JOURNAL OF VIROLOGY, Mar. 1997, p. 2495–2499 Vol. 71, No. 3
0022-538X/97/$04.00+0
Copyright © 1997, American Society for Microbiology

Molecular Determinants for Cellular Uptake of Tat Protein of Human Immunodeficiency Virus Type 1 in Brain Cells

MEIHUI MA[1] AND AVINDRA NATH[1,2]*

Department of Medical Microbiology[1] and Section of Neurology, Department of Internal Medicine,[2] University of Manitoba, Winnipeg, Manitoba, Canada R3E 0W3

Received 17 July 1996/Accepted 26 November 1996

We measured the cellular uptake of ^{125}I-labeled full-length Tat (amino acids 1 to 86) (^{125}I-Tat$_{1–86}$) and ^{125}I-Tat$_{1–72}$ (first exon) in human fetal astrocytes, neuroblastoma cells, and human fetal neurons and demonstrated that the uptake of ^{125}I-Tat$_{1–72}$ without the second exon was much lower than that of ^{125}I-Tat$_{1–86}$ ($P < 0.01$). This suggests an important role for the C-terminal region of Tat for its cellular uptake. ^{125}I-Tat uptake could be inhibited by dextran sulfate and competitively inhibited by unlabeled Tat but not by overlapping 15-mer peptides, suggesting that Tat internalization is charge and conformationally dependent. Interestingly, one of 15-mer peptides, Tat$_{28–42}$, greatly enhanced ^{125}I-Tat uptake. These findings are important for understanding the neuropathogenesis of human immunodeficiency virus type 1 infection and in the potential application of Tat for drug delivery to cells.

The Tat protein of human immunodeficiency virus type 1 (HIV-1) is a regulatory protein which transactivates HIV-1 expression (7). However, Tat can be released from productively infected cells (8, 9). Extracellular Tat is internalized by cells and localized in the nucleus (14, 16), where it can affect a variety of cellular functions (3, 10, 13, 17, 18, 27, 31, 33). For example, Tat can also act on neuronal cell membranes to produce neuronal excitation, elevation of intracellular calcium, and toxicity (20, 24, 28). It also causes aggregation of neurons (25). In astrocytes, extracellular Tat increases both NF-κB binding and protein kinase C activity (6). Tat also transactivates JC virus T antigen expression in astrocytes (5). However, it remains to be determined if these actions are due to effects of Tat on the cell membrane or following internalization of Tat.

The ability of exogenous Tat to be taken up by cells has generated considerable interest due to the potential biotechnological applications whereby Tat can be used as a vehicle for delivering heterogeneous proteins and drugs that would otherwise not have access to the intracellular environment (11, 23). In fact, substances conjugated to Tat have been shown to be localized in the nucleus (11). Hence, it is important to determine the regions of Tat that are responsible for its uptake versus those that are responsible for its functional activities following internalization.

Tat is formed from two exons. The first exon encodes amino acids 1 to 72 (Tat$_{1–72}$), and the second is of variable length, encoding another 14 to 32 amino acids. Tat$_{1–72}$ is sufficient for transactivation, which is regulated by the basic region of Tat between amino acid residues 49 and 57 (1, 15, 16, 30). The biological function of the C-terminal region encoded by the second exon is still unclear. However, the C-terminal region has an integrin receptor binding sequence (Arg-Gly-Asp) (3, 32). In this study, we define the regions of Tat that regulate its uptake into brain cells.

Uptake of Tat$_{1–86}$ and Tat$_{1–72}$. Human fetal brain tissue (gestational age, 13 to 16 weeks) was obtained with written consent from women undergoing elective termination of pregnancy and with approval of the University of Manitoba Human Ethics Committee. Human fetal astrocyte and neuron cultures were prepared as described previously (19, 20). Purity of astrocytes (>95%) and neurons (>70%) was determined by immunostaining for glial fibrillary acidic protein and microtubule-associated protein 2, respectively. Prior to the binding assay, the cells were plated into 24-well plates and grown to 100% confluence (5×10^5 cells per well).

The Tat gene encoding the first 72 amino acids (first exon) was inserted into an *Escherichia coli* vector, PinPoint Xa-2 (Promega), expressed as a fusion protein. Tat$_{1–72}$ was enzymatically cleaved from the fusion protein and purified as described previously (6). The Tat protein was >95% pure as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining. The purified product was further confirmed by Western blot analysis using polyclonal antisera to Tat (AIDS repository, National Institutes of Health). Recombinant HIV-1$_{BRU}$ Tat$_{1–86}$ was obtained from Repligen and was >98% pure. Purified Tat$_{1–86}$ or Tat$_{1–72}$ was labeled with Na^{125}I by using Iodo-beads (Pierce). The labeled protein was separated from free ^{125}I with a PD-10 column (Pharmacia). The specific activity of ^{125}I-Tat was 2.4×10^4 cpm/ng.

