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RESEARCH ARTICLE

Immobilized HIV-1 Tat protein promotes gene transfer via a transactivation-independent mechanism which requires binding of Tat to viral particles

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Abstract

Background Retroviral transduction of cells is improved upon virus adsorption onto immobilized fibronectin (FN) fragments. Because HIV-1 Tat possesses the same functional domains that lead to increased transduction efficiency in FN by colocalization of bound virus and cells, we hypothesized that Tat could enhance gene transfer by a similar mechanism.

Methods Single-cycle replication retro- or lentivirus carrying green fluorescent protein or cloramphenicol acetyltransferase as reporter genes were added to wells coated with Tat or Tat peptides. Wells were extensively washed to remove unbound virus and levels of transduction were detected by measuring reporter gene expression. Virus adsorption to immobilized Tat was measured using a p24 antigen capture assay.

Results Immobilized Tat efficiently binds retro- and lentiviral particles and mediates virus transmission at virus input doses that were otherwise unable to transduce susceptible cells. Virus adsorption to Tat is not mediated by envelope glycoprotein (Env) because immobilized Tat binds and retains vesicular stomatitis virus G (VSV-G) pseudotypes as well as envelope-free particles. HIV-1 Env or VSV-G are required for Tat-assisted transduction, which is abrogated by an antibody blocking the HIV-1 Env–CD4 interaction. Tat-assisted transduction is mediated by the cysteine-rich region of Tat, which is known to be essential for Tat transactivation activity. However, Tat transactivation is not required for Tat-assisted transduction, as indicated by the enhancement of transduction by transactivation-silent Tat mutants.

Conclusions Immobilized Tat promotes virus transduction by a transactivation-independent mechanism, which requires binding of virus to Tat. Recombinant Tat or Tat fragments provide a new method to increase efficiency of retro- and lentiviral based gene transfer and gene therapy. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords gene therapy; immobilized virus; Tat; viral transduction

Introduction

Virus-mediated gene transfer into mammalian cells is crucial to study the function of genes and to correct genetic diseases by replacing malfunctioning

genes with functional gene copies. It has been previously demonstrated that gene transduction can be efficiently improved by incubating retroviruses and target cells on specific immobilized fibronectin (FN) domains and this technology is now used in gene therapy trials [1,2]. The most recent optimized protocols utilize a recombinant FN fragment (CH-296 or Retronectin) which allows for high-efficiency gene transfer [3]. This FN fragment is composed of three functional domains comprising virion and cell attachment domains. In particular, the fragment recognizes the cell integrin receptors VLA-4 and VLA-5 at the same time as binding virions via the FN heparin-binding domain. Colocalization of retrovirus particles and cells has been proposed as the mechanism responsible for Retronectin-assisted retrovirus transduction [1,2].

Tat is the transactivating protein of HIV-1 that is produced early upon infection and is required for HIV-1 gene expression and viral replication [4–7]. Tat is detected prior to virus integration and it also induces cell activation, which favors virus replication [8]. In acutely infected cells or Tat-transfected cells, high expression of Tat leads to the release of the protein in the extracellular environment in the absence of cell death or cell permeability changes [9–13]. Upon release from infected or transfected cells, Tat binds with its basic region to heparan sulfate proteoglycan (HSPG) and is rapidly stored into the extracellular matrix, whereas only a smaller fraction of the protein remains in a soluble form [10,12]. Consequently, extracellular Tat is also detected in tissues of HIV infected patients, where it is mostly bound to cells and vessels, and in sera of infected individuals [14–18]. Soluble Tat is taken up by cells and localizes into the nucleus, where it affects, either directly or indirectly, viral or cellular gene expression [9,11,12,19–24]. On the other hand, immobilized Tat can mimic the effect of adhesion molecules by binding integrins [25–27]. Furthermore, similar to FN, Tat can increase the responsiveness of vascular cells to mitogens [14,26]. Nothing, however, is known on the role of extracellular immobilized Tat on virus infection.

The evidence that Tat possesses the same functional domains mediating FN-assisted retrovirus gene transfer suggests that Tat might promote gene transfer to target cells. In the present study, we show that immobilized Tat promotes virus transduction by a transactivation-independent mechanism, which requires binding of viral particles to Tat.

Materials and methods

Production of recombinant replication-defective viruses and virus titration

The recombinant, MLV- or HIV-based, replication-defective viruses, carrying green fluorescent protein (GFP) [28] or cloramphenicol acetyltransferase (CAT) as reporter gene, were generated by transient transfection of 293 cells [29] by the calcium phosphate method [30,31] using pEF.GFP [32], pMLVgagpol [33] and vesicular stomatitis virus G protein (VSV-G) expression vector [34] for retroviral vector production (MLV); pEF.GFP, pCMVΔR8.2 [35] and VSV-G expression vector for lentivirus production (LV); and HIV-CAT reporter plasmid and the plasmid encoding the envelope protein from the laboratory adapted X4-tropic isolate (HXBc2) [22] or the VSV-G [34] for the HXBc2/HIV-CAT and VSV-G/HIV-CAT viruses. The HIV-CAT reporter plasmid contains an HIV-1 provirus deleted in the envelope glycoprotein (Env) gene (Δ-Env/HIV-CAT) and in which the nef gene has been replaced with the CAT gene [36].

The supernatants harvested from the transfected 293 cells containing the recombinant GFP or CAT viruses were collected and filtered through 0.45-μm pore-size filters (Millipore, Billerica, MA, USA).

HIV-CAT supernatants were assayed for reverse transcriptase (RT) activity [37] and/or p24 production to determine virus titers. HIV-CAT titers were expressed as RT activity (cpm/ml) and/or pg of p24.

To estimate virus-GFP titers, 293 cells were plated at 10^5 cells/well in six-well plates. Cells were incubated for 2 h at 37 °C in a 5% CO_2 incubator with serial dilutions of VSV-G/MLV-GFP or VSV-G/LV-GFP supernatants. Cells were cultured for 3 days, incubated with trypsin, and analyzed for GFP expression by fluorescence-activated cell sorting (FACS) analysis, using a FACSCalibur analyzer (Becton Dickinson, Oxford, UK). GFP-virus titers were expressed as transduction units per milliliter (TU/ml).

