

## Increased Cellular Uptake of the Human Immunodeficiency Virus-1 Tat Protein after Modification with Biotin

Ling Ling Chen,\* Alan D. Frankel,†‡ Jennifer L. Harder,‡ Stephen Fawell,\* James Barsoum,\* and Blake Pepinsky\*

\*Biogen Incorporated, 14 Cambridge Center, Cambridge, Massachusetts 02142; †Department of Biochemistry and Biophysics, and Gladstone Institute of Virology and Immunology, University of California at San Francisco, P.O. Box 419100, San Francisco, California 94141; and ‡Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, Massachusetts 02142

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**The human immunodeficiency virus-1 Tat protein can efficiently enter cells when added exogenously in tissue culture. Using the transactivation activity of Tat as a measure of intracellular delivery, we found that the addition of hydrophobic groups to Tat potentiated its uptake. Biotin was the most promising of the reagents tested and we characterized this effect in more detail. When coupled through a cysteine thiol, the addition of a single biotin to Tat increased activity by about six-fold. Increased activity was only seen with reducible biotin analogs, as modification with noncleavable analogs is known to block Tat transactivation activity. Biotin had no effect on Tat uptake when mixed with Tat without cross-linking. Recently, Tat was used as a carrier to direct the uptake of heterologous proteins into cells. We have used RNase as a model system for studying Tat-mediated uptake and found that biotin also increased the delivery of a Tat<sub>37-58</sub>-RNase conjugate. The increased uptake of Tat and Tat conjugates by addition of hydrophobic groups may significantly enhance the usefulness of Tat as a delivery vehicle, and the approach may be applicable to other systems.** © 1995 Academic Press, Inc.

The Tat protein from HIV<sup>1</sup> is a transactivator that

<sup>1</sup> Abbreviations used: HIV, human immunodeficiency virus; TAR, transactivation response element; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; biotin-HPDP, *N*-(6-[biotinamido]hexyl)-3'-(2'-pyridyldithio)propionamide; AMCA-HPDP, *N*-(6-[7-amino-4-methylcoumarin-3-acetamido]-hexyl)-3'-(2'-pyridyldithio)propionamide; DPDPB, 1,4-di(3'-(2'-pyridyldithio)propionamido)butane; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); MBB, maleimidobutylbiocytin; SMCC, succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline.

modulates transcription through its interaction with the TAR element in the HIV LTR (1–4). The full-length Tat protein is encoded by two exons and ranges in size from 86 to 101 amino acids. The first exon is highly conserved among virus isolates. The product of the first exon, Tat<sub>1-72</sub>, is sufficient for full Tat activity (1,5–7). The significance of the poorly conserved second exon is unclear. Tat<sub>1-72</sub> contains four important features: an acidic N-terminal domain (residues 1–9) which is needed for efficient transactivation (8); a cysteine-rich region (residues 22–37) which is essential for function (9–12); a basic domain (residues 49–57) which is required for nuclear localization and TAR RNA binding (13–15); and a stretch of highly conserved residues between the basic and cysteine-rich regions (see review by Jones (16) for additional references).

In studying the HIV Tat protein, it was discovered that Tat could efficiently enter cells when added exogenously to tissue culture medium and promote a dose-dependent biological response (10). A quantitative assay for the cellular uptake of Tat was developed using stable cell lines containing a CAT gene whose transcription is directed by the HIV LTR (17,18). In this system, the expression of CAT is coupled to delivery of Tat to the nucleus. CAT expression in the cellular assay can be induced by as much as 1000-fold after addition of Tat to the medium. Increases in CAT activity can be detected when as little as 1 ng of Tat is added.

The Tat protein binds efficiently to cells with greater than 10<sup>7</sup> binding sites per cell (18). By chemically conjugating Tat with reporter molecules such as  $\beta$ -galactosidase and horseradish peroxidase and then monitoring the movement of the conjugates into cells, it has been possible to study the uptake process in greater detail by histology (19). After incubating cells with the reporter

conjugates for 0–20 min, staining was predominantly cell-surface associated with progressive accumulation of intracellular staining over 30 min to 6 h. Highest levels of staining were observed after 18 h. Cells from chase periods of 48 h retained intracellular staining. A striking result from these studies was that all of the treated cells were labeled. In addition to full-length Tat, peptides from Tat that contain the basic sequence RKKRRQRRR retain membrane binding and uptake activity (20–22). Tat-based peptides also can be used as carriers, directing the uptake of conjugated heterologous proteins (19,21,23). While the detailed mechanism of Tat uptake is still unclear, binding, penetration, and release from the cell membrane are critical features of this process and therefore are likely to be influenced by its hydrophobicity. Here we show that the uptake activity of Tat can be increased by the addition of hydrophobic groups. We have used biotin reagents to characterize this effect in some detail and show that the addition of a single biotin moiety increases uptake of Tat or Tat conjugates.