Cellular uptake assays of ^{125}I-Tat were performed as previously described (14, 20). Briefly, cells were incubated with various concentrations of ^{125}I-Tat$_{1–86}$ or ^{125}I-Tat$_{1–72}$ (2 to 64 nM) in 0.2 ml of binding medium (Dulbecco's modified Eagle medium [DMEM] and 0.1% bovine serum albumin) at room temperature for 30 min to 4 h. The cells were washed in ice-cold DMEM and treated with 0.2 ml of trypsin-EDTA (GIBCO BRL) for 10 min at 37°C. The cells were centrifuged, and supernatant was removed as a membrane fraction. The cell pellet was washed twice in DMEM with 10% fetal bovine serum. The nuclear and cytoplasmic fractions were isolated by lysis in 0.2 ml of 0.5% Nonidet P-40 (vol/vol) as described previously (2). The fractions were counted in a gamma counter. Uptake of both ^{125}I-Tat$_{1–86}$ and ^{125}I-Tat$_{1–72}$ was time and dose dependent (Fig. 1). However, the uptake of ^{125}I-Tat$_{1–72}$ was much lower than that of ^{125}I-Tat$_{1–86}$ (0.08 ± 0.01 nM versus 0.42 ± 0.009 nM at 4 h; $P < 0.01$) (Fig. 1A). At the maximal concentration (64 nM) of ^{125}I-Tat$_{1–86}$ or ^{125}I-Tat$_{1–72}$ tested, the uptake of Tat could not be saturated (Fig. 1B).

* Corresponding author. Mailing address: 523-730 William Ave., Department of Medical Microbiology, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0W3. Phone: (204) 789-3273. Fax: (204) 783-5255. E-mail: Nath@bldghsc.lan1.umanitoba.ca.

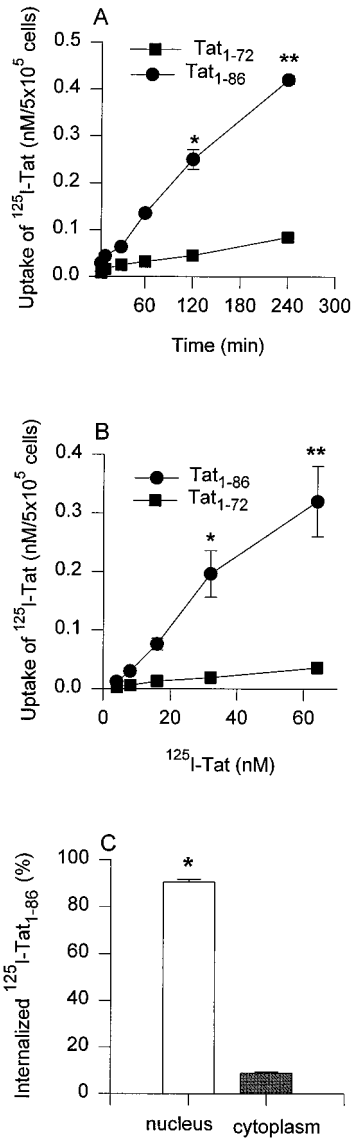

FIG. 1. Uptake of Tat by astrocytes. (A) Astrocytes were incubated with 10 nM ^{125}I-Tat$_{1–86}$ or ^{125}I-Tat$_{1–72}$ in 0.2 ml of binding medium at room temperature for 30 min to 4 h. The cells were washed in ice-cold DMEM and treated with 0.2 ml of trypsin-EDTA for 10 min at 37°C. The cells were centrifuged, and supernatant was removed as a membrane fraction. The cell pellet was washed twice in DMEM with 10% fetal bovine serum. The nuclear and cytoplasmic fractions were isolated by lysis in 0.2 ml of 0.5% Nonidet P-40 (vol/vol). The fractions were counted in a gamma counter. Both ^{125}I-Tat$_{1–86}$ and ^{125}I-Tat$_{1–72}$ were internalized by astrocytes. Uptake of Tat$_{1–86}$ was much more rapid and occurred in larger amounts (*, $P < 0.05$; **, $P < 0.01$). (B) Astrocytes were incubated with various concentrations of ^{125}I-Tat$_{1–86}$ or ^{125}I-Tat$_{1–72}$ (2 to 64 nM) in 0.2 ml of binding medium at room temperature for 2 h and harvested as described above. ^{125}I-Tat$_{1–86}$ and ^{125}I-Tat$_{1–72}$ were internalized dose dependently in astrocytes. Uptake of Tat$_{1-86}$ was more efficient (*, $P < 0.05$; **, $P < 0.01$). (C) Internalized ^{125}Tat$_{1-86}$ was predominantly present in the nuclear fraction of astrocytes (*, $P < 0.005$). Values in all panels represent the means ± standard errors (error bars) of two experiments, each done in triplicate.