Recombinant HIV-1 Tat protein expression and purification

The Tat proteins (Tat and Tat_{22}) (amino acids 1–86) from HTLV-IIIB isolate, BH-10 clone (clade B), were expressed in *Escherichia coli*, purified to homogeneity by heparin-affinity chromatography and high-performance liquid chromatography (HPLC) and stored in lyophilized form at −80 °C in the dark as described previously [10,38]. The purified wild-type Tat protein was fully monomeric, as determined by sodium dodecyl sulfate-polyacrilamide gel electrophoresis and Comassie blue staining, silver staining, western blotting and HPLC, and had full biological activity, as assessed by virus transactivation assay [12], by assays with endothelial cells [26] and by uptake studies with dendritic cells [38]. Because of the presence of seven cystein residues, Tat sticks easily to surfaces and is particularly sensitive to oxidative stresses that modify its conformation, inducing protein multimerization aggregates with loss of biological activity [38]. Therefore, the lyophilized protein was resuspended just before use in a degassed phosphate-buffered saline (PBS) containing 0.1% bovine serum albumine (BSA) (Sigma-Aldrich, St Louis, MO, USA). In addition, all procedures were performed on ice and in the dark, as previously described [10,38].

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Cell cultures

Jurkat and CEMss cell lines were grown in RPMI 1640 medium (Life Technologies, Paisley, UK) supplemented with 10% FBS. 293 cells were grown in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% FBS.

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density-gradient separation (Ficoll-Paque Research Grade, Pharmacia Biotech, Uppsala, Sweden), depleted of $CD8^+$ cells by using magnetically labelled anti-$CD8^+$ beads (Dynal ASA, Oslo, Norway) in accordance with the manufacturer's instructions and cultured in a complete medium supplemented with 10% FBS, 5 U/ml human recombinant interleukin-2 (BD Labware, Bedford, MA, USA) and 2 μg/ml of phytohemaglutinin (Murex Diagnostic, Chatillon, France).

Virus transduction on wells coated with proteins, peptides or control buffer

Twenty-four-well nontissue-culture treated plates were coated with the indicated concentrations of Tat (1–86), Retronectin (Takara, Shiga, Japan), FN (Roche, Mannheim, Germany), BSA, glutathione-*S*-transferase (GST)-Tat (22–86), GST-Tat (13Tyr), GST-Tat (1–72), GST-Tat (1–86) protein or GST alone [39,40], or with the Tat peptides (1–38), (21–58), (47–86), (1–20), (11–25), (16–30), (21–40), (26–40), (31–45), (46–60), (56–70) and (65–80) or with the buffer (PBS–0.1% BSA) in which proteins or peptides were resuspended (buffer) and incubated overnight at 37 °C. The next day, wells were washed with PBS–0.1% BSA and blocked with the same buffer for 2 h at 37 °C. Wells were then incubated for 90 min at 37 °C with equal amounts of the replication-deficient HXBc2, VSV-G or Δ-Env reporter viruses. Unbound viral particles were then removed by washing the plates three times with PBS, and either CEMss or Jurkat cells (2×10^5 cells/well) or primary human $CD8^+$-depleted PBMCs ($CD8^-$ PBMCs) (2×10^6 cells/well) were added to the wells. Alternatively, the virus that remained bound to Tat- or buffer-coated wells after washing was measured (see below). After 48 h, HIV-CAT transduced cells were lysed, cell extracts made and normalized to the total protein content and used for measurement of CAT activity by thin layer chromatography, as described previously [11,12]. CAT activity was expressed as the percentage of conversion of chloramphenicol to its acetylated forms/100 μg of total protein.

After 72 h, MLV- or LV-GFP transduced cells were extensively washed and analyzed for GFP expression by FACS analysis, using a FACSCalibur analyzer (Becton Dickinson).

Single-cycle virus infection in the presence of soluble Tat

CEMss cells (4×10^5 cells/ml) were incubated in culture medium containing Tat (5 μg/ml) for 30 min at room temperature. After treatment, cells were centrifuged and suspended in 0.5 ml of HXBc2/HIV-CAT supernatants (100 000 cpm/ml). After 48 h, cells were lysed and cleared cell extracts, normalized to the total protein content, were used for measurement of CAT activity by thin layer chromatography. CAT activity was expressed as the percentage of conversion of chloramphenicol to its acetylated forms/100 μg of total protein.

Quantification of virus bound to the wells

To quantify virus bound to the coated wells, virions were lysed with 0.1M TrisHCl, 0.5% Triton X-100 (pH 7.4) and p24 content was determined by an antigen capture assay (Innogenetics, Ghent, Belgium). Values were expressed as the mean ± SD of p24 per well (bound virus; p24 pg/well).

Blocking of Tat-assisted transduction with anti-Tat antibodies or anti-CD4 antibodies

For blocking experiments with anti-Tat antibodies, Tat-coated wells were incubated with 200 μl of affinity purified rabbit anti-Tat antibodies (450 μg/ml) (Diatheva, Fano, Italy) or with an equal concentration of rabbit immunoglobulin (Ig)G, or with PBS for 1 h at 37 °C. After extensive washing, wells were incubated with HXBc2/HIV-CAT supernatants (100 000 cpm/ml) and either CEMss cells (2×10^5 cells) were added, and CAT assays performed on cell lysates 48 h post infection, as described above, or bound virus was quantified by measuring p24 content as described above.

For blocking experiments with anti-CD4 antibodies, CEMss cells (2×10^5 cells/well) were incubated with 4.5 μg of anti-human CD4 (Dako A/S, Glostrup, Denmark) or with anti-HHV8 ORF73 (ABI, Advanced Biotechnologies, Columbia, MD, USA) for 1 h at 4 °C and plated on Tat-coated wells previously incubated with HXBc2/HIV-CAT supernatants (100 000 cpm/ml) and CAT activity was determined and expressed as described above.

Detection of GST-Tat proteins coated onto wells

After overnight coating and incubation with PBS–0.1% BSA, GST-Tat proteins coated onto nontissue-culture treated 96-well plates were washed three times with 200 μl of PBS–0.1% Tween-20 and incubated for 30 min at 37 °C with anti-mouse GST antibody (Amersham-Pharmacia, Piscataway, NJ, USA). After incubation,

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plates were washed and incubated for 30 min at 37 °C with horseradish peroxidase conjugated goat anti-(mouse IgG) (Amersham-Pharmacia). After washing, wells were incubated with 100 µl of peroxidase substrate solution (Roche) for 15 min at room temperature. The reaction was stopped with 100 µl of 1 N H_2SO_4, and the optical density measured at 450 nm using an enzyme-linked immunosorbent assay (ELISA) reader. The results were expressed as optical density values.

