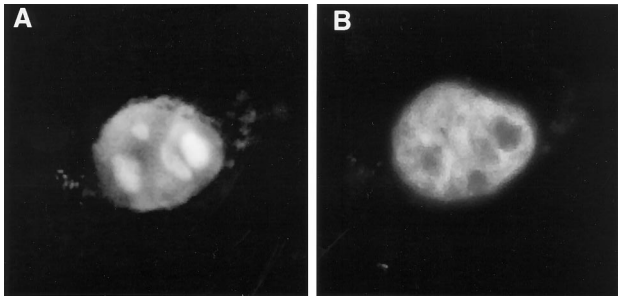

FIG. 6. **Peptide detection on unfixed cells.** HeLa cells were incubated with 500 nM fluorescein-labeled Tat-(48–60) peptide. After incubation, cells were rinsed three times before coloration of nuclei with Hoechst's reagent and direct mounting in PBS/glycerol containing antifading reagent. *Panel A* shows the fluorescein-Tat peptide internalized in the nucleus with nucleolar concentration; *panel B* shows the cell nucleus stained with Hoechst's reagent.

(37–53) are required to recover uptake. All the experiments described in the present paper were performed on HeLa cells. Similar data have been obtained with other cell lines such as CCL39 and HL116 (data not shown). Two other protocols of fixation were used with no differences in the subcellular localization of the peptides (data not shown). These included a fixation with ethanol/glacial acetic acid (95/5) for 5 min at −20 °C or with 2% formaldehyde, 0.2% glutaraldehyde for 5 min at room temperature. In addition cells were incubated with fluorescein-labeled peptides and observed without any fixation (Fig. 6). Most of the peptide was found in the nucleus, with a nucleolar concentration in living cells as well.

Mechanism of Cell Internalization—The kinetics of internalization were studied by varying the time of incubation of the cells with the peptides at a final concentration of 1 μM. Cells were incubated from 15 s up to 1 h before three quick washes with PBS to remove free peptide and immediate cell fixation. Peptides were detected in the cell after as little as 1 min of incubation (data not shown). The internalization of cell-bound peptides during the washing steps and the early time of the fixation procedure cannot be excluded, however.

To further study the mechanism through which these peptides could be internalized, experiments were performed at 4 °C until the end of the cell fixation procedure. Cells were preincubated for 30 min at 4 °C before being incubated with the peptide solution. Low temperature incubation did not alter cellular uptake and nuclear accumulation (Fig. 7), which precludes an endocytic mechanism. These data are in agreement with the internalization process recently proposed for the Antennapedia homeodomain peptide (1).

Although the translocating activity of the Tat peptides was not inhibited at 4 °C, uptake via potocytosis has been reported to be temperature insensitive (13). This phenomenon involves caveolae or non-coated plasmalemmal vesicles, which are specialized invaginations of the plasma membrane. This pathway

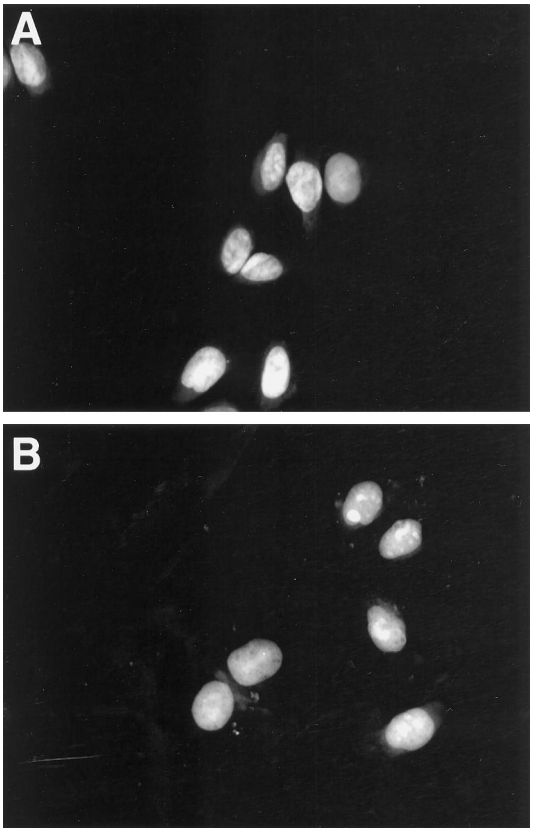

FIG. 7. **Effect of temperature on Tat-(48–60) peptide internalization.** HeLa cells were incubated with 5 μM Tat-(48–60) peptide for 15 min at 37 °C (*panel A*) or at 4 °C (*panel B*). Peptide detection was performed by indirect immunofluorescence.

can be greatly inhibited by okadaic acid at the concentration of 1 μM (14) through the reversible inhibition of PP1 and PP2A without directly affecting other known phosphatases or kinases (15). To further investigate whether this pathway could be involved, cells were treated by okadaic acid before incubation with the peptides. Preincubation with okadaic acid for 1 h led to the detachment of a large majority of the HeLa cells from the culture chamber slide. Fluorescence microscopy on the residual cells indicated that the fluorescein-labeled Tat-(48–60) peptide was taken up normally in okadaic acid-treated cells (data not shown). A more quantitative evaluation of a possible effect of okadaic acid was performed by FACS analysis. As shown in Fig. 8*A*, no significant differences between treated and untreated cells was observed.