## MATERIALS AND METHODS

**Purification of Tat.** Tat<sub>1-72</sub> was expressed in *Escherichia coli* using a T7 expression vector (24). Plasmid pTat72 (10) was derived from the pET-3a expression vector by insertion of a synthetic gene encoding amino acids 1–72 of HIV-1 Tat. The Tat coding region employs *E. coli* codon usage and is driven by the bacteriophage T7 polymerase promoter. The promoter is induced with isopropyl  $\beta$ -D-thiogalactopyranoside. To purify Tat, cells were pelleted in a Sorval RC3B centrifuge (4000 rpm, 30 min) and stored at  $-70^{\circ}\text{C}$ . Cells (6 g) were suspended in 60 ml of 25 mM Tris-HCl, pH 8.0, 10 mM DTT, 5 mM EDTA and lysed in a French press (12,000 psi). Cellular debris was removed by centrifugation at 10,000g for 1 h. The supernatant was loaded onto a 20-ml Q-Sepharose (Pharmacia) ion-exchange column that was equilibrated in 25 mM Tris-HCl, pH 7.5. The flow-through fraction, which contained Tat, was treated with 0.5 M NaCl. This step caused the Tat protein to precipitate. Tat was collected by centrifugation at 35,000 rpm for 1 h in a 50.2 Ti rotor and extracted from the pellet with 6 M guanidine-HCl, 50 mM sodium phosphate, pH 5.4, 10 mM DTT. Cellular debris was removed by centrifugation at 35,000 rpm for 1 h in a 70.1 Ti rotor. One-milliliter aliquots of the extract were subjected to gel filtration at 2 cm/h on an A.5M agarose column (Bio-Rad, column dimensions 1.0  $\times$  40 cm) that was equilibrated in the 6 M guanidine-HCl containing extraction buffer. The eluate was monitored for absorbance at 280 nm. Tat-containing fractions were identified by SDS-PAGE using ethanol precipitation (25) to remove the guanidine for gel samples (10  $\mu$ l of sample was treated with 400  $\mu$ l of ethanol for 5 min at  $23^{\circ}\text{C}$ ). Tat-containing fractions

from the gel filtration column were then directly subjected to reverse-phase HPLC on a Vydac C<sub>4</sub> column (column dimensions, 0.45  $\times$  25 cm). The column was developed with a 30-min gradient from 0 to 70% acetonitrile, in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. The column effluent was monitored for absorbance at 214 nm. Fractions (0.5 min) were collected. The peak fractions were pooled, aliquoted, and stored at  $-70^{\circ}\text{C}$  in the elution buffer. Prior to use, samples were lyophilized and suspended at 1 mg/ml in 25 mM Hepes, pH 7.5. The product was greater than 95% pure as judged by analysis on SDS-PAGE. Protein concentrations were estimated using an extinction coefficient at 280 nm of 1.16 for 1 mg/ml Tat.

**Production of Tat conjugates.** A series of protein modification reagents were used to test the effects of added hydrophobic groups on Tat uptake. *N*-(6-[Biotinamido]hexyl)-3'-(2'-pyridyldithio)propionamide (biotin-HPDP), *N*-(6-[7-amino-4-methylcoumarin-3-acetamido]hexyl)-3'-(2'-pyridyldithio)propionamide (AMCA-HPDP), 1,4-di(3'-(2'-pyridyldithio)propionamido)butane (DPDPB), and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Pierce. Maleimidobutyrylbioctin (MBB) was obtained from Calbiochem. Each reagent was dissolved in DMSO at 10 mM and then diluted to the concentrations indicated with Tat that had been suspended at 150  $\mu\text{g}/\text{ml}$  in 100 mM Tris-HCl, pH 7.6. Samples were incubated at  $25^{\circ}\text{C}$  for 60 min and then diluted to the desired concentration with cell culture medium.

The extent of modification for the Tat-biotin conjugates was evaluated by Western blotting using a streptavidin-alkaline phosphatase conjugate (Zymed) to detect the biotin. The blots were quantified by densitometry on a Molecular Dynamics Image Quant (Sunnyvale, CA). Two different biotinylated standards were used to assess cross-linking: the biotinylated Tat peptide-RNase conjugate described below and a genetically altered form of HPV E2R that was designed to contain only a single reactive cysteine (23). The E2 was treated with a 100-fold excess biotin-HPDP to ensure efficient conjugation. Relative conjugate ratios for Tat samples treated with a 1.6-, 3.2-, and 16-fold excess of biotin-HPDP were 1:2:4, respectively. Estimates for the absolute number of biotins per Tat for the 1.6 $\times$  sample ranged from 0.3 using the RNase standard to 1 using the E2 standard.