Competition of Tat-assisted HIV single-cycle infection by Tat_{22}

Full length Tat_{22}, at different molar concentrations compared to Tat (5 µg/ml assumed as 1X) or PBS–0.1% BSA (buffer) were preincubated with the HIV/HXBc2-CAT virus (100 000 cpm/ml) for 1 h at 4 °C. The mixture was then added to Tat-coated wells as described above. CEMss cell infection was performed, evaluated and the results expressed as described above.

Statistical analysis

The percentage of fluorescent cells transduced on Tat- or Retronectin-coated cells was compared with the percentage of fluorescent cells transduced on buffer-coated wells using the Cochran–Mantel–Haenszel test; the expression of the percentage of acetylation was evaluated by the *t*-test and the association between the percentage of acetylation and Tat concentration or input virus was assessed with Pearson's correlation coefficient.

Statistical analyses were performed using SAS software (SAS Institute, Cary, NC, USA) and $p < 0.05$ was considered statistically significant.

Results

Immobilized Tat protein enhances gene transfer by retroviral and lentiviral vectors

To analyze the capability of Tat in promoting gene transfer, single-cycle replication defective lentiviruses (VSV-G/LV-GFP, 2.4×10^6 TU/ml) or retroviruses (VSV-G/MLV-GFP, 1.6×10^5 TU/ml) carrying GFP [28] as a reporter gene were used in transduction experiments. Retrovirus and lentivirus containing supernatants were generated upon three plasmid transfection of 293 cells and added to wells coated with native, biologically active and monomeric Tat protein (10 µg/ml), Retronectin (20 µg/ml) or the protein resuspension buffer (buffer). Wells were extensively washed prior to addition of CEMss cells and virus infection was monitored at 72 h post-infection by measuring GFP expression at FACS. As shown in Figure 1, transduction was detected in cells plated on Tat or Retronectin, but not in wells coated with the resuspension buffer (buffer). Specifically, $67.5 \pm 1.8\%$ and $83.0 \pm 2.3\%$ of GFP positive cells were detected when VSV-G pseudotyped lentivirus were used to transduce CEMss cells in plates coated with Tat or Retronectin, respectively, compared to $1.8 \pm 0.5\%$ of GFP positive cells in buffer-coated plates ($p < 0.0001$; Cochran–Mantel–Haenszel test). Analogously, 5.8 ± 0.3 and $10.6 \pm 0.5\%$ of GFP positive cells were detected when VSV-G pseudotyped retrovirus was used in the same experiment compared to $0.7 \pm 0.4\%$ of GFP positive cells in buffer-coated plates ($p < 0.0001$; Cochran–Mantel–Haenszel test).

Thus, both immobilized Tat and Retronectin can bind and retain VSV-G pseudotyped-retroviruses and -lentiviruses and consequently promote infection of CEMss when plates were previously incubated with infectious supernatants.

Immobilized Tat binds HIV in a gp120-independent fashion but does not substitute for receptor requirement

To investigate the mechanism by which immobilized Tat enhances gene transfer, we used single-cycle replication-defective HIV-1 encoding CAT as a reporter gene, which was pseudotyped with the Env of the X4-tropic HXBc2 isolate (HXBc2/HIV-CAT) or lacking Env expression (Δ-Env/HIV-CAT). These viruses were then used in a quantitative single-round infection assay.

The same amounts of Δ-Env or HXBc2 [22]/HIV-CAT viruses (100 000 cpm/ml) [36], which were produced in the same cell type, were incubated on 5 µg/ml Tat- or buffer-coated wells. Wells were then extensively washed and either cells were added to the wells and virus infection monitored by CAT activity, or the virus retained in the wells measured by p24 determination. As shown in Figure 2A, similar levels of p24 were detected on Tat-coated wells with both the HXBc2/HIV-CAT or the Δ-Env/HIV-CAT virus, corresponding to approximately 10% of the p24 input, with no statistically significant difference. However, no infection was observed with the Δ-Env/virus, whereas high levels of acetylation could be detected on Tat-coated plates (106.5%) with HXBc2/HIV-CAT virus compared to buffer (0%) ($p = 0.0137$; *t*-test). This suggested that, although Tat-assisted infection does not require binding of Tat to the HIV Env, immobilization of the lentivirus does not overcome its receptor requirement. To verify this, CEMss cells were incubated with anti-CD4 antibodies or with an irrelevant antibody (anti-HHV8 ORF73) and then added to HXBc2/HIV-CAT virus-adsorbed-Tat-coated wells. As shown in Figure 2B, anti-CD4 antibodies totally blocked (0%) Tat-assisted single-cycle infection of $CD4^+$ cells with the HXBc2/HIV-CAT virus compared to control (74.54%) ($p = 0.0011$; *t*-test). Thus, Tat-assisted single-cycle infection of $CD4^+$ T cells by HIV requires the binding of Tat to viral particles but does not substitute for Env–CD4 interaction.

