Internalization of HIV-1 Tat Requires Cell Surface Heparan Sulfate Proteoglycans*

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Tat, the transactivator protein of human immunodeficiency virus-1, has the unusual capacity of being internalized by cells when present in the extracellular milieu. This property can be exploited for the cellular delivery of heterologous proteins fused to Tat both in cell culture and in living animals. Here we provide genetic and biochemical evidence that cell membrane heparan sulfate (HS) proteoglycans act as receptors for extracellular Tat uptake. Cells genetically defective in the biosynthesis of fully sulfated HS are selectively impaired in the internalization of recombinant Tat fused to the green fluorescent protein, as evaluated by both flow cytometry and functional assays. In wild type cells, Tat uptake is competitively inhibited by soluble heparin and by treatment with glycosaminoglycan lyases specifically degrading HS chains. Cell surface HS proteoglycans also mediate physiological internalization of Tat green fluorescent protein released from neighboring producing cells. In contrast to extracellular Tat uptake, both wild type cells and cells genetically impaired in proteoglycan synthesis are equally proficient in the extracellular release of Tat, thus indicating that proteoglycans are not required for this process. The ubiquitous distribution of HS proteoglycans is consistent with the efficient intracellular delivery of heterologous proteins fused with Tat to different mammalian cell types.

The Tat protein of human immunodeficiency virus-1 (HIV-1)[1] is a powerful transcriptional activator of the integrated viral genome. The protein binds to a highly structured RNA element located at the 5′ end of all viral transcripts (1), and from there it increases the rates of both transcriptional initiation and elongation from the long terminal repeat (LTR) promoter. These two functions are mediated by the interaction of Tat with nuclear proteins possessing chromatin remodeling activity (2–4) and with cellular kinases phosphorylating the C-terminal domain of RNA polymerase II (5–9), respectively.

In addition to these transcriptional functions at the HIV promoter, the Tat polypeptide exerts pleiotropic biological activities when present in the extracellular compartment. Extracellular Tat promotes the production of cytokines (10–15) and cytokine receptors (16–18); modulates the survival, proliferation, and migration of different cell types (19–24); exerts angiogenic activity *in vitro* and *in vivo* (25–27); inhibits antigen-specific lymphocyte proliferation (28–30); and induces neurotoxicity in the central nervous system (31–36). Since a growing body of evidence exists that Tat could be released by producing cells (37–40), it is likely that some of the above-mentioned effects of extracellular Tat could have important implications for the pathogenesis of HIV disease in an autocrine or paracrine fashion.

Besides its interaction with cell surface receptors and the consequent activation of intracellular signal transduction pathways (41–43), most of the activities of extracellular Tat are mediated by its unique property of being rapidly internalized by a variety of cell types, as originally shown more than 10 years ago (44–46). One of the consequences of Tat internalization is the activation of cellular transcription factor NF-κB; several of the pleiotropic functions of extracellular Tat could be mediated by this pathway (47–50).

The uptake, internalization, and nuclear translocation of extracellular Tat can also be exploited as a biotechnological tool for intracellular protein delivery. Chemical cross-linking of Tat peptides with heterologous proteins (51) or, more efficiently, production of recombinant proteins containing the protein transduction domain of Tat (52, 53) facilitate the intracellular delivery of these proteins. In particular, it has been recently reported that the intraperitoneal injection of the 120-kDa β-galactosidase protein fused to 11 amino acids encompassing the arginine-rich region of Tat results in delivery of the fusion protein to virtually all tissues in mice (53).

Despite the large body of evidence available about the functions of extracellular Tat and its recent use, a biotechnological vector for protein transduction, the cellular mechanisms for Tat uptake and internalization, are still largely unexplored. Inspired by the observation that Tat can enter a variety of different cell types both *in vitro* and *in vivo* and by the possibility of modulating several of the biological properties of extracellular Tat by soluble heparin (54–56), we started investigating the role of cell surface proteoglycans in the process of Tat translocation through the plasma membrane. Here we provide genetic and biochemical evidences that cell membrane heparan sulfate (HS) proteoglycans are the receptors for extracellular Tat internalization.