**Monitoring Tat uptake by transactivation.** HL3T1 cells (a HeLa cell line derivative containing an integrated LTR-CAT reporter; 18) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% donor calf serum. The cells were grown at  $37^{\circ}\text{C}$  in 5% CO<sub>2</sub> in a tissue culture incubator. For the uptake assay, cells were plated into 24-well tissue culture dishes in 0.5 ml/well DMEM plus serum at  $2.0 \times 10^5$  cells/well.

After the cells adhered, the growth medium was replaced with 0.5 ml of chloroquine-containing medium (parallel sets of samples were assayed with both 70 and 140  $\mu\text{M}$  chloroquine present) and the diluted Tat conjugates were added at 30, 100, and 300 ng/ml. The cells were incubated overnight at 37°C after which the medium was replaced with fresh medium (without chloroquine) and the cells were incubated for an additional 24 h. Cells were then harvested and assayed for CAT activity. To assay for CAT activity, cells were washed twice with PBS and 50  $\mu\text{l}$ /well of lysis buffer (0.65% Nonidet-P40 in 10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0) was added. After 2–4 min, the cell lysate was transferred to an eppendorf tube and heated at 60°C for 5 min. The samples were centrifuged for 5 min and the supernatants were transferred to fresh tubes. Sample volumes were adjusted to 60  $\mu\text{l}$  with 0.25 M Tris, pH 7.8. Cocktail (40  $\mu\text{l}$ ) containing 4 mM chloramphenicol, 0.25 mM [<sup>14</sup>C]-acetyl-CoA was added and the samples were incubated for 60 min at 37°C. Ice-cold ethyl acetate (100  $\mu\text{l}$ ) was added and the samples were mixed vigorously and centrifuged for 1 min in an eppendorf centrifuge. Upper organic phase (80  $\mu\text{l}$ ) was transferred to a scintillation vial, mixed with 2.0 ml of scintillation fluid, and counted.

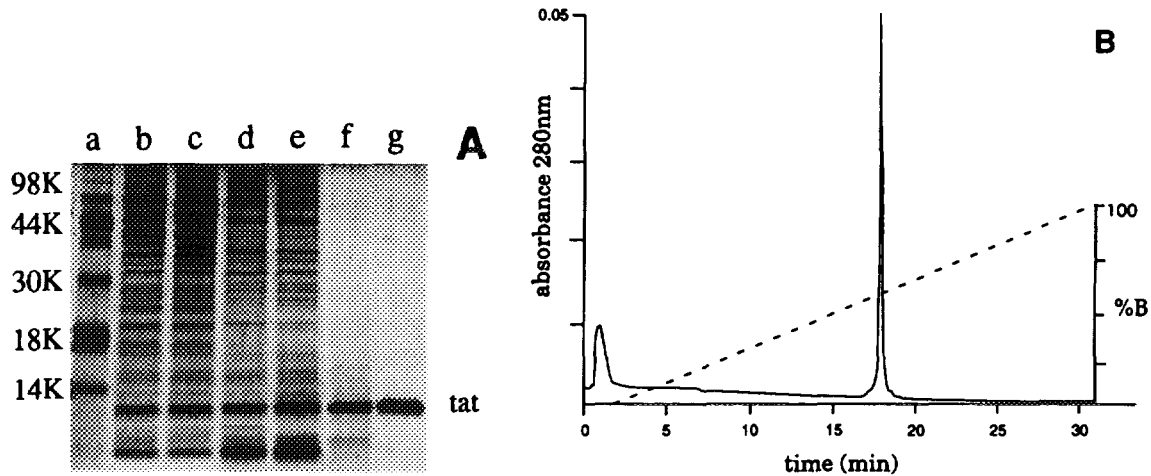
*Preparation and analysis of Tat-RNase conjugates.* Tat<sub>37-58</sub> (CFITKALGISYGRKKRRRQRRP) and the same peptide with biotin added at its N-terminus were synthesized by Research Genetics (Huntsville, AL). The peptides were purified by reverse-phase HPLC. Bovine pancreatic ribonuclease A Type 12 A (10 mg) (Sigma, Cat. No. R5500) in 500  $\mu\text{l}$  of 100 mM Na<sub>3</sub>PO<sub>4</sub>, pH 8.6, was incubated with 10 mM sulfo-SMCC (Pierce) for 40 min at 25°C. Unreacted SMCC was removed on a 5-ml P6DG (Bio-Rad) desalting column that had been equilibrated in 25 mM Hepes, pH 6.0, 50 mM NaCl. The RNase-SMCC containing fractions were identified by monitoring the absorbance at 280 nm. Peak fractions were pooled and stored at -70°C. To prepare RNase-Tat conjugates, 300  $\mu\text{l}$  of RNase-SMCC was treated with 38  $\mu\text{l}$  of 1 M Hepes, pH 7.5. Dilutions of Tat<sub>37-58</sub> or biotin-Tat<sub>37-58</sub> in 400  $\mu\text{l}$  of water were added and the samples were incubated at 25°C for 60 min. The extent of conjugation was determined by SDS-PAGE. RNase activity was assessed both *in vitro* and in a cell-based assay. In the *in vitro* assay, the conversion of a tRNA substrate from CCl<sub>3</sub>COOH-insoluble to -soluble material was monitored. For the cell-based assay we measured the ability of RNase to inhibit protein synthesis in HL3T1 cells by monitoring [<sup>35</sup>S]methionine incorporation into CCl<sub>3</sub>COOH-insoluble material. Cells were plated at 10<sup>5</sup> cells/well in a 24-well tissue culture plate and cultured overnight. The cells were washed with PBS and the conjugate was added at the concentration indicated (300  $\mu\text{l}$ /well) in PBS plus 160  $\mu\text{M}$  chloroquine. The cells were incubated at 37°C for 75 min. The PBS/chlo-