Further, >90% of internalized ^{125}I-Tat$_{1–86}$ was localized in the nucleus while the cytoplasmic fraction represented only <10% in the astrocytes (Fig. 1C). To further confirm the nuclear localization and the fate of Tat within the nucleus, nuclear fractions were prepared from ^{125}I-Tat$_{1–86}$- or ^{125}I-Tat$_{1–72}$-treated astrocytes and analyzed by SDS-PAGE followed by autoradiography. By 2 h, prominent bands for both Tat$_{1–86}$ and

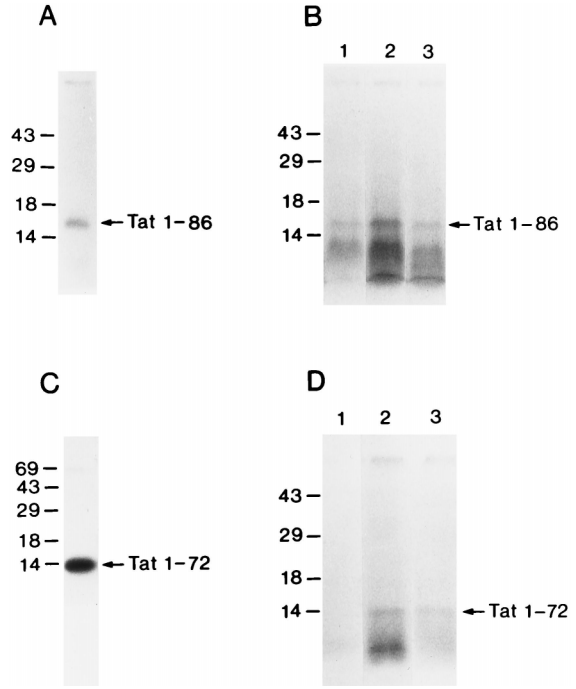

FIG. 2. Analysis of nuclear fractions. Nuclear fractions were prepared from ^{125}I-Tat$_{1–86}$- or ^{125}I-Tat$_{1–72}$-treated astrocytes and analyzed by SDS-PAGE and autoradiography. The numbers to the left side of each gel indicate the positions of molecular mass markers (in kilodaltons). (A) Purified ^{125}I-Tat$_{1–86}$ alone shows a single band at 15 kDa. (B) Lanes 1, 2, and 3 represent internalized ^{125}I-Tat$_{1–86}$ in nuclear fractions of astrocytes at 0.5, 2, and 4 h, respectively, which show increasing amounts of Tat$_{1–86}$ degradation products. (C) Purified ^{125}I-Tat$_{1–72}$ alone shows a single band at 14 kDa. (D) Lanes 1, 2, and 3 represent internalized ^{125}I-Tat$_{1–72}$ in nuclear fractions of astrocytes at 0.5, 2, and 4 h, respectively, which show the presence of degradation products.

Tat$_{1–72}$ were detected (Fig. 2B and D). Bands with lower molecular weights were also noted (Fig. 2B and D), and these represent breakdown products since the purified ^{125}I-Tat$_{1–86}$ or ^{125}I-Tat$_{1–72}$ prior to treatment with astrocytes showed a single band in Fig. 2A and C. Similar results were observed in neurons, NB41 cells, and SupT-1 cells (data not shown). All subsequent experiments were done with astrocytes only.

To further determine the specificity of Tat uptake, immunoabsorption of Tat was performed as described previously (20). Briefly, a 1:100 dilution of rabbit anti-Tat serum or normal rabbit serum was bound to protein A-Sepharose (Pharmacia) and incubated with ^{125}I-Tat for 1 h at room temperature followed by centrifugation. The supernatants were collected and used for Tat uptake assays. The uptake of ^{125}I-Tat$_{1–86}$ or ^{125}I-Tat$_{1–72}$ treated with normal rabbit sera was normalized to 100%. As shown in Fig. 3, cellular uptake of ^{125}I-Tat$_{1–86}$ and ^{125}I-Tat$_{1–72}$ treated with anti-Tat sera was decreased by 79% ± 11% and 78% ± 3%, respectively, demonstrating that the uptake of Tat was specific.

Competition of Tat$_{1–86}$ and Tat$_{1–72}$ uptake by unlabeled Tat and dextran sulfate. Astrocytes were treated with 10 nM ^{125}I-Tat$_{1–72}$ in the presence of increasing concentrations of unlabeled Tat$_{1–72}$, ranging from 200 to 1,000 nM, for 2 h at room temperature. Binding of ^{125}I-Tat$_{1–72}$ was inhibited dose dependently with maximal ^{125}I-Tat$_{1–72}$ inhibition (60%) achieved with 800 nM unlabeled Tat$_{1–72}$ (Fig. 4A). To determine if Tat uptake was charge dependent, human fetal astrocytes were treated with ^{125}I-Tat$_{1–86}$ and ^{125}I-Tat$_{1–72}$ in the presence of increasing concentrations of dextran sulfate (0.25 to 3.2 μM) (Sigma). Dextran sulfate inhibited ^{125}I-Tat$_{1–86}$ or ^{125}I-Tat$_{1–72}$

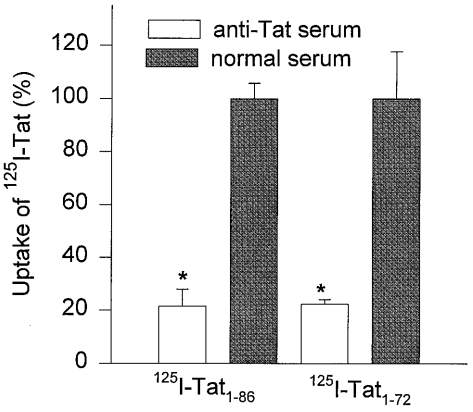

FIG. 3. Specificity of ^{125}I-Tat$_{1–86}$ and ^{125}I-Tat$_{1–72}$ uptake by astrocytes. Rabbit anti-Tat serum or normal rabbit serum was bound to protein A-Sepharose and incubated with ^{125}I-Tat for 1 h at room temperature followed by centrifugation. The supernatants were collected and used for the Tat uptake assay. The uptake of ^{125}I-Tat$_{1–86}$ or ^{125}I-Tat$_{1–72}$ treated with normal rabbit serum was considered to be 100%. Uptake of both of ^{125}I-Tat$_{1–86}$ and ^{125}I-Tat$_{1–72}$ is abolished following preabsorption of Tat with polyclonal Tat antisera conjugated with protein A-Sepharose (*, $P < 0.005$). Each value represents the mean ± standard error (error bar) of two experiments, each done in triplicate.