§ Supported by a pre-doctoral fellowship of the International School for Advanced Studies (ISAS) of Trieste, Italy.

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[1] The abbreviations used are: HIV-1, human immunodeficiency virus-1; LTR, long terminal repeat; HS, heparan sulfate; CHO, Chinese hamster ovary; GAG, glycosaminoglycan; CAT, chloramphenicol transferase; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; GST, glutathione S-transferase.

EXPERIMENTAL PROCEDURES

Cell Lines—The wild type CHO K1 and CHO K1 mutants deficient in proteoglycan biosynthesis (57) were obtained from the American Type Culture Collection (Manassas, VA). The pgs A-745 cell line does not produce detectable levels of proteoglycans since it lacks xylosyltransferase, an enzyme necessary for the initiation of glycosaminoglycan (GAG) synthesis. Mutant pgs B-618 has a defect in the galactosyltransferase-I enzyme gene and produces about 15% the amount of proteoglycans synthesized by wild type cells. Cell line pgs E-606 is partially deficient in HS *N*-sulfotransferase and produces an undersulfated form of HS proteoglycan. The cell line pgs D-677 has a single mutation that affects both *N*-acetylglucosaminyltransferase and glucuronosyltransferase activities, which are necessary for the polymerization of HS disaccharide chains and does not synthesize any HS proteoglycan. This mutant cell line also produces approximately three times more chondroitin sulfate than wild type cells. Finally, mutant cell line pgs C-605 has a defect in a saturable, 4-acetamido-4-isothiocyanostilbene-2,2′-disulfonic acid-sensitive transport system required for sulfate uptake. Despite a dramatic reduction in $^{35}SO_4$ incorporation, the mutant synthesizes sulfated heparan and chondroitin chains by using the inorganic sulfate produced from oxidative metabolism of cysteine and methionine (58).

HL3T1 cells (a HeLa derivative containing an integrated LTR-CAT cassette) were a kind gift of B. Felber. Cell lines constitutively expressing Tat-green fluorescent protein (GFP) were obtained by selection for neomycin-resistant clones with 500 μg/ml G418 (Life Technologies, Inc.) after transfection of pCDNA3-Tat-GFP; single clones were collected and propagated.

Recombinant Proteins—Recombinant glutathione *S*-transferase (GST)-Tat containing the 86-amino acid Tat protein of HIV-1 clone HXB2 fused to glutathione *S*-transferase and its mutated derivative containing alanine substitutions at arginines 49, 52, 53, 55, 56, and 57, GST-Tat R (49–57)A, were produced and purified by glutathione-agarose affinity chromatography as already described (3, 48). The plasmid expressing GST-Tat-GFP was obtained by cloning a polymerase chain reaction-amplified fragment into the *Bam*HI and *Eco*RI sites of the commercial vector pGEX2T (Amersham Pharmacia Biotech). The fragment was obtained by the separate amplifications of HXB2 Tat using primers 5′-GTGGATCCATGGAGCCAGTAGATCCTA-3′ and 5′-CCCT-TGCTCACCATAAGCTTTTCCTTCGGGCC-3′ and of enhanced green fluorescent protein (GFP) using primers 5′-GGCCCGAAGGAAAAGCT-TATGGTGAGCAAGGG-3′ and 5′-GGCGAATTCTCTAGAGTCGCGGC-CGCTTTA-3′. Templates for amplification were plasmid pGEX2T-Tat (3) and pEGFP-N1 (CLONTECH, Palo Alto CA) respectively. The two amplification products contain complementary sequences at the 3′ and 5′ regions of the coding strands of Tat and GFP, respectively. They were gel purified, mixed, annealed, and amplified with the external primers to obtain a single amplification products, which contains *Bam*HI and *Eco*RI sites at the extremities.