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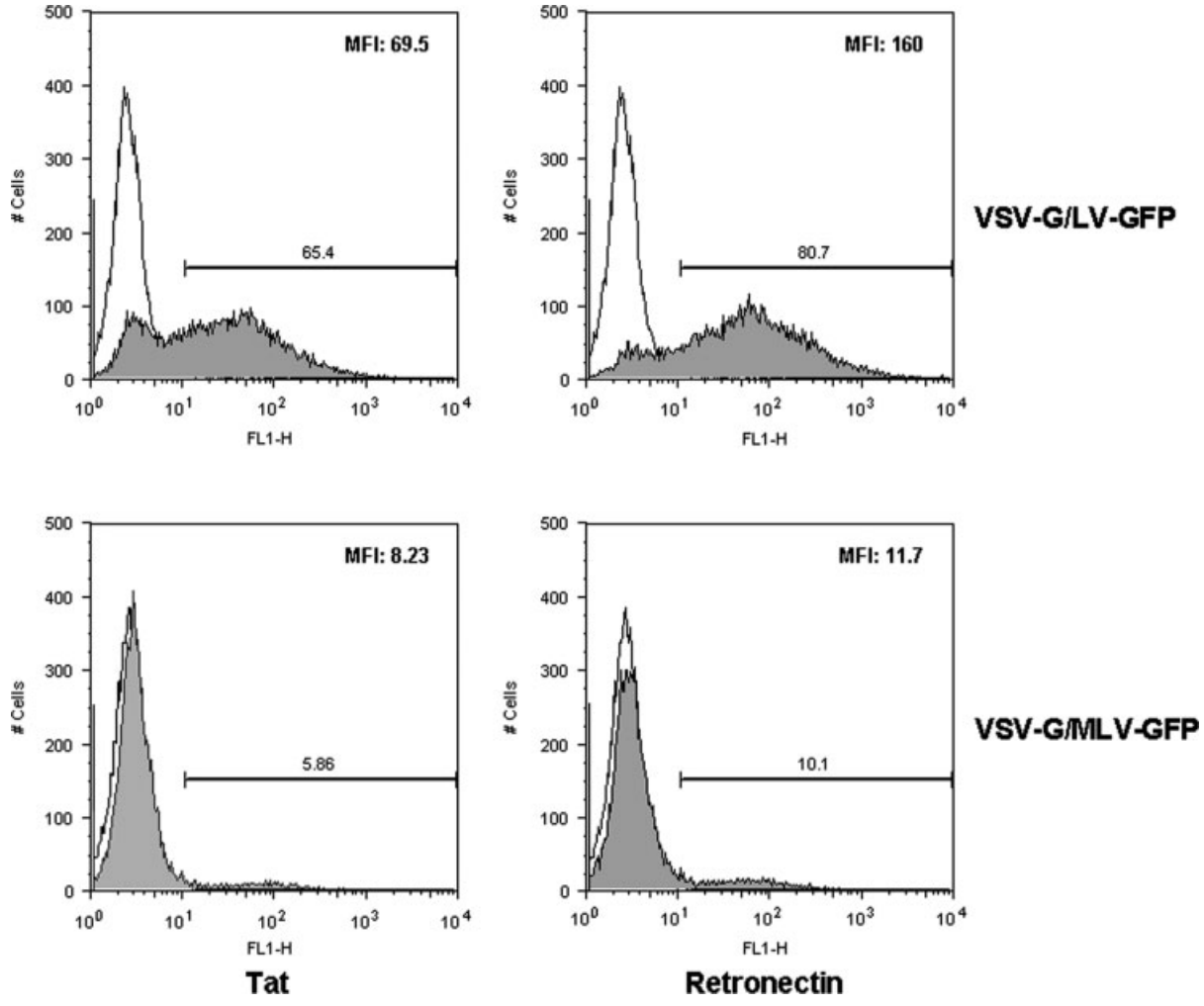

Figure 1. Immobilized Tat assists transduction of CEMss cells by lentivirus and retrovirus vectors. Supernatant containing retrovirus based (VSV-G/MLV-GFP) and lentivirus based (VSV-G/LV-GFP) vectors were added to buffer- or 10 μg/ml Tat-coated (Tat) or 20 μg/ml Retronectin-coated (Retronectin) wells. Histograms show GFP expression for cells transduced on the buffer used to resuspend the proteins (white area) or on immobilized Tat or Retronectin (gray area). The percentage and mean fluorescent intensity (MFI) of cells infected on Tat and Retronectin plates are shown (MFI of cells infected on buffer: 3.1). The results are representative of one of three independent experiments. The differences in the percentage of fluorescent cells between Tat and Retronectin or buffer, respectively, were statistically significant ($p < 0.0001$; Cochran–Mantel–Haenszel test)

Immobilized Tat mediates infection of CD4$^+$ susceptible cells in a dose-dependent fashion

To further investigate the role of extracellular immobilized HIV Tat on cell transduction, the HXBc2/HIV-CAT virus was added to wells coated with scalar amounts of Tat, its resuspension buffer or corresponding equimolar quantities of FN or of BSA (used as negative controls). Plates were then extensively washed, cells were added to the wells and virus replication monitored at 48 h post-infection by measuring CAT activity. As shown in Figure 3A, the level of infection in CEMss cells was dependent on the amount of the immobilized protein. Indeed, a statistically significant positive correlation was observed ($p = 0.0105$) between the percentage of acetylation and Tat concentration ($r = 0.7$; Pearson's correlation coefficient). By contrast, no CAT activity was detected in cells incubated in wells coated with the Tat resuspension buffer, with FN or with BSA. Tat-assisted infection was also observed with Jurkat cells or CD8$^+$-depleted PBMCs (Figure 3B). These data suggested that immobilized Tat can retain virus particles and allow infection of CD4$^+$ T cell lines and primary T cells in a dose-dependent fashion.

Immobilized Tat, but not soluble Tat, increases HIV transduction

To determine whether soluble Tat also could promote the gene transfer mechanism, HXBc2/HIV-CAT supernatants were added to buffer-coated or Tat-coated wells. CEMss cells were added after extensive washing of the plates. At the same time, the same virus input was used for direct infection of cells, which were preincubated with soluble Tat (5 μg/ml) (cells/sTat + HIV) (i.e. by the direct addition of the virus to the cells in the absence of any pre-adsorption onto wells). Untreated cells were used

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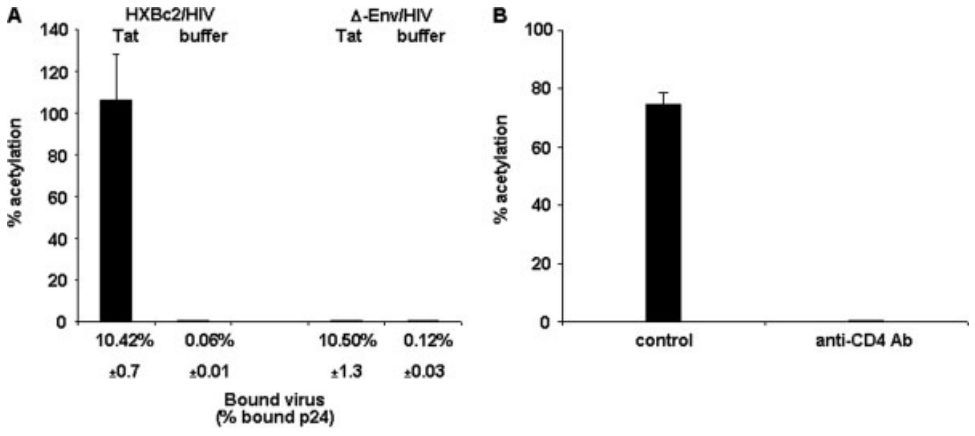