roquine medium was diluted with 750  $\mu\text{l}$  of the growth medium and the cells were further incubated overnight. The cells were washed once with PBS and incubated for 1 h in 250  $\mu\text{l}$ /well of minimal essential medium without methionine containing 1  $\mu\text{Ci}$  [<sup>35</sup>S]methionine/well. The label was removed, the cells were washed three times each with 1 ml of 5% trichloroacetic acid/well, and 250  $\mu\text{l}$  of 0.5 M NaOH was added per well. After 1 h at 25°C, 100  $\mu\text{l}$  of each sample was pipetted into a scintillation vial, mixed with 50  $\mu\text{l}$  1 M HCl + 4 ml scintillation fluid, and uptake quantified by scintillation counting.

*SDS-PAGE.* Samples were subjected to SDS-PAGE using the Laemmli buffer system. Tat<sub>1-72</sub> was analyzed on 18% gels from Novex (San Diego, CA). Tat-RNase conjugates were analyzed on 10–20% gradient gels purchased from Integrated Separation Systems (Natick, MA). Prior to analysis, samples were treated with electrophoresis sample buffer and heated to 60°C for 10 min. Tat-RNase conjugates were treated with sample buffer containing 2% 2-mercaptoethanol. Tat<sub>1-72</sub>-containing samples were treated for 10 min at 60°C with sample buffer containing 10 mM DTT and then with 50 mM iodoacetamide. Alkylation of the cysteines was needed to block disulfide-induced aggregation of Tat which occurred during electrophoresis. Tat that had been modified with biotin-HPDP was treated with iodoacetamide without reduction. Proteins were visualized by staining with Coomassie brilliant blue, silver staining (26), or Western blotting as indicated.

## RESULTS

*Purification and characterization of Tat.* Expression of the HIV-1 Tat protein in *E. coli* using the pTat72 vector results in an expression level of 5% of the total protein. Several purification protocols for Tat have been published (27–29), but these methods often have low yields, typically less than 10%. We have developed an alternative method that routinely yields 10 mg of Tat per liter of cells with an overall yield of greater than 50%. Results from a typical purification are summarized in Fig. 1. Cells were lysed at low ionic strength in a French press. After centrifugation for 30 min at 10,000g (lane c), Tat was found in the supernatant. The 10K supernatant was loaded onto a Q-Sepharose anion-exchange column and Tat was recovered in the flow-through fraction. Although Tat was present in the 10K lysate, the protein behaved as if it were in an aggregated state (probably due to association with nucleic acids) and we were unable to define a chromatographic step for further purification. However, Tat could be quantitatively precipitated upon addition of 0.5 M NaCl and we have used this property both as a concentration step and for further purification. Lane e shows the 0.5 M NaCl precipitate. Tat was extracted from the precipitate with 6 M guanidine-HCl and fractionated by gel filtration in the presence of guanidine. This step yielded protein that was approximately