Since it has been suggested that NEM-sensitive factors are involved in caveolae-mediated endocytosis (16), similar experiments were performed on NEM-treated cells. FACS analysis did not reveal any significant differences between treated and untreated cells (Fig. 8*B*).

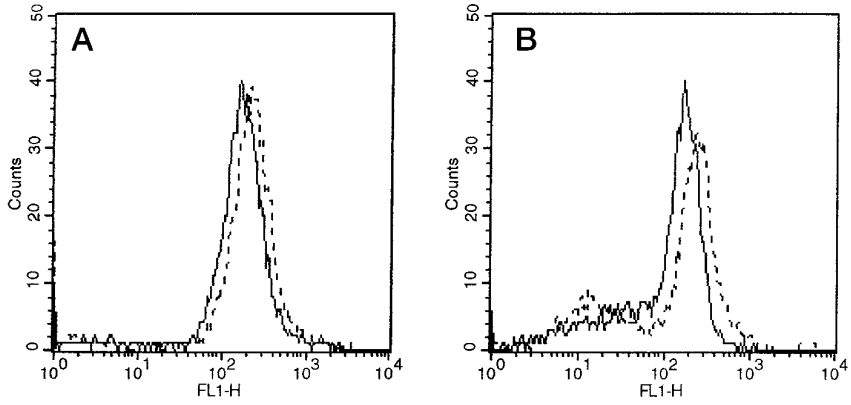

FIG. 8. **Effect of okadaic acid and NEM treatment on peptide uptake.** FACScan analysis of HeLa cells treated (——) for 1 h with okadaic acid (*panel A*) or 5 min with NEM (*panel B*) at 37 °C, before incubation with fluorescein-labeled Tat-(48–60) peptide for 15 min. Corresponding untreated cells are shown for each panel (- - -).

To investigate a possible disruption of the plasma membrane associated with peptide translocation or even responsible for the translocation process, the Tat-(37–60) basic peptide was co-incubated with a 50-fold excess of a peptide corresponding to the N-terminal region of the Tat protein, *e.g.* a Glu-Pro-Val-Asp-Pro-Arg-Leu-Glu-Pro-Trp-Lys-His-Pro-Gly-Ser-Gln-Pro-Lys-Thr-Ala-Cys-Thr sequence extending from residues 2 to 23 (8). The internalization of the Tat-(2–23) peptide was monitored with a monoclonal antibody directed against the N-terminal region of this Tat-(2–23) peptide (kindly provided by Dr Bahraoui, UPS Toulouse). Tat basic peptides do not promote a detectable internalization of this unrelated non-conjugated peptide unless cells were deliberately permeabilized with saponin (data not shown). Cells were also incubated with various doses of the Tat-(48–60) peptide in the presence of a large excess of tetramethylrhodamine succinimidyl ester (TAMRA-SE). None of the rhodamine dye was detected in the cells (Fig. 9*A*) unless the cells were deliberately permeabilized with saponin (20 μg/ml) (Fig. 9*B*). Likewise, no fluorescent dye was incorporated in cells co-incubated with rhodamine and peptide concentrations up to 100 μM under the standard conditions (data not shown). The TAMRA-SE fluorochrome could interfere with peptide translocation. To rule this out, cells were incubated with fluorescein-labeled peptides and an excess of TAMRA-SE. No rhodamine was taken up by cells (Fig. 9*D*) while the fluorescein-labeled peptide was internalized normally (Fig. 9*C*). The peptide translocation process does not appear to involve dramatic changes in membrane conformation leading to the internalization of rhodamine molecules. Similar results were obtained when coincubating an unlabeled peptide with a large excess of fluorescein maleimide. Evidence for the fusogenic or membrane destabilizing properties of various amphipathic peptides has been obtained upon incubation with human erythrocytes (3) or calcein-filled lipid vesicles (4). An erythrocyte leakage assay was performed with Tat-derived peptides at concentrations up to 50 μM at neutral (pH 7.2) or acidic pH (pH 5.5). No hemoglobin release was observed for all synthesized peptides at both pH (data not shown). Again these data do not support a membrane destabilization or permeabilization mechanism as an explanation for the uptake of the Tat-derived basic peptide.