Figure 2. The HIV Env is required for virus entry and for infection of CD4$^+$ T cells, but not for virus particle binding to Tat. (A) The HXBc2/HIV-CAT or the Env-deleted Δ-Env/HIV-CAT viruses were added to buffer- (buffer) or 5 μg/ml Tat-coated (Tat) wells and either CEMss cells (2×10^5 cells/well) were seeded on the wells and CAT activity measured, or the amount of viral particles attached to the wells quantified by measuring p24 content. The results are expressed as the percentage of conversion of chloramphenicol to its acetylated forms per 100 μg of total protein and the percentage of total input virus p24 bound to the well. Values are the mean ± SD of three experiments; the difference in the percentage of acetylation for Tat and buffer was statistically significant (HXBc2/HIV-CAT virus; $p = 0.0137$; *t*-test). (B) Infection of CEMss cells preincubated with anti-human CD4 (anti-CD4 Ab) or with anti-HHV8 ORF73 (control) on 5 μg/ml Tat-coated wells previously incubated with HXbc2/HIV-CAT supernatants. The results are the mean ± SD of three experiments. The percentage of acetylation of the control was significantly higher compared to anti-CD4 Ab ($p = 0.0011$; *t*-test)

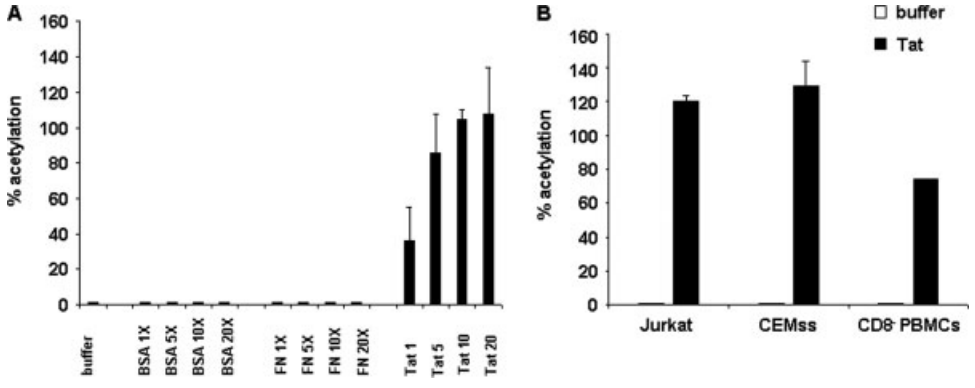

Figure 3. Immobilized Tat protein mediates infection of CD4$^+$ T cell lines and primary T cells by replication-defective HIV/CAT virus in a dose-dependent fashion. (A) CEMss cell infection on plates coated with increasing concentrations (1, 5, 10, 20 μg/ml) of the Tat (1–86) protein (Tat1, Tat5, Tat10, Tat20) or with equimolar concentrations of BSA (1X, 5X, 10X, 20X corresponding to 6.78, 33.9, 67.8, 135.6 μg/ml of BSA, respectively) or FN (1X, 5X, 10X, 20X corresponding to 31.43, 157.14, 314.29 and 628.57 μg/ml of FN, respectively), or with Tat resuspension buffer (buffer). The results are expressed as the percentage of acetylation per 100 μg of total protein. A statistically significant positive correlation was observed between the percentage of acetylation and the concentration of Tat ($p = 0.0105$; $r = 0.7$; Pearson's correlation coefficient). (B) Infection of Jurkat cells, CEMss cells and human CD8$^-$ PBMCs on buffer- (white bars) or 5 μg/ml Tat-coated wells (black bars). The results are expressed as the percentage of acetylation per 100 μg of total protein. The Results in shown in (A) and (B) are the mean ± SD of three experiments

as negative control (cells + HIV). Single-cycle infection levels were measured by CAT activity in cell lysates after 48 h. As shown in Figure 4, soluble Tat did not affect cell transduction. Furthermore, cells transduced on immobilized Tat (coated Tat) showed a higher level of transduction compared to the cells treated with soluble Tat prior to infection ($p = 0.0513$; *t*-test).

Immobilized Tat increases HIV transduction efficiency

To further determine whether binding of the virus to Tat also increased single-cycle infection efficiency, serial dilutions of the HXBc2/HIV-CAT virus stock (278 833; 24 655; 1482 and 141 pg of p24, respectively) were added to Tat-coated or buffer-coated wells. After washing the non-adherent virus, either CEMss cells were added to the wells and then infection monitored by measuring CAT activity, or the virus that remained bound to Tat- or buffer-coated wells was lysed and the p24 content determined by an ELISA assay. At the same time, the same serial-dilutions of the input viruses were used for direct infection of the cells (i.e. by the direct addition of virus to the cells, in the absence of any pre-adsorption onto wells). As shown in Figure 5A, very low levels of HIV p24 were detected on buffer-coated wells (253; 18; 2 and 1 pg, respectively)

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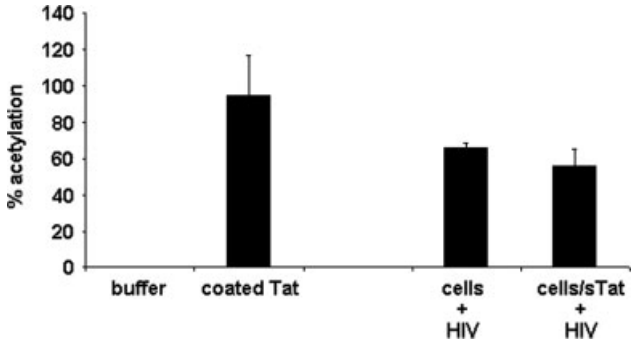