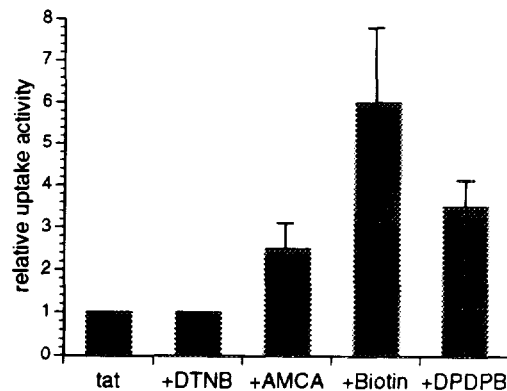
**FIG. 1.** Purification of the HIV-1 Tat protein. Tat was expressed in *E. coli* and purified by sequential steps on Q-Sepharose, gel filtration, and reverse-phase HPLC. Samples from each step in the purification were subjected to SDS-PAGE and visualized by silver staining. (A) Lane a, BRL high-molecular-weight standards; lane b, french press lysate; lane c, 10K supernatant; lane d, Q-Sepharose flow through; lane e, 0.5 M NaCl precipitate; lane f, peak fraction from gel filtration; lane g, peak from HPLC. (B) Analysis of the purified Tat by C<sub>4</sub> HPLC. 100% buffer B = 0.1% TFA, 70% acetonitrile.

80% pure (lane f). Finally, the Tat was submitted to reverse-phase HPLC. Tat eluted as a single sharp peak at 32% acetonitrile. The final product after HPLC was greater than 95% pure (lane g). By N-terminal sequencing, the expected sequence (MEPVDPRLPWKHPG) was obtained.

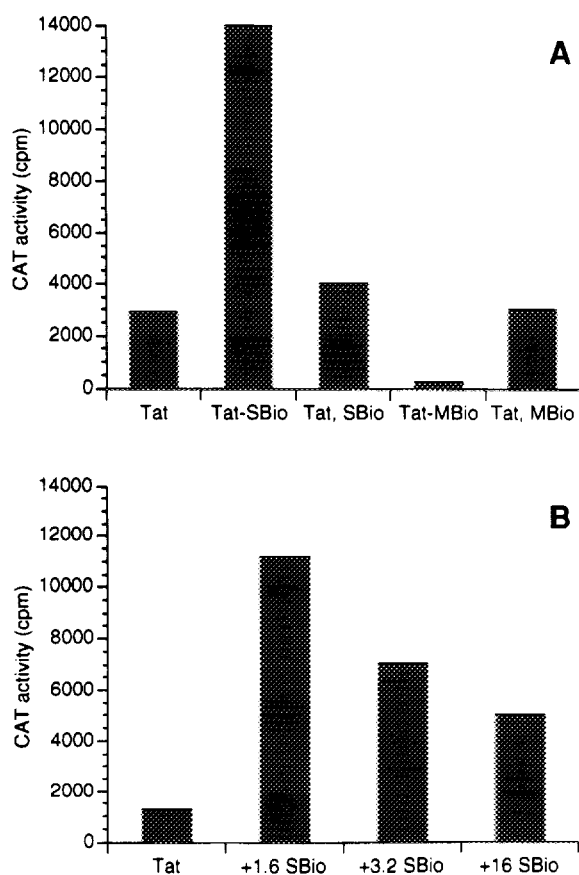
Purified Tat was tested for function in an uptake assay. This assay requires that Tat be taken up into cells and induce CAT expression by transactivating the HIV LTR. The recombinant Tat protein was active in the nanomolar range, consistent with previously reported data (18). In a side by side comparison of Tat purified by the previous (27) and current protocols, the specific activities of Tat were identical (data not shown). Uptake activity was strongly enhanced by chloroquine as previously reported (10,18). The percentage of Tat competent for uptake has been previously quantified by iodinating Tat and then monitoring cell-associated radioactivity. With this procedure only 3% of the Tat was active. We have prepared Tat by metabolic labeling with [<sup>35</sup>S]sulfate and demonstrated that greater than 60% of the material was competent (S. Fawell, unpublished results). The added radioactivity was rapidly converted into a trypsin-insensitive form, suggesting that Tat had been internalized.

**Hydrophobic groups increase uptake activity.** A series of hydrophobic protein modification reagents were tested for their effects on the uptake activity of Tat. Since the sulfhydryls of Tat are critical for transactivation activity, modification of the sulfhydryls might be expected to inactivate Tat. To minimize this possibility, we selected reversible reagents that could be released by reduction and therefore would be expected to dissociate

from Tat upon entering the reducing environment of the cytoplasm. Figure 2 shows results obtained with four reagents; DTNB, AMCA-HPDP, biotin-HPDP, and DPDPB. DTNB had no effect on uptake, while all other reagents caused significant improvements. AMCA and DPDPB caused about a threefold improvement, whereas biotin produced about a sixfold improvement. Because biotin appeared to be the most promising of the reagents, the characteristics of the modification were investigated in more detail (Fig. 3). To test whether the increased activity with biotin-HPDP required modification of



**FIG. 2.** Effect of hydrophobic groups on uptake of Tat. Tat was treated with DTNB, AMCA-HPDP, biotin-HPDP, and DPDPB and then 30 ng/ml of each conjugate was assayed for uptake activity. Background CAT activity in this series of studies for HL3T1 cells in the absence of Tat was less than 100 cpm. Values obtained for Tat alone, Tat-DTNB, Tat-AMCA, Tat-biotin, and Tat-DPDPB were 1465, 1435, 6465, 9042, and 3638 cpm, respectively. Error bars denote averages from three experiments.