Peptide Cytotoxicity—The cytotoxicity of all peptides was investigated after incubation of HeLa cells with peptide concentrations up to 100 μM for 1 h and 24 h. Interestingly, the peptides had different effects on cell viability when incubated for 24 h (Fig. 10). Peptides with the full α-helix moiety (*i.e.* Tat-(37–60) and Tat-(37–53)) decreased cell viability at high concentrations, while peptides Tat-(43–60) and Tat-(48–60) did not induce any significant toxicity (10–15% of cell death) even at 100 μM (Fig. 10). This peptide concentration largely exceeds the dose expected to be used in a vectorization process.

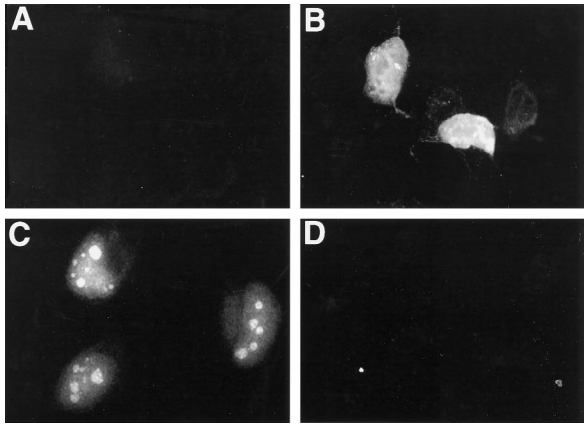

FIG. 9. **Coincubation of Tat peptide with rhodamine fluorochrome.** Cells were incubated with a large excess of TAMRA-SE. No rhodamine dye was detected in the cells (*A*) unless the cells were deliberately permeabilized with saponin (20 μg/ml) (*B*). Cells were co-incubated with 100 μM Tat-(48–60) peptide (*D*) in the presence of the same amount of fluorescent dye as shown in *A* and *B*. Control experiment for the translocation activity of the Tat peptide in presence of TAMRA-SE was performed using 1 μM fluorescein-labeled peptide co-incubated with TAMRA-SE (*C*).

No toxicity was observed for 1-h incubation times, even at 100 μM, with these two latter peptides (data not shown).

DISCUSSION

Several strategies have been proposed to improve the cellular uptake of proteins or nucleic acids. Some of these are based on the use of peptide sequences from proteins known to translocate through the plasma membrane. Along these lines, the HIV-1 Tat protein is able to cross the plasma membrane and to reach the cell nucleus to transactivate the viral genome (6–8). Moreover, a 35-amino acid peptide from Tat is able to promote the intracellular delivery of covalently bound proteins such as β-galactosidase, RNase A, or horseradish peroxidase in several cell lines and tissues (9). This peptide contains a cluster of basic amino acids extending from residues 49 to 58 and a sequence assumed to adopt an α-helical configuration (11).

The present study aimed at delineating whether shorter domains from this Tat peptide would be sufficient for cell internalization. The main determinant required for translocation was identified as the cluster of basic amino acids while the putative α-helix domain appeared dispensable. The full basic domain is required since a peptide deleted from the three arginine residues at its C-terminal end is not taken up by cells, even at high concentration. In keeping with a requirement for the full complement of positive charges in the Tat basic domain, any deletion or substitution of basic charges within the

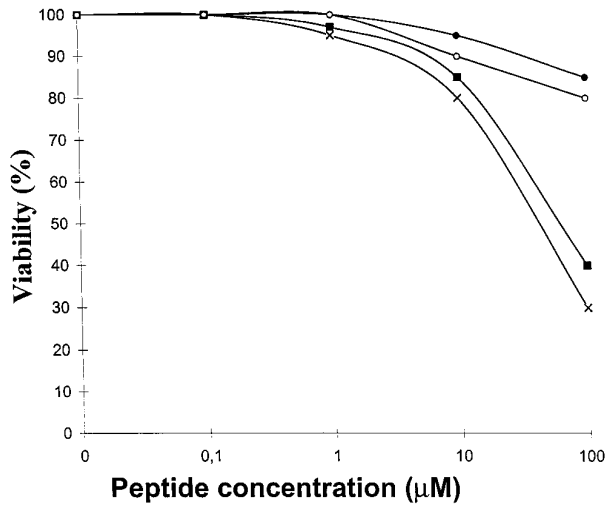

FIG. 10. **Toxicity of the Tat peptides.** HeLa cells were incubated for 24 h with increasing concentrations of the different Tat peptides. Cell viability was measured following a standard MTT assay procedure and was expressed as the ratio of A_{570} of cells treated with peptide over control sample. ○, Tat-(48–60); ●, Tat-(43–60); ×, Tat-(37–53); ■, Tat-(37–60).