Figure 4. Immobilized Tat, but not soluble Tat, increases HIV single-cycle infection efficiency. HXBc2/HIV-CAT supernatants were added to wells coated with buffer (buffer) or with 5 μg/ml Tat (Tat-coated), and CEMss cells (2×10^5 cells/well) were seeded on the wells. The same virus input was used to resuspend CEMss cells treated (cells/sTat + HIV) or untreated (cells + HIV) with soluble Tat (5 μg/ml). CAT activity was measured in cell lysates. The results are expressed as the percentage of acetylation per 100 μg of total protein

and CAT activity was undetectable. By contrast, part of the p24 input was recovered from the Tat-coated wells (44 985; 5114; 231 and 1 pg, respectively) and, even if its amount was much less than that used by the direct method of infection (278 833; 24 665; 1482 and 141 pg, respectively) (Figure 5B), virus infection was much more efficient when cells were seeded on virus-bound Tat-coated wells compared to the direct infection method (106.46%, 14.83%, 1.78% and 0% versus 57.68%, 4.51%, 0.69% and 0% of CAT activity, for Tat-assisted infection and direct infection, respectively). A positive correlation was observed between the percentage of acetylation and the Tat-bound virus ($p = 0.0003$; $r = 0.9$; Pearson's correlation coefficient). These data indicate that the efficiency of transduction is related to the amount of the virus retained on Tat coated plates. Thus, after binding to virus particles, immobilized Tat increases infection efficiency and allows infection at levels of input virus that are usually insufficient to infect susceptible cells.

Tat-assisted infection requires specific binding of virus particles to Tat

To determine whether the binding of the virus to immobilized Tat protein is specific, blocking and competition experiments were performed, respectively, with anti-Tat antibodies or with a mutated Tat protein (Tat_{22}) expressed and purified as the wild-type Tat. For the blocking experiments, Tat-coated wells were incubated with anti-Tat antibodies, extensively washed and then incubated with the virus. After extensive washing, either CEMss cells were added to the wells and CAT activity measured after 48 h or bound virus was measured by determining the p24 content by an ELISA assay. As shown in Figure 6A, anti-Tat antibodies dramatically reduced the amount of Tat-bound virus and totally inhibited Tat-assisted infection compared to buffer or rabbit IgGs ($p = 0.0028$ and $p = 0.0089$, respectively; *t*-test).

For the competition experiments, the virus was first incubated with different molar ratios (0.1X, 1X, 10X) of Tat_{22} and added to Tat coated wells. Tat_{22} contains a mutation in cysteine 22 (substituted with a glycine), which abolishes Tat transactivating activity [9]. After extensive washing to remove unbound virus, either cells were added to wells and CAT assay performed

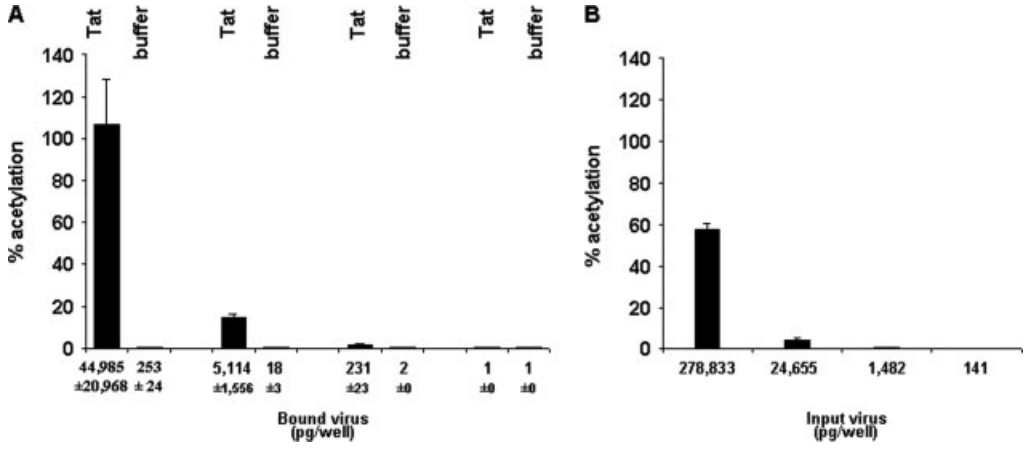

Figure 5. Immobilized Tat increases HIV single-cycle infection efficiency. (A) HIV infection of CEMss cells with serial dilutions of HXBc2/HIV-CAT corresponding to 278 333, 24 665, 1482 and 141 pg of p24, respectively, on Tat- (5 μg/ml) or buffer- (buffer) coated wells and quantification of the virus bound to the wells. The results of CEMss infection are expressed as CAT activity per 100 μg of total protein and values of well-bound virus are expressed as the mean ± SD p24 pg/well. A statistically significant positive correlation was observed between the percentage of acetylation and the amount of bound virus ($p = 0.0003$; $r = 0.9$; Pearson's correlation coefficient). (B) Direct infection of CEMss cells with the same virus input as in (A) in the absence of virus pre-adsorption onto Tat-coated wells. The same virus inputs as in (A) (278 333, 24 665, 1482 and 141 pg of p24, respectively) were directly added to CEM cells to verify the level of infection. Cells were then harvested and processed as described in (A). The results are expressed as CAT activity per 100 μg of total protein. A statistically significant positive correlation was detected between the percentage of acetylation and virus input ($p < 0.0001$; $r = 1.0$; Pearson's correlation coefficient). The results shown in (A) and (B) are the mean ± SD of three experiments

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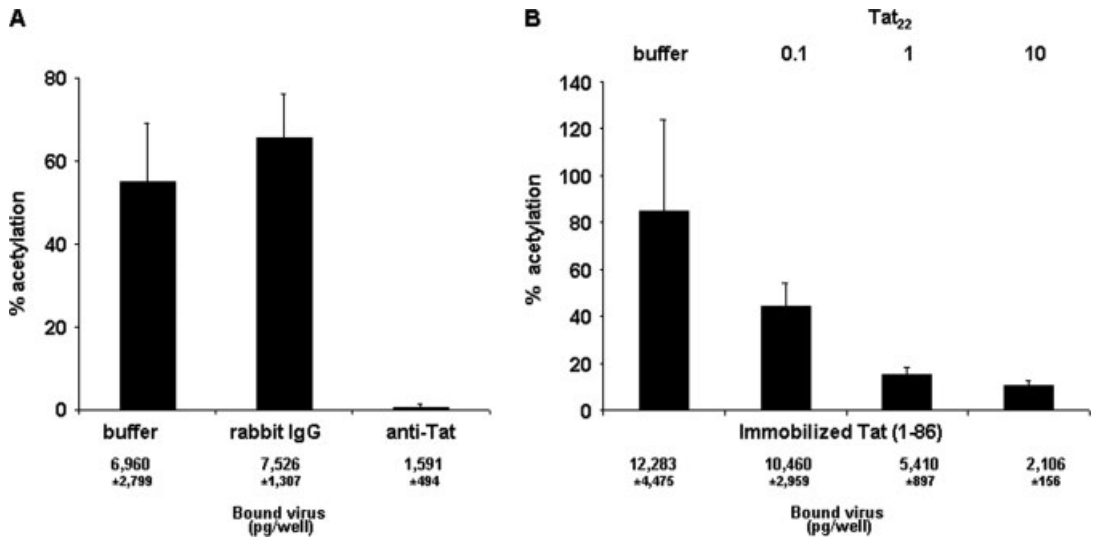