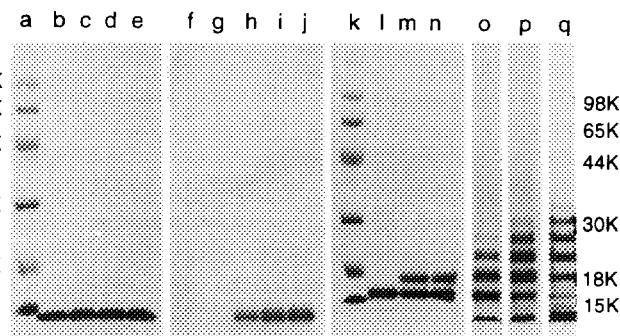
**FIG. 3.** Evaluation of the effects of biotin on Tat delivery. Cleavable and noncleavable analogues of biotin were added to Tat at a ratio of 1.6 molecules of biotin per Tat and then tested for their effects on Tat uptake in HL3T1 cells (A). Parallel samples each containing 30 ng/ml of Tat were analyzed after modification or under conditions where they were simultaneously added to the assay without modification of Tat. Background CAT activity in these studies was less than 100 cpm. (B) Tat was treated with a 1.6-, 3.2-, and 16-fold molar excess of biotin-HPDP and assayed in the Tat uptake assay. SBio refers to treatment with biotin-HPDP. MBio refers to treatment with MBB.

Tat, we performed a simple mixing experiment in which equivalent amounts of reagent were added to Tat under modifying and nonmodifying conditions. No increase in Tat activity was observed without modification (Fig. 3A). A similar experiment was performed with the noncleavable HPDP-biotin analogue MBB. Modifying Tat with MBB destroyed activity (Fig. 3A). These results suggest that hydrophobic groups attached to Tat can enhance uptake and confirm that the cysteine thiols are necessary for Tat transactivation activity.

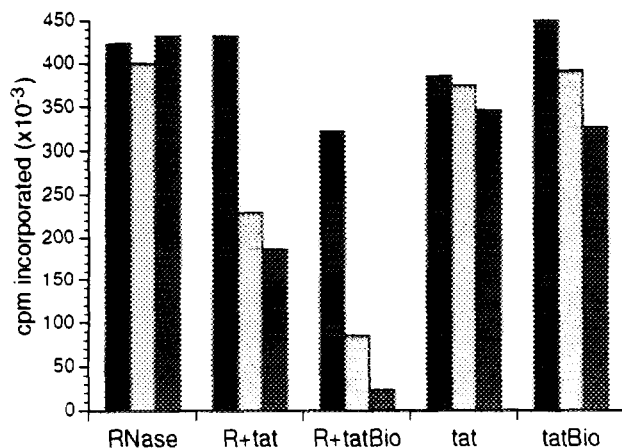
Since Tat contains seven cysteines, we examined whether the extent of modification would influence uptake activity. Tat was modified at three different biotin ratios. The extent of modification was verified by Western blotting using streptavidin-alkaline phosphatase to visualize the biotin moiety (see Fig. 4). At the lowest biotin concentration tested, which resulted in the addition

of one or less biotins on the average per Tat, we achieved the highest level of activity (Fig. 3B). At higher biotin concentrations, we observed a dose-dependent reduction in transactivation activity. The cause of the drop in activity at the high ratios of modification is unclear. It is possible that higher levels of modification may hinder the release of Tat from the cell membrane or interfere with the ability to reduce the conjugate back to free Tat once it enters the cell.

*The effect of biotin on the uptake of Tat-RNase conjugates.* Previously, Tat-mediated delivery of RNase was used as a method to study Tat uptake (19). It was shown that RNase alone had no cytotoxicity, whereas RNase-Tat conjugates had an  $IC_{50}$  for cellular protein synthesis of 15  $\mu$ g/ml, indicating that conjugation with Tat had potentiated its uptake. RNase is an attractive model because it (i) is commercially available, (ii) has low cytotoxicity, (iii) has an easily measurable enzymatic activity, and (iv) can be heavily modified with no effect on its activity. By conjugating Tat-derived peptides with RNase and then measuring cytotoxic activity, we found that the basic region of Tat is the minimal sequence needed for uptake (data not shown). To test the effect of biotin on uptake of Tat-RNase, we obtained peptides corresponding to Tat<sub>37-58</sub> that had been synthesized with and without biotin groups at their N-termini. The peptides also contained a single thiol group to be used for conjugation. Conjugates were synthesized by first reacting RNase with SMCC and then reacting the RNase-SMCC adduct with Tat<sub>37-58</sub>. By varying the Tat to RNase-SMCC ratio, the extent of modification could be varied from one Tat per RNase to five. The Tat<sub>37-58</sub>-