Tat Protein Transduction—To study the kinetics of recombinant Tat internalization, HL3T1 cells were seeded in 24-well or 10-cm-diameter dishes at the density of 1–2 × 10⁴ cells/cm² in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. After 24 h, cell cultures were washed twice and incubated for an additional 24 h in fresh medium containing 10% fetal calf serum, 100 μM chloroquine, and recombinant Tat protein. Incubation in the presence of chloroquine favors Tat uptake by modifying the pH of endolysosomal vesicles and preventing protein degradation (46). After 24 h, the medium was changed to Dulbecco's modified Eagle's medium, 10% fetal calf serum, and cells were incubated for an additional 24 h. At the end of incubation, cells were extracted, and the amount of CAT protein present in the cell extracts was determined by the CAT enzyme-linked immunosorbent assay kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. Alternatively, cells were collected and analyzed by FACS (see below).

Internalization of ^{125}I-*GST-Tat*—Recombinant GST-Tat was labeled with ^{125}I (17 Ci/mg, PerkinElmer Life Sciences) using iodogen (Pierce) to a specific radioactivity of 400 cpm/fmol as described previously (55). HL3T1 cells were seeded in 24-well dishes at the density of 45,000 cells/cm² in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. After 24 h, cell cultures were washed twice with Tris-buffered saline and incubated for 16 h at 37 °C in binding medium (serum-free medium containing 0.15% gelatin and 20 mM Hepes buffer, pH 7.5) with the addition of 20 ng/ml [^{125}I]GST-Tat plus 200 ng/ml unlabeled GST-Tat as a carrier and in the presence of 100 μM chloroquine. After incubation, medium was removed, cells were washed three times with cold Tris-buffered saline and lysed by incubation with 0.5%

Triton X-100 in 0.1 M sodium phosphate, pH 8.1. Radioactivity of the cell lysates was measured, and nonspecific binding, determined in the presence of a 200-fold molar excess of unlabeled GST-Tat (4 μg/ml), was subtracted.

Immunocytochemistry—For immunocytochemistry, HL3T1 cells were grown to about 60% confluency on glass coverslips. The GST-Tat protein (1 μg/ml) was added to the cell culture medium in the presence of 100 μM chloroquine. After different time intervals, cells were washed 6 times with PBS and fixed with a cold acetone:methanol mixture (50:50) for 15 min. Cells were then washed 3 times with PBS containing 0.2% Triton X-100 (PBS-Triton X-100) and then 5 times with PBS for 5 min each. Cells were then incubated with an anti-Tat monoclonal antibody (ADP352/NT3, obtained from the Medical Research Council repository for AIDS research) for 1 h, washed 5 times with warm PBS (25 to 28 °C), and incubated with rhodamine-conjugated secondary antibody (Sigma) for 30 min. Cells were then washed three times with warm PBS/Triton X-100 and with warm PBS for 5 min each time. For each immunostaining, one coverslip was incubated in secondary antibody alone as a negative control for background immunofluorescence. Nuclei were counterstained with Hoechst 33342 (10 μg/ml in PBS) for 5 min, and coverslips were washed three times with PBS and mounted on glass slides. Slides were observed using Zeiss Axiophot fluorescence microscope.

Flow Cytometry—To analyze GFP-Tat internalization by cell cytometry, cells were washed four times with PBS, trypsinized, again washed with PBS, and analyzed with a FACScan flow cytometer (Becton Dickinson). A total of 10,000 events per sample were considered.

Cell Treatment with Soluble GAGs—The soluble GAGs (heparin, from porcine intestinal mucosa; chondroitin sulfate A, from bovine trachea; chondroitin sulfate B, from porcine intestinal mucosa; and chondroitin sulfate C, from shark cartilage) and dextran sulfate (molecular weight, 5000) were all purchased from Sigma. In the FACS experiments with soluble GAG analogues, 5 × 10⁵ CHO K1 cells were incubated in fresh culture medium with the addition of 1 μg/ml GST-Tat-GFP protein, 100 μM chloroquine, and the appropriate amount of GAG dissolved in PBS.