Figure 6. Tat-assisted cell infection requires a specific binding of virus particles to Tat and is blocked by anti-Tat antibodies or by Tat_{22} used as a competitor. (A) CEMss infection on Tat-coated wells preincubated with anti-Tat antibodies, with rabbit IgGs or with PBS (buffer). The difference in the percentage of acetylation of rabbit IgG and anti-Tat antibodies was statistically significant ($p = 0.0089$; *t*-test). (B) CEMss infection with HIV/HXBc2-CAT virus preincubated with different molar ratios of Tat_{22} [0.1X, 1X and 10X (0.1, 1, 10)] or with buffer and added to Tat-coated wells (5 μg/ml assumed as 1X) [immobilized Tat (1–86)]. The results of CEMss infection are expressed as the percentage of acetylation/100 μg of total protein and values of well-bound virus are expressed as the mean ± SD of p24 per well (bound virus; p24 pg/well). A negative correlation ($r = -0.5$ Pearson's correlation coefficient) was detected between the percentage of acetylation and Tat_{22} concentration. The results shown in (A) and (B) are the mean ± SD of three experiments

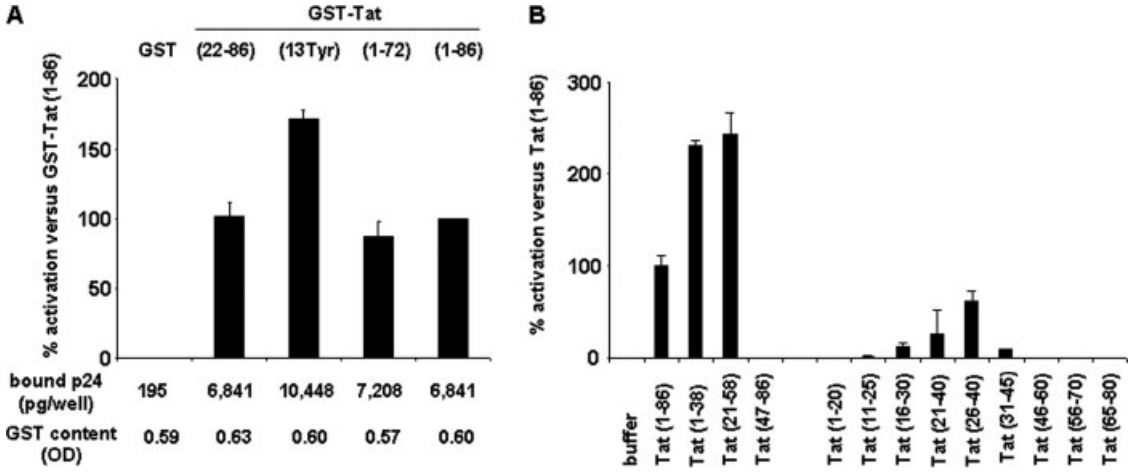

Figure 7. The transactivating activity of Tat is not required to assist HIV infection, which is mediated by the cysteine-rich region of Tat. (A) HIV infection of CEMss cells seeded on wells coated with 5 μg/ml of GST, GST-Tat (22–86), GST-Tat (13Tyr), GST-Tat (1–72) or GST-Tat (1–86) proteins. CAT activity is expressed as the percentage of acetylation versus the wild-type GST-Tat (1–86) protein, which was given a value of 100%. Levels of bound virus are expressed as p24 per well (bound p24). GST content was measured in each well to ensure that all proteins bound equally to the wells and is expressed as the optical density value. (B) Infection of CEMss cells on full length Tat (1–86) (5 μg/ml) or onto an equimolar concentration of Tat (21–58), or a ten-fold molar excess of Tat (1–38), (21–58), (47–86), (1–20), (11–25), (16–30), (21–40), (26–40), (31–45), (46–60), (56–70) or (65–80) peptides. Values are expressed as the percentage of activation versus Tat (1–86), which was given a value of 100%. The results shown in (A) and (B) are the mean ± SD of three experiments

after 48 h or bound virus was lysed and p24 levels measured by an ELISA assay. As shown in Figure 6B, Tat_{22} inhibited Tat-assisted infection very efficiently and in a dose-dependent fashion by competing the binding of the virus to Tat-coated wells ($r = -0.5$; Pearson's correlation coefficient). Thus, Tat-assisted single-cycle infection requires a specific binding of virus particles to immobilized Tat.

Tat-assisted infection does not require Tat-transactivation activity and is mediated by the cysteine-rich region of Tat

To determine the Tat domains required for Tat-assisted infection, initial experiments were performed with mutated recombinant Tat proteins. Specifically, wells

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were coated with the transactivation-silent GST-Tat fusion proteins GST-Tat_{22-86} (lacking the aminoterminal region) and the point mutant GST-Tat_{13Tyr}, or with the GST-Tat_{1-72} protein (lacking the product of Tat II exon), and compared with the wild-type protein GST-Tat_{1-86} [39,40]. Virus was then added, wells were extensively washed, and either cells were added and CAT activity monitored, or the amount of virus bound to the wells measured by determining p24 content. In addition, to determine whether all proteins were equally bound to the wells, the GST content was also measured. As shown in Figure 7A, all proteins bound to the wells with a similar efficiency. In addition, the amount of virus attached to Tat-coated wells, measured as p24 levels, was similar for all the GST-Tat proteins tested and was even higher with the transactivation-silent 13Tyr mutant, indicating that all proteins were capable of binding the virus at least as well as the wild-type Tat. Finally, CAT activity was detected with all GST-proteins used, including the transactivation-defective GST-Tat_{22-86} and was correspondingly higher (compared to the attached virus) with the GST-Tat_{13Tyr} protein (Figure 7A), indicating that the transactivating function of Tat is not required for Tat-assisted infection. Similarly, the product of Tat II exon was not required for the binding of Tat to the virus. Taken together, these data indicated that Tat transactivation activity is not required for Tat-assisted infection.