**FIG. 4.** Analysis of conjugates by SDS-PAGE. Biotin-Tat<sub>1-72</sub> (lanes a-j) and RNase-Tat<sub>37-58</sub> (lanes k-q) conjugates that were used to assess delivery were evaluated by SDS-PAGE. Tat-biotin conjugates at ratios of 1.6-, 3.2-, and 16-fold molar excesses of biotin were analyzed by staining with Coomassie blue (lanes a-e) and Western blotting to detect the biotin (lanes f-j). RNase samples (lanes k-q) were visualized by Coomassie blue staining. Lanes a, f, and k, BRL molecular weight markers; lanes b and g, Tat<sub>1-72</sub>-0 biotin; lanes c and h, Tat<sub>1-72</sub>-1.6 $\times$  biotin; lanes d and i, Tat<sub>1-72</sub>-3.2 $\times$  biotin; lanes e and j, Tat<sub>1-72</sub>-16 $\times$  biotin; lane l, RNase-SMCC; lane m, Tat<sub>37-58</sub>-RNase; lane n, Tat<sub>37-58</sub>-biotin-RNase; lanes o-q, Tat<sub>37-58</sub>-RNase with increasing amounts of Tat.



**FIG. 5.** Biotinylated Tat stimulates the uptake of RNase. Tat peptide 37-58 was synthesized with and without biotin and cross-linked to RNase, and the conjugates were assayed for cytotoxic activity on HL3T1 cells. The cells were treated with the conjugate and then assayed for total protein synthesis by measuring the incorporation of [<sup>35</sup>S]methionine into TCA-precipitable counts. Black, light grey, and dark grey refer to treatments with 26, 78, and 260  $\mu$ g/ml SMCC-RNase (RNase) and with 2.6, 7.8, and 26  $\mu$ g/ml of the Tat-RNase conjugates (R + tat, R + tatBio). The pure peptides Tat<sub>37-58</sub> (tat) and biotinylated Tat<sub>37-58</sub> (tatBio) were tested at the same concentration that had been used for generating the conjugates.

RNase conjugates were fully active for *in vitro* RNase activity (data not shown). When Tat<sub>37-58</sub>-RNase was assayed for cytotoxic activity, both Tat<sub>37-58</sub>-RNase and the biotinylated adduct were cytotoxic (Fig. 5). However, the biotinylated conjugate was approximately threefold more active than the control conjugate. RNase modified by addition of the cross-linking reagent SMCC but lacking Tat had no cytotoxic activity at 10-fold higher concentrations than that used for the conjugates. The peptides used for conjugation had only slight activity. In these experiments, the Tat to RNase ratio was adjusted such that about 70% of the RNase was free and 30% received a single Tat (see Fig. 4). This eliminated possible complications arising from RNase molecules having different modification states. Experiments with more highly substituted RNase gave similar results (data not shown). Since only a third of the RNase was actually biotinylated, we infer that the effect of the biotin on delivery is substantially greater than that measured in this experiment.

## DISCUSSION

We have shown that the uptake activity of Tat can be increased by addition of hydrophobic groups. Biotin, AMCA, and DPDPB all improved the uptake activity while DTNB had no effect. Biotin was most effective and the characteristics of modification were analyzed in more detail. Using a reversible Tat-biotin-HPDP conjugate, we found that Tat delivery was improved by as

much as sixfold. Biotin-HPDP had no effect on Tat uptake when it was simply mixed with Tat without modification. Interestingly, the increase in activity was optimal at the lowest biotin:Tat ratio tested. The reason for this is unclear, although it is possible that the presence of additional biotins on Tat may interfere with the release of the conjugates from the cell membrane or in the reduction step once they enter the cell. To further control for the effects of biotin on Tat activity, a Tat-biotin conjugate was prepared using the noncleavable biotin analog MBB. Modification with MBB should inactivate Tat since the cysteines in Tat are required for transactivation (9-12). Indeed when Tat was treated with the noncleavable biotin analogue MBB, the Tat-MBB adduct was inactive. Two important conclusions could be drawn from this control. First, since cysteine modification blocked Tat transactivation activity, the stimulatory effect of biotin on CAT activity is likely to be a direct consequence of its effect on Tat uptake. Second, we can infer that the Tat-HPDP-biotin adduct is reduced back to free biotin once it's delivered inside the cell, since the conjugate would be inactive without conversion. The conversion to free cysteine is expected to occur since the cell cytoplasm is a reducing environment (30).