uptake dose dependently (Fig. 4B). Inhibition of ^{125}I-Tat$_{1–86}$ and ^{125}I-Tat$_{1–72}$ uptake (80 and 75%, respectively) was noted in the presence of 0.4 and 1.5 μM dextran sulfate, respectively (Fig. 4B).

Role of integrin binding on uptake of Tat$_{1–86}$. To determine if the basic region of Tat (Tat$_{49–57}$) or the RGD sequence in the C-terminal region of Tat previously shown to bind to integrins $\alpha_v\beta_5$ and $\alpha_5\beta_1$ or $\alpha_v\beta_3$ (3, 33) mediates cellular uptake of Tat, we treated astrocytes with 10 nM ^{125}I-Tat$_{1–86}$ in the presence of a 600-fold concentration of either Tat$_{31–72}$ containing the basic region or Tat$_{48–86}$ containing the basic region and the RGD sequence (AIDS Reagent Program of the Medical Research Council of the United Kingdom) (Table 1). Both peptides failed to inhibit ^{125}I-Tat$_{1–86}$ internalization in astrocytes (Fig. 4C). Conversely, when astrocytes were treated similarly with ^{125}I-Tat$_{1–72}$ in the presence of Tat$_{31–72}$, a fivefold enhancement of Tat uptake was observed ($P < 0.01$) while Tat$_{48–86}$ had no effect on ^{125}I-Tat$_{1–72}$ uptake by astrocytes (Fig. 4D). The cells were also pretreated with polyclonal antisera to $\alpha_v\beta_3$, $\alpha_v\beta_5$ (kindly provided by J. Wilkins) and $\alpha_5\beta_1$ (Chemicon) at 1:100 and 1:200 dilutions (vol/vol) for 90 min followed by ^{125}I-Tat$_{1–72}$ uptake assay. None of above-mentioned antibodies blocked the uptake of Tat$_{1–72}$ (data not shown).

Effect of 15-mer Tat peptides on Tat$_{1–72}$ uptake. To further determine the region responsible for Tat$_{1–72}$ uptake, we synthesized and purified 15-mer Tat peptides completely spanning the 86-amino-acid sequence of Tat HIV$_{BRU}$ (Table 1) (24). Astrocytes were incubated with ^{125}I-Tat$_{1–72}$ in the presence of various 15-mer Tat peptides (500 M excess) for 2 h at room temperature. None of the peptides blocked Tat$_{1–72}$ uptake. Instead, a 35-fold enhancement of Tat$_{1–72}$ uptake by Tat$_{28–42}$ (KKCCFHCQVCFTTKA) was observed. Tat$_{23–37}$ and Tat$_{18–32}$ also produced a 10-fold and a 2-fold enhancement of ^{125}I-Tat$_{1–72}$ uptake, respectively, while the remaining 15-mer peptides had no significant effect (Fig. 5A). Similarly, Tat$_{72–86}$, which sup-