Inhibition of Tat transactivation by soluble GAGs was studied in 3 × 10⁵ CHO K1 cells transfected with 1 μg of pBlue-LTR-CAT (containing an LTR-CAT cassette, a kind gift of B. Berkhout) using Lipofectin (Life Technologies, Inc.). Forty-eight hours after transfection, cells were treated with different concentrations of GAGs along with 1 μg/ml GST-Tat-GFP and 100 μM chloroquine in fresh culture medium for 14 h. After this time period, cells were washed four times with PBS, and fresh culture medium was added. CAT assays were performed after 36 h according to standard protocols (59) and after normalization for transfection efficiency. Each experimental point was performed in triplicate.

Cell Treatment with GAG Lyases—Enzymatic treatment with GAG lyases was performed as described (60). Briefly, 5 × 10⁵ CHO K1 cells were incubated with the GAG lyases (Sigma; dissolved in PBS) in PBS containing 0.1% bovine serum albumin, 0.2% gelatin, and 0.1% glucose for 40 min at 37 °C in a CO_2 incubator. Cells were then washed gently 6 times with PBS and incubated in Ham's F-10 medium without serum in the presence of 1 μg/ml GST-Tat-GFP protein and 100 μM chloroquine for 5 h. Cells were then washed 4 times with PBS, trypsinized, washed again with complete Ham's F-10 medium, resuspended, and used for FACS analysis. Each experimental point was performed in triplicate.

HIV-1 LTR Transactivation Assays in Cell Mutants—For CAT assays, 1 × 10⁶ cells were seeded in 10-cm-diameter dishes. After 24 h, cells were transfected with either pBlue-LTR-CAT (1 μg) alone or together with pCDNA3-Tat-GFP (1 μg). The latter plasmid was obtained by recovering the Tat-GFP cassette of plasmid pGEX2T-Tat-GFP using *Eco*RI and *Bam*HI and cloning into the respective sites of vector pCDNA3 (Invitrogen, Carlsbad, CA). In this plasmid, Tat-GFP is expressed under the control of the cytomegalovirus promoter. After lipofection, cells were washed twice with PBS and incubated in fresh culture medium for additional 24 h. Cells transfected with pBlue-LTR-CAT alone were supplied with 1 μg/ml GST-Tat-GFP in the presence of 100 μM chloroquine and incubated for 24 h. Cells were then washed twice with PBS and incubated for additional 24 h in fresh medium. Finally, cells were scraped off the dishes using a rubber policeman, and cell extracts were used for CAT assays.

Transcellular Transactivation—Wild type CHO K1 cells, the mutated pgs A-745 clone, and HeLa cells were transfected by Lipofectin with 1 μg/ml pBlue-LTR-CAT. After 48 h, cells were trypsinized and plated in equal number with CHO and A-745 cell clones expressing Tat-GFP, with the combinations reported in Fig. 7A. Cells were incubated in complete medium containing 100 μM chloroquine. Seventy-two

hours later, cells were scraped off the plate with a rubber policeman, and cell lysates were analyzed for CAT activity.

<div align="center">RESULTS</div>

Kinetics of Extracellular Tat Internalization—To explore the mechanisms of HIV-1 LTR transactivation by Tat, in the last few years we have taken advantage of the property of a GST-Tat fusion protein to enter cells when added to the cell culture medium (3, 48). This protein contains the first 86 amino acids of Tat fused at the C terminus of the GST protein (~34 kDa total). The kinetics of HIV-1 LTR transactivation by GST-Tat and by a fusion protein additionally containing the GFP at the C terminus (~60 kDa total) are shown in Fig. 1*A*. LTR transactivation starts to be observed a few hours after the addition of GST-Tat to the culture medium of HL3T1 cells (containing an integrated LTR-CAT reporter gene that is silent in the absence of stimulation) and peaks at 10–15 h. The kinetics of LTR transactivation by the GST-Tat-GFP protein is delayed by a few hours but still reaches the same levels after 15–20 h. These data are consistent with previous findings showing a peak of LTR-CAT mRNA expression ~5 h after the addition of exogenous GST-Tat protein to the culture medium (48). As shown in Fig. 1*B*, LTR transactivation is already appreciable at a concentration of GST-Tat protein equal to 50 ng/ml (~1.5 nM) and reaches a plateau at 200 ng/ml. A similar dose response curve is evident for the GST-Tat-GFP protein when considering the molar differences between the two preparations. In contrast to these recombinant proteins, which contain the wild type Tat amino acid sequence, fusion of GST to the Tat R (49–57)A mutant, in which arginines at positions 49, 52, 53, 55, 56, and 57 were mutated to alanines (47), produces a protein that is completely inefficient in driving LTR activation, even at the highest concentrations (Figs. 1, *A* and *B*).