To identify the region(s) of Tat directly binding to the virions and mediating Tat-assisted infection, different peptides spanning the Tat sequence within amino acids 1–86 were coated onto wells and tested for their capability to assist CEMss cell infection by HIV. As shown in Figure 7B, peptides 1–38 and 21–58 (containing both the cysteine and the basic region of Tat) were capable of assisting the infection of CEMss cells. Smaller peptides spanning the cysteine-rich region (Tat 11–25, 16–30, 21–40, 26–40 and 31–45) had different levels of activity (2%, 13%, 28%, 62% and 10% of activity, respectively, compared to Tat 100%).

Thus, the cysteine region of Tat is required for retention of virus particles and transmission to target cells.

Discussion

It has been suggested that increased infection on recombinant FN fragment depends on the ability of the different domains of the FN molecule to specifically bind retroviral particles and target cells to immobilized molecules, thereby serving as a molecular bridge to increase the likelihood that the viral vector and the cell will interact, compared to the situation where both cell and viral particles would be in suspension [1,2].

It has also previously been shown that immobilization of HIV results in an increased infectivity of the virus. A high increase of local concentration of viral particles at the interface between the surface containing the bound virus and the membrane of the interacting cells, engagement of multiple virus receptors by immobilized virus, optimization of virus-cell post binding events or selection of the most fit particles and elimination of transduction inhibitors and toxic metabolites from the viral supernatant have all been proposed as mechanisms to explain the increased infection efficiency [1,41–43]. The data obtained in the present study indicate that, similarly to recombinant coated FN fragments, immobilized Tat binds lentivirus or retrovirus particles very efficiently and specifically, and increases gene transfer mechanisms in target cells in a dose-dependent fashion and at HIV input doses that are otherwise unable to infect susceptible cells.

The binding of virus particles to Tat and particles retention by Tat are specific because they are blocked by anti-Tat antibodies or by a mutant Tat protein used as a competitor.

It has been demonstrated that sFN, a multimeric form of FN, but not FN, enhances HIV infection of $CD4^+$ T lymphocytes. The interaction of HIV and sFN was shown to be partly mediated by the III_1-C epitope of sFN and the gp120 envelope protein of HIV-1 [44]. The data obtained in the present study demonstrate that HIV can bind immobilized Tat but not immobilized FN; however, similarly to retrovirus gp70, which is not involved in retrovirus binding to FN [45], gp120 of HIV is not involved in the binding of the virus to Tat. It has also been demonstrated the second exon of Tat can specifically interact with HIV through a direct binding with the V1/V2 loop of gp120. This interaction enhances virus attachment and entry into cells [46]. Although specific binding between low doses of soluble Tat and HIV associated gp120 cannot be excluded, our data demonstrate that retention of viral particles by immobilized Tat does not require the HIV Env protein because it also occurs with HIV not expressing gp120 or with VSV-G-pseudotyped HIV or MLV. In addition, preincubation of cells with soluble Tat prior to exposure to the virus did not increase viral transduction. It was hypothesized that sFN can retain HIV particles by exposure of a cryptic fragment that becomes available for HIV binding after FN multimerization [44]. A similar mechanism may explain the different effect of immobilized Tat compared to soluble Tat. Another hypothesis is that soluble Tat may be not available for interacting with viral particles because it is rapidly internalized by cells.

Furthermore, Tat has been shown to bind HSPG, the vascular endothelial growth factor receptors Flt-1 and Flk-1/KDR through its basic domain [10–12,47], the $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrin receptors through its RGD domain [25,26], or CCR2 and CCR3 chemokine receptors via its cysteine domain [48]. However, experiments performed to verify all of these interactions yielded negative results. Specifically, treatment of the virus particles with heparinase, which releases Tat from HSPG of the extracellular matrix [10,26], or with heparitinase, keratanase or chondroitinase ABC, did not inhibit Tat-assisted infection (data not shown), indicating that HSPG does not mediate binding of Tat to the virus. Similarly,

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immobilized vascular endothelial growth factor, the Flt-1 and KDR receptor ligand, did not assist infection of CEMss cells (data not shown). In addition, infection was not blocked by antibodies directed against $\alpha_5\beta_1$ and $\alpha_v\beta_3$ that bind the Tat RGD region (data not shown), and FN (the receptor ligand) [25,26] had no effects on virus infection when coated onto wells [44]. Furthermore, the CCR2 and CCR3 chemokine receptors are not expressed by the 293 cells used to produce the HIV-CAT virus.

Consistent with these data, we found that the heparin binding domain of Tat (amino acids 46–60) that binds to HSPG and the RGD region (amino acids 78–80) are dispensable for Tat-assisted transduction. By contrast, the cysteine-rich region of Tat mediated Tat-assisted transduction.

The transactivating activity of Tat is not involved in Tat-assisted transduction. Indeed, key deletions or amino acid substitutions that abolish Tat-promoted transactivation of HIV-1 gene expression do not affect Tat-assisted transduction.

It has also been demonstrated that the colocalization function of FN fragments can be reproduced by other strategies that simply provide a positively-charged solid surface [49]. The data obtained in the present study clearly indicate that the cysteine-rich region of Tat is involved in the binding of viral particles, suggesting a specific interaction between Tat and the virus membrane. Because similar results were obtained with virus particles produced in different cell types, it is conceivable that Tat may bind to noncell type-specific structures of cellular origin present on virus particles. These may be acquired during virus budding, as found previously for the lymphocyte function-associated antigen 1 and the very late antigen 4, which are present on the virus surface and can bind the immobilized counterreceptor [41].

Taken together, these data indicate that plate-coated Tat protein increases virus transduction. This may also provide a new tool to allow and improve gene transfer protocols with HIV-based lentivectors, especially in experimental settings where the absence of any integrin receptor triggering is recommended.

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