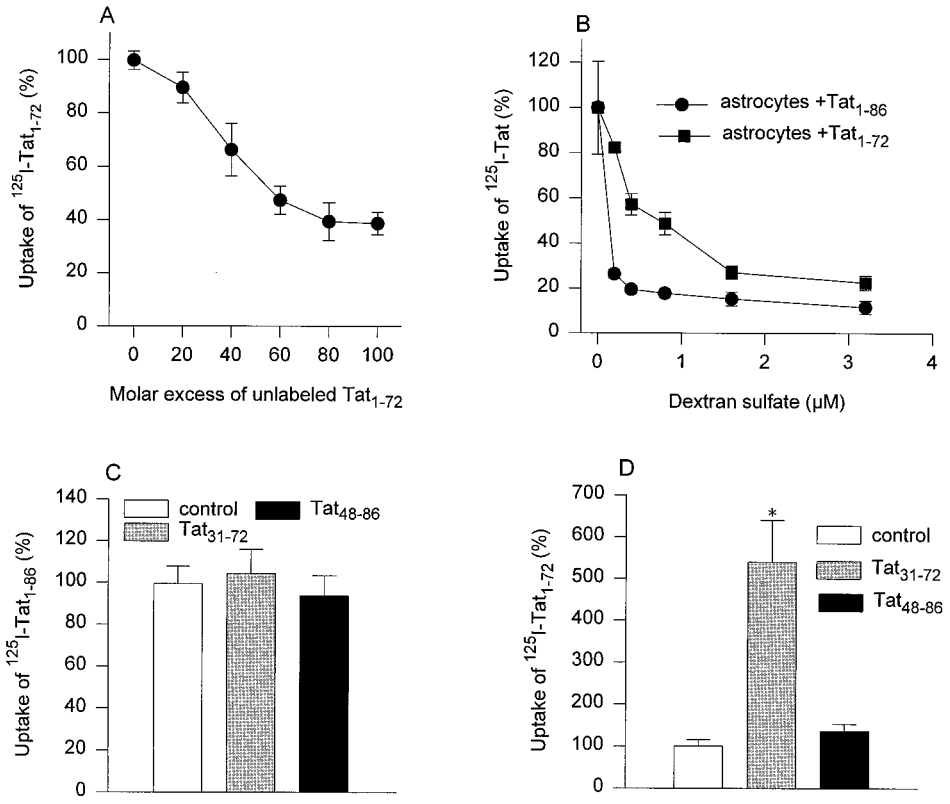

FIG. 4. (A) Competition of ^{125}I-Tat$_{1–72}$ uptake by unlabeled Tat$_{1–72}$ in astrocytes. Increasing concentrations (fold) of unlabeled Tat$_{1–72}$ inhibited ^{125}I-Tat$_{1–72}$ (10 nM) uptake dose dependently. (B) Effect of dextran sulfate on uptake of ^{125}I-Tat$_{1–86}$ and ^{125}I-Tat$_{1–72}$. Increasing concentrations of dextran sulfate (0.2 to 3.2 μM) blocked ^{125}I-Tat$_{1–86}$ (10 nM) or ^{125}I-Tat$_{1–72}$ (10 nM) uptake dose dependently. (C) Internalization of ^{125}I-Tat$_{1–86}$ (10 nM) by astrocytes was not affected by Tat$_{31–72}$ (6 μM) or Tat$_{48–86}$ (6 μM). (D) Uptake of ^{125}I-Tat$_{1–72}$ (10 nM) in astrocytes was enhanced fivefold (*, $P < 0.01$) by Tat$_{31–72}$ (6 μM), while Tat$_{49–86}$ had no effect. Values in all panels represent the means ± standard errors (error bars) of two experiments, each done in triplicate.

TABLE 1. HIV-1 Tat peptides derived from Tat_{BRU} used in this study

Tat peptide	Amino acid sequence
$Tat_{31–71}$	FHCQVCFTTKALGISYGRKKRRQRRRPPQESQTHQVSLSKQ
$Tat_{31–61}$	FHCQVCFTTKALGISYGRKKRRQRRRPPQES
$Tat_{48–85}$	RKKRRQRRRPPQESQTHQVSLSKQPTSQSRGDPTEPKK
$Tat_{1–15}$	MEPVDPRLEPWKHPG
$Tat_{3–17}$	PVDPRLEPWKHPGSQ
$Tat_{8–22}$	LEPWKHPGSQPKTAC
$Tat_{13–27}$	HPGSQPKTACTNCYC
$Tat_{18–32}$	PKTACTNCYCKKCCF
$Tat_{23–37}$	TNCYCKKCCFHCQVC
$Tat_{28–42}$	KKCCFHCQVCFTTKA
$Tat_{33–47}$	HCQVCFTTKALGISY
$Tat_{38–52}$	FTTKALGISYGRKKR
$Tat_{43–57}$	LGISYGRKKRRQRRR
$Tat_{48–62}$	GRKKRRQRRRPPQGS
$Tat_{53–67}$	RQRRRPPQGSQTHQV
$Tat_{58–72}$	PPQGSQTHQVSLSKQ
$Tat_{63–77}$	QTHQVSLSKQPTSQP
$Tat_{68–82}$	SLSKQPTSQPRGDPT
$Tat_{72–86}$	QPTSQPRGDPTGPKE

plements the deleted region of the second exon, had no effect on ^{125}I-$Tat_{1–72}$ uptake (Fig. 5A).

To determine if internalized $Tat_{1–72}$ retains its functional properties, transactivation of the HIV long terminal repeat (LTR) was determined in HL3T1 cells containing the LTR-chloramphenicol acetyltransferase (CAT) (AIDS Repository, National Institutes of Health) treated with 0.5 μM $Tat_{1–72}$ without scrape-loading in the presence or absence of 6 μM $Tat_{1–15}$, $Tat_{28–42}$, or $Tat_{72–86}$. The Tat transactivation assay was performed as previously described (12, 14), and CAT activity was measured by a simple phase extraction assay (29). As shown in Fig. 5B, $Tat_{28–42}$ produced a ninefold increase in $Tat_{1–72}$-induced LTR transactivation while $Tat_{1–15}$ or $Tat_{72–86}$ had no effect. $Tat_{28–42}$ alone did not show any transactivation activity.

Not only is understanding the precise mechanisms underlying the internalization of extracellular Tat important in determining its role in regulating viral and host function, but this phenomenon could also potentially be exploited to deliver heterologous proteins or drugs into cells. We demonstrate here that both full-length $Tat_{1–86}$ and to a lesser degree $Tat_{1–72}$ are internalized by brain cells and that following entry, they are predominantly localized in the nucleus. Consistent with previous observations that $Tat_{1–86}$ uptake in lymphoid and monocytoid cells could be inhibited by polyanions such as heparin or dextran sulfate (21), we found that $Tat_{1–72}$ uptake in astrocytes could also be blocked by dextran sulfate, suggesting that internalization of Tat is charge dependent. We demonstrate that uptake of ^{125}I-$Tat_{1–86}$ and ^{125}I-$Tat_{1–72}$ by human fetal astrocytes was competitively inhibited dose dependently by an excess of unlabeled $Tat_{1–72}$, suggesting that uptake of ^{125}I-$Tat_{1–72}$ is receptor mediated.