As an independent measure of the role of biotin on Tat uptake, we investigated the effect of Tat-biotin on the delivery of RNase. In this system cytotoxicity by RNase was monitored rather than transactivation by Tat. For modification of RNase we used a peptide from Tat that had been synthesized with and without biotin added at its N-terminus. Uptake of the Tat-RNase conjugate was also improved by the addition of biotin, supporting the hypothesis that biotin increases Tat delivery. Since the biotin linkage in the Tat<sub>37-58</sub>-biotin-RNase conjugate is noncleavable, the results show that removal of biotin is not obligatory for delivery. Although we used chemical cross-linking to incorporate biotin in these studies, it is possible to direct biotinylation of proteins in *E. coli* by incorporating biotin attachment sites into the protein of interest (31). The genetic approach to biotinylation should allow for the design of better defined conjugates, although the existing method requires the addition of a 75-amino acid attachment site which may perturb the target protein.

While the mechanism of uptake of Tat is not well understood, Tat efficiently enters cells when added alone and can serve as a carrier for heterologous proteins when tested as conjugates (18,19,21,23). Despite this, the actual percentage of product that reaches its intracellular target is still quite low (21). Part of the loss in activity is likely due to proteolysis (10) and we have observed partial digestion of some of the conjugates (data not shown). However, the major factor is likely the need for a molecule to have sufficient hydrophobic character to pass through the membrane and at the same time have sufficient water solubility that it can be released from

the membrane. The addition of hydrophobic groups represents one method for altering these characteristics. The increase in CAT activity is likely a consequence of improving the delivery of Tat into intracellular compartments, since the binding of Tat to cells is already highly efficient (18,21).

Two labs recently demonstrated that Tat binds to specific cell-surface proteins, raising the possibility that delivery is receptor mediated (20,32). Whether these specific interactions are involved in uptake of Tat remains to be determined. By measuring chromium release, others have shown that Tat can punch holes in cells when added at micromolar concentrations (22). At these high concentrations the protein is cytotoxic. Tat is not cytotoxic under the conditions used in our assay, and in fact the transactivation assay requires living cells.

The efficient delivery of macromolecules into cells is important for structure-function studies that aim to truly mimic intracellular conditions. While many methods exist for protein delivery into cells (microinjection, scrape loading, electroporation, liposomes, bacterial toxins, and receptor-mediated endocytosis, see 33-36; and see 19 for additional references), most of these methods are inefficient, time consuming, cause appreciable cell death, or result in uptake into intracellular vesicles without delivery. Furthermore, many assays used to measure uptake do not actually measure delivery into the cytoplasm. The transactivation and cytotoxicity assays used here require cytoplasmic delivery and therefore monitor the desired endpoint. While we have limited our studies to Tat-mediated delivery, biotin may improve the effectiveness of other delivery systems. It is interesting to note that in plant cells a receptor-mediated delivery system for biotin-conjugated proteins was developed; however, the approach was not applicable to mammalian cells (34,37). Because biotin is frequently used as a tag for studying protein structure-function (38), it will be important to understand how biotin effects Tat uptake and to determine whether it perturbs the cellular distribution of other proteins to which it is conjugated.

The HIV Tat protein used in this study was purified from *E. coli* using a new method that relies on precipitation of Tat with 0.5 M NaCl. This was a critical step for obtaining high yields because the bacterially expressed Tat appeared to be aggregated in the cell lysate despite the observation that Tat was in the 10,000g supernatant after centrifugation. Any attempt to fractionate the protein had low yields or resulted in no purification. By concentrating the protein by precipitation and then immediately denaturing and purifying the protein in denaturant we were able to remove the bacterial components to which Tat was bound. Typical yields with this procedure were greater than 50% and we recovered 10 mg of product from 1 liter (6 g) of cells. Recently, a method for purifying Tat<sub>1-86</sub> was described in which it was suggested that the Tat purified was 100 times more active than Tat

generated by other methods (29). We have purified Tat<sub>1-72</sub> using the Slice *et al.* (29) method and have found no difference in activity (data not shown). The uptake of HIV Tat is ideally suited as a model system for investigating the intracellular delivery of macromolecules because Tat can efficiently enter cells when simply added to the growth medium, it can direct the uptake of heterologous molecules, and uptake can be quantified by monitoring transactivation. The Tat protein should serve as a valuable tool for studying delivery mechanisms and for elucidating methods for modulating uptake.

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