Entry of GST-Tat into the cells was also directly appreciated by labeling the protein with [125I]iodine. As shown in Fig. 1*C*, the protein was rapidly internalized, reaching significant intracellular levels already 4 h after the addition to the medium. By staining with an antibody specific for Tat, after 2 h the protein began to be found inside the cells with a distribution compatible with its presence inside endosomal vesicles, as already described (Ref. 46 and data not shown). At 4 h, the anti-Tat antibody stained the nuclei of most cells, consistent with the transactivating activity of the protein at this time point (Fig. 1*E*). At 24 h, the protein could be still visualized inside the cells, but it was mostly excluded from nuclei. Contrary to the GST-Tat fusion protein, treatment of cells with 1 µg/ml recombinant GST protein for 24 h did not result in any internalization of this protein that could be appreciable with anti-GST antibody (Fig. 1*E*, *upper panels*).

Cell entry of GST-Tat-GFP was also visualized by flow cytometric analysis of the treated cells (Fig. 1*D*). A time course study showed that increased cell fluorescence started to be detected 2 h after the addition of the protein to the cell culture medium and increased during the first 24 h. At each time point, the relatively narrow FACS peak width indicates the uniform uptake of exogenous Tat by all exposed cells, consistent with the uniform pattern of staining with the anti-Tat antibody of Fig. 1*E*. Again, a 24-h treatment with the same amount of a recombinant GST-GFP protein did not result in any appreciable increase of intracellular fluorescence. Altogether, these data indicate that functional recombinant Tat is able to specifically enter most treated cells with relatively rapid kinetics and direct the entry of larger protein cargos inside the cells.

Effect of Soluble Glycosaminoglycans on Tat Internalization—What is the receptor for Tat internalization? Earlier studies indicate that cellular entry and HIV-1-LTR transacti-

vation activity of exogenous Tat are inhibited by heparin and that heparin-Tat interaction involves the basic domain of Tat (25, 46, 54–56). Heparin is a close structural homologue of the HS GAG, a major constituent of cell surface and extracellular matrix proteoglycans (reviewed in Refs. 61–63), thus suggesting that heparin could compete with cell surface HS proteoglycans for binding to Tat, as observed for other heparin binding growth factors. We therefore tested whether other soluble GAGs could inhibit Tat internalization. Hamster CHO K1 cells were incubated with recombinant GST-Tat-GFP in the presence of different GAGs, and internalization of the fluorescent protein was visualized by flow cytometry. The CHO K1 cell line was chosen since mutants genetically impaired in GAGs biosynthesis are available. As shown in Fig. 2, heparin (average molecular mass 13.6 kDa) almost completely prevented Tat entry at a concentration as low as 1 µg/ml. In contrast, chondroitin sulfate B (dermatan sulfate), chondroitin sulfate A, and chondroitin sulfate C caused only a very limited inhibition at the highest concentrations tested.

GAGs are characterized by differences in their negative charge density and saccharide composition. In previous experiments, we observed that high affinity interaction with Tat is dependent on the extent of sulfation of the heparin chain, which imparts an average charge density higher than that of chondroitin sulfates (55). To understand whether inhibition of Tat entry by heparin depends on specific structural requirements of the GAG chain or simply by its ionic charge, competitive inhibition studies were also performed using dextran sulfate (average molecular mass 5 kDa), a homogeneous highly sulfated polysaccharide. As shown in Fig. 2, high concentrations of dextran sulfate (>10 µg/ml) were necessary to inhibit Tat internalization. Thus, on a molar basis heparin appears to be a Tat antagonist at least 30 times more potent than dextran sulfate, indicating that both saccharide composition and charge distribution of the heparin chain play an essential role in Tat interaction.