Transactivation of HIV-2 Tat requires full-length protein (26), while the HIV-1 $Tat_{1–72}$ has the same efficiency for transactivation as HIV-1 $Tat_{1–86}$ (16). The role of the C-terminal region formed by the second exon of the *tat* gene in HIV-1 infection was not previously determined. We observed that even though both $Tat_{1–86}$ and $Tat_{1–72}$ could be internalized by several cell types, $Tat_{1–86}$ internalization was up to 10 times more efficient than that by $Tat_{1–72}$, suggesting that the region encoded by the second exon is important in mediating Tat internalization. However, $Tat_{1–72}$ uptake was not affected by

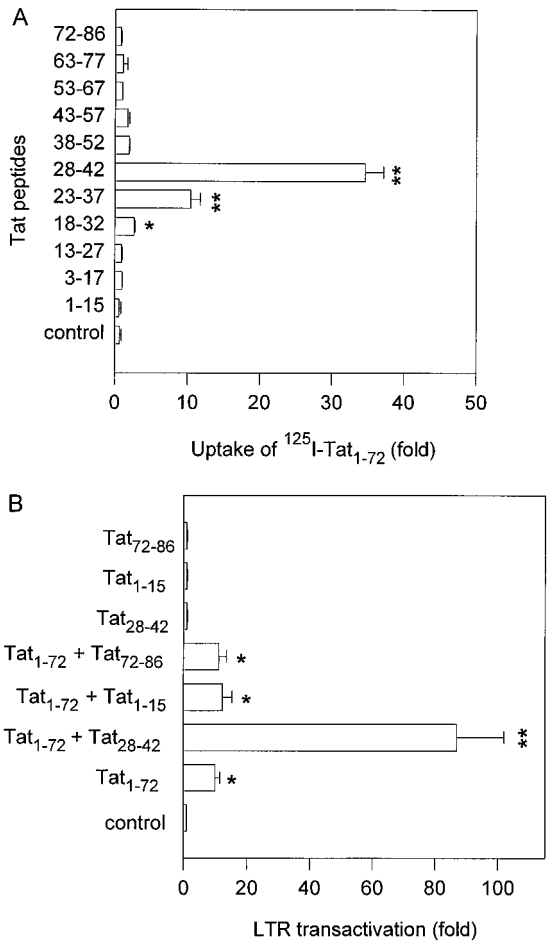

FIG. 5. (A) Effect of 15-mer Tat peptides on $Tat_{1–72}$ uptake. Astrocytes were treated with ^{125}I-$Tat_{1–72}$ (10 nM) in the presence of various 15-mer peptides (6 μM each). ^{125}I-$Tat_{1–72}$ (10 nM) uptake was enhanced 35-fold (**, $P < 0.005$), 10-fold (**, $P < 0.005$), or 2-fold (*, $P < 0.01$) in the presence of $Tat_{28–42}$, $Tat_{23–37}$, or $Tat_{18–32}$ respectively, while the rest of the 15-mer peptides had no effect. (B) Effect of 15-mer Tat peptides on transactivation of HIV-1 LTR in HL3T1 cells by $Tat_{1–72}$. HL3T1 cells were treated with 0.1 to 0.5 μM $Tat_{1–72}$ and 100 μM chloroquine without scrape-loading in the presence or absence of 6 μM $Tat_{1–15}$, $Tat_{28–42}$, or $Tat_{72–86}$. The Tat transactivation assay was performed, and CAT activity was measured by a simple phase extraction assay. $Tat_{1–72}$ transactivated LTR-CAT significantly compared to the control (*, $P < 0.01$). $Tat_{1–72}$-induced transactivation was enhanced ninefold (**, $P < 0.005$) by $Tat_{28–42}$. Neither $Tat_{1–15}$ nor $Tat_{72–86}$ had any effect on $Tat_{1–72}$-induced transactivation. $Tat_{28–42}$ alone did not have any transactivation activity. Values in both panels represent the means ± standard errors (error bars) of two experiments, each done in triplicate.

coincubating with peptides including the second exon ($Tat_{72–86}$ or $Tat_{48–86}$). Thus, peptide bond linkage of the peptides encoded by the two exons is essential for the efficient uptake of Tat. This linkage most likely influences the tertiary configuration of the molecule. The importance of the tertiary configuration for Tat uptake is further supported by the results of Bonifaci et al., which demonstrate that the Tat molecule unfolds before entering the cells (4). However, the peptide encoded by the second exon is not exclusively involved in Tat uptake since significant uptake of $Tat_{1–72}$ was also observed.