Tat-(48–60) peptide led to a reduced membrane translocating activity.[2]

Shorter peptides such as Tat-(37–58) or Tat-(47–58) were less efficient carriers of proteins than the original 35-amino acid peptide (9). A steric hindrance between such short peptides and the bound protein could have reduced their availability for translocation.

Along the same lines, a 16-amino acid peptide from the Antennapedia third helix homeodomain was described as having a good translocation ability through the plasma membrane, and it was initially assumed that its α-helix structure was important (1). Likewise, the fusogenic properties of several viral peptides have been ascribed to α-helical determinants (17). However, it was recently established that the insertion of proline residues, known to disrupt α-helical structures, did not abolish the translocation of the Antennapedia peptide (18). The sequence of this active Antennapedia peptide analogue is Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys. It contains five positive charges (two Arg and three Lys) within a linear sequence of seven residues at its C-terminal end. Similarly, the short Tat basic peptide described here contains eight positive charges within a sequence of nine residues. It is noteworthy that a shorter peptide from Antennapedia deleted of the C-terminal Trp-Lys-Lys residues (*i.e.* lacking two positive charges) was not internalized (1). Although the tryptophan residue was shown to play a role in the translocation event (1), an additional effect of the two Lys residues was not evaluated independently. The data reported with the Antennapedia peptide and those obtained in the present study with the Tat peptide strongly suggest that internalization could be linked to the presence of a high density of positive charges within a short sequence.

Both the full-length Antennapedia (1) and the Tat-(48–60) peptides (Fig. 1) are taken up efficiently at 4 °C. Likewise, several drugs known to interfere with caveolae-mediated uptake did not affect Tat uptake in our studies. Altogether, these studies strongly suggest that endocytosis is not involved in the uptake of these short basic peptides. On the other hand, the incubation of the Tat peptides with various unbound fluorochromes or with a non-permeant peptide did not induce their uptake. Taken together, these experiments do not support the involvement of a significant membrane disruption by Tat basic peptides or a well defined internalization pathway.

The mechanism of translocation of the Tat basic peptide could be analogous to the model proposed for the Antennapedia homeodomain peptide (18). A tight ionic interaction between the basic groups of the peptide side chains and the negative charges of the phospholipid heads could induce a local invagination of the plasma membrane. The local reorganization of the phospholipid bilayer would then lead to the formation of inverted micelles with the peptide enclosed in the hydrophilic cavity and ultimately to the cytoplasmic release of the peptide. Because of the presence of a nuclear localization signal, the Tat peptide is rapidly translocated and concentrates in the nucleus. This would limit its release from the cell by the same mechanism. Further experiments are in progress to assess the reality of this working hypothesis. Additional studies will be required to define more accurately the structural requirements for this translocation activity and to uncover the mechanism by which Tat and possibly other basic peptides cross the plasma membrane.

The translocation activity of such small Tat-derived peptides is powerful, as nuclear localization was observed after a few minutes of incubation with the cells. Internalization could be monitored in the micromolar concentration range by indirect immunofluorescence with peptide-specific antibodies or even at an order of magnitude lower by direct labeling of the peptide with a fluorochrome. Previous studies with the Antennapedia peptide made use of incubation times of several hours and routine concentrations of 20 μM (1). These differences were confirmed in a comparative study using the same fluorescein-labeling method for both peptides with Antennapedia peptide kindly provided by G. Chassaing and A. Prochiantz (CNRS URA1414, Ecole Normale Supérieure, Paris, France) (data not shown).

The indirect immunodetection of the Tat peptide ensures that its ability to translocate through the plasma membrane was not altered by the reporter group itself. In most published studies, the possible influence of the fluorochrome reporter group or of the biotin-linking arm on the behavior of the peptide was not assessed. Along these lines, the biotinylation of a Tat peptide increased by 6-fold its uptake as compared with the non-biotinylated peptide (19).

The internalization properties of these small Tat basic peptides could then be exploited for the intracellular delivery and for the nuclear targeting of conjugated non-permeant molecules. Ongoing work in our group aims at establishing whether the covalent conjugation through various linking arms of short Tat basic peptides to antisense oligonucleotides, to peptide nucleic acids, or to peptides will lead to their nuclear accumulation. Preliminary data indicate that a peptide which did not enter the cell by itself could be efficiently internalized when conjugated to the shorter Tat peptide.[2] Along the same lines the covalent linking of a 15-mer oligonucleotides to the 16-amino acid Antennapedia peptide led to improved intracellular delivery and to a significant increase in biological activity (20).

Peptides bearing a high density of basic residues might also improve hybridization properties of antisense oligonucleotides. Previous studies have indeed described the enhanced affinities and kinetics of hybridization for its target sequence of an oligonucleotide covalently linked to a polyarginine sequence (21).

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[2] E. Vivès, C. Granier, P. Prévot, and B. Lebleu (1997) *Lett. Pept. Sci.*, in press.

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