$Tat_{1–72}$ is highly conserved between different strains of HIV-1. However, the second exon shows heterogeneity in the amino acid sequence and is of variable length. Since this region is a major determinant of Tat uptake into the cell, it could play an important role in regulating strain-to-strain variability of

the intra- versus extracellular action of Tat on the host cell and hence effect virulence. We have previously shown that extracellular Tat causes neurotoxicity by acting on cell surface excitatory amino acid receptors (20) and that the neurotoxic epitope of Tat resides in the first exon (24). It is thus likely that Tat molecules with low cellular uptake as determined by their second exon might be more neurotoxic since higher levels will be achieved extracellularly.

Integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ function as receptors for Tat and mediate Tat effects on Kaposi's sarcoma cells or cytokine-activated endothelial cells (11) by binding to the RGD sequence of the Tat protein. Further, 12-mer peptides containing the basic region of Tat have been shown to bind to another integrin molecule, $\alpha_v\beta_5$ (32). However, it is unlikely that binding of Tat to these integrin receptors influences Tat uptake since $Tat_{49–86}$ containing the RGD sequence, $Tat_{31–62}$ containing the basic domain, or antisera to integrins $\alpha_5\beta_1$, $\alpha_v\beta_3$, or $\alpha_v\beta_5$ could not inhibit Tat uptake.

Our results show that $Tat_{28–42}$ also greatly increased $Tat_{1–72}$ uptake and transactivation. This peptide contains only four basic amino acids and increased Tat uptake by 35-fold, while $Tat_{38–52}$, which contains five basic amino acids had no significant effect on Tat uptake, indicating that a mechanism of enhancement other than positive charge dependence might also be involved.

The phenomenon by which one protein facilitates the internalization of another protein has also been observed in anthrax toxins. The protective antigen of anthrax toxin can form ion-conductive channels in biological membranes and convey the edema factor and lethal factor of the toxin into the cytoplasm by inducing changes in the membrane permeability (22). Presumably, the Tat peptides can use a similar mechanism to facilitate the internalization of Tat protein.

We thus conclude that the cellular uptake of Tat (i) is determined by the tertiary configuration of the molecule, (ii) is dependent upon the C-terminal region and the basic region of Tat, and (iii) is perhaps independent of integrin binding. Further, Tat uptake can perhaps be enhanced by an autologous peptide through yet-unknown mechanisms.

We thank Mark Bernier and Carol Martin for technical assistance and J. D. Geiger, J. Wilkins, and M. McGavin for helpful comments.

This study was supported by a grant from the National Health and Research Development Program (NHRDP). M. Ma is a recipient of a studentship from the NHRDP, and A. Nath is an NHRDP AIDS Scholar.

REFERENCES

1. **Arya, S. K., S. Guo, S. F. Josephs, and F. Wong-Staal.** 1985. Trans-activator gene of human T-lymphotropic virus type III (HTLV-III). Science **229:**69–73.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
3. **Barillari, G., R. Gendelman, R. C. Gallo, and B. Ensoli.** 1993. The Tat protein of human immunodeficiency virus type 1, a growth factor for AIDS Kaposi sarcoma and cytokine-activated vascular cells, induces adhesion of the same cell types by using integrin receptors recognizing the RGD amino acid sequence. Proc. Natl. Acad. Sci. USA **90:**7941–7945.
4. **Bonifaci, N., R. Sitia, and A. Rubartelli.** 1995. Nuclear translocation of an exogenous fusion protein containing HIV Tat requires unfolding. AIDS **9:**995–1000.
5. **Chowdaury, M., J. P. Taylor, H. Tada, J. Rappaport, F. Wong-Staal, S. Amini, and K. Khalili.** 1990. Regulation of the human neurotropic promotor by JCV-T antigen and HIV-1 tat protein. Oncogene **5:**1737–1742.
6. **Conant, K., M. Ma, A. Nath, and E. O. Major.** 1996. Extracellular HIV-1 Tat protein is associated with an increase in both NF-κB binding and protein kinase C activity in primary astrocytes. J. Virol. **70:**1384–1389.
7. **Dingwall, C., I. Ernberg, M. J. Gait, S. M. Green, S. Heaphy, J. Karn, A. D. Lowe, M. Singh, M. A. Skinner, and R. Valerio.** 1989. Human immunodeficiency virus 1 Tat protein binds trans-activation-responsive region (TAR) in vitro. Proc. Natl. Acad. Sci. USA **86:**6925–6929.
8. **Ensoli, B., G. Barillari, and S. Salahuddin.** 1990. Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. Nature **345:**84–86.
9. **Ensoli, B., L. Buonaguro, G. Barillari, V. Fiorelli, R. Gendelman, R. Morgan, P. Wingfield, and R. Gallo.** 1992. Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation. J. Virol. **67:**277–287.
10. **Ensoli, B., R. Gendelman, P. Markham, V. Fiorelli, S. Colombini, M. Raffeld, A. Cafaro, H. Chang, J. N. Brady, and R. C. Gallo.** 1994. Synergy between basic fibroblast growth factor and HIV-1 Tat protein in induction of Kaposi's sarcoma. Nature **371:**674–680.
11. **Fawell, S., J. Seery, Y. Daikh, C. Moore, L. L. Chen, B. Pepinsky, and J. Barsoum.** 1994. Tat-mediated delivery of heterologous proteins into cells. Proc. Natl. Acad. Sci. USA **91:**664–668.
12. **Felber, B. K., and G. Pavlaski.** 1988. A quantitative bioassay for HIV-1 based on transactivation. Science **239:**184–187.
13. **Flores, S. C., J. C. Marecki, K. P. Harper, S. K. Bose, S. K. Nelson, and J. M. McCord.** 1993. Tat protein of human immunodeficiency virus type 1 represses expression of manganese superoxide dismutase in HeLa cells. Proc. Natl. Acad. Sci. USA **90:**7632–7636.
14. **Frankel, A. D., and C. O. Pabo.** 1988. Cellular uptake of the Tat protein from human immunodeficiency virus. Cell **55:**1189–1193.
15. **Garcia, J., D. Harrich, L. Pearson, R. Mitsuyasu, and R. B. Gaynor.** 1988. Functional domains required for tat-induced transcriptional activation of the HIV-1 long terminal repeat. EMBO J. **7:**3143–3147.
16. **Green, M., and P. M. Loewenstein.** 1988. Autonomous functional domains of chemically synthesized human immunodeficiency virus Tat trans-activator protein. Cell **55:**1179–1188.
17. **Howcroft, T. K., K. Strebel, M. A. Martin, and D. S. Singer.** 1993. Repression of MHC class I gene promotor activity by two-exon tat of HIV. Science **260:**1320–1322.
18. **Kim, C.-M., J. Vogel, G. Jay, and J. S. Rhim.** 1992. The HIV gene transforms human keratinocytes. Oncogene **7:**1525–1529.
19. **Ma, M., J. D. Geiger, and A. Nath.** 1994. Characterization of a novel binding site for the human immunodeficiency virus type 1 envelope gp120 on human fetal astrocytes. J. Virol. **68:**6824–6828.
20. **Magnuson, D., B. E. Knudsen, J. D. Geiger, R. M. Brownstone, and A. Nath.** 1995. Human immunodeficiency virus type 1 Tat activates non-N-methyl-D-aspartate excitatory amino acid receptors and causes neurotoxicity. Ann. Neurol. **37:**373–380.
21. **Mann, D. A., and A. D. Frankel.** 1991. Endocytosis and targeting of exogenous HIV-1 Tat. EMBO J. **10:**1733–1739.
22. **Milne, J. C., D. Furlong, P. C. Hanna, J. S. Wall, and R. J. Collier.** 1994. Anthrax protective antigen forms oligomers during intoxication of mammalian cells. J. Biol. Chem. **269:**20607–20612.
23. **Miyazaki, Y., T. Takamatsu, T. Nosaka, S. Fujita, and M. Hatanaka.** 1992. Intranuclear topological distribution of HIV-1 trans-activators. FEBS Lett. **305:**1–5.
24. **Nath, A., K. Psooy, C. Martin, B. Knudsen, D. S. K. Magnuson, N. Haughey, and J. D. Geiger.** 1996. Identification of a human immunodeficiency virus type 1 Tat epitope that is neuroexcitatory and neurotoxic. J. Virol. **70:**1475–1480.
25. **Orsini, M. J., C. M. Debouck, C. L. Webb, and P. G. Lysko.** 1996. Extracellular human immunodeficiency virus type 1 Tat protein promotes aggregation and adhesion of cerebellar neurons. J. Neurosci. **16:**2546–2552.
26. **Pagtakhan, A. S., and S. E. Tong-Starken.** 1995. Function of exon2 in optimal trans-activation by Tat of HIV-2. AIDS Res. Hum. Retrovirol. **11:**1367–1372.
27. **Puri, R. K., and B. B. Aggarwal.** 1992. Human immunodeficiency virus type 1 Tat gene up-regulates interleukin 4 receptors on a human B-lymphoblastoma cell line. Cancer Res. **52:**3787–3790.
28. **Sabatier, J.-M., E. Vives, K. Mabrouk, A. Benjouad, H. Rochat, A. Duval, B. Hue, and E. Bahraoui.** 1991. Evidence for neurotoxicity of tat from HIV. J. Virol. **65:**961–967.
29. **Seed, B., and J. Y. Sheen.** 1988. A simple phase-extraction assay for chloramphenicol acetyltransferase activity. Gene **67:**271–277.
30. **Sodroski, J., R. Patarca, C. Rosen, F. Wong-Staal, and W. Haseltine.** 1985. Location of the trans-activating region on the genome of human T-cell lymphotropic virus type III. Science **229:**74–77.
31. **Taylor, J. P., C. Cupp, A. Diaz, M. Chowdhury, K. Khalili, S. A. Jimenez, and S. Amini.** 1992. Activation of expression of genes coding for extracellular matrix proteins in Tat-producing glioblastoma cells. Proc. Natl. Acad. Sci. USA **89:**9617–9621.
32. **Vogel, B. E., S. Lee, A. Hildebrand, W. Craig, M. D. Pierschbacher, F. Wong-Staal, and E. Ruoslahti.** 1993. A novel integrin specificity exemplified by binding of the $\alpha_v\beta_5$ integrin to the basic domain of the HIV Tat protein and vitronectin. J. Cell Biol. **121:**461–468.
33. **Westendorp, M., M. Li-Weber, R. W. Frank, and P. H. Krammer.** 1994. Human immunodeficiency virus type 1 Tat upregulates interleukin-2 secretion in activated T cells. J. Virol. **68:**4177–4185.