The inhibition of Tat internalization by soluble GAGs was further confirmed by determining the ability of different GAGs to inhibit HIV-1 LTR promoter transactivation by the recombinant Tat-GFP protein. CHO K1 cells were transiently transfected with a plasmid containing the reporter CAT gene under the control of the HIV-1 LTR promoter, and after 48 h, they were incubated with the Tat protein in the presence of different concentrations of GAGs. In keeping with the flow cytometric analysis data, heparin showed a dose-dependent inhibition of LTR transactivation by extracellular Tat, whereas all the chondroitin sulfates were ineffective, and dextran sulfate inhibited LTR transactivation only at 100 µg/ml (Fig. 3). It is interesting to note that heparin appears to be more potent in inhibiting cell internalization (dose inhibiting 50% of uptake < 1 µg/ml) than LTR transactivation activity (ID_{50} = 5 µg/ml), promoted by saturating concentrations of extracellular Tat (1 µg/ml). This observation is in agreement with the notion that limited amounts of Tat are sufficient to exert a still potent transactivation activity (see Fig. 1*B*).

Tat Uptake Requires Cell Surface Heparan Sulfate Proteoglycans—Altogether, the above-reported observations indicate that the interaction between Tat and heparin/HS is specific and suggest a role for cell surface HS proteoglycans in Tat uptake. A large body of evidence indicate that cell surface HS proteoglycans are rapidly internalized through an endocytic pathway and may directly internalize ligands that bind to their GAG chains. Such a mechanism of entry has already been described for other HS proteoglycan ligands, including basic fibroblast growth factor, bacteria, and animal viruses (57, 63). To test whether this was the case also for Tat, we studied Tat inter-
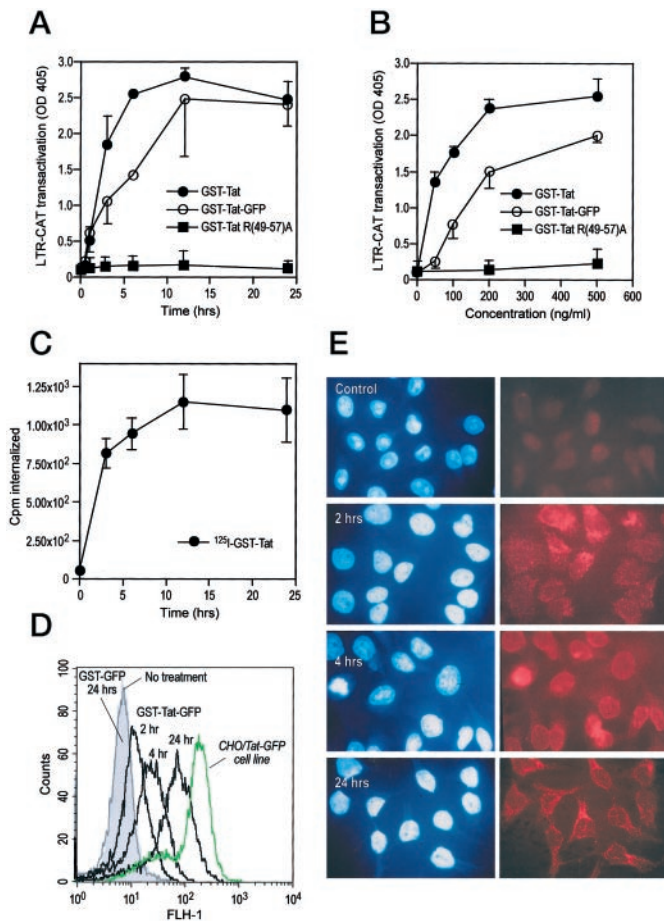
FIG. 1. **Kinetics of uptake of extracellular Tat fusion proteins.** *A*, time-dependent transactivation activity of exogenous recombinant GST-Tat and GST-Tat-GFP. The recombinant proteins (200 ng/ml), purified as GST fusions, were added to the culture medium of HL3T1 cells containing an integrated LTR-CAT construct. At the indicated time points, cells were extensively washed, and fresh medium was added. CAT activity was determined by enzyme-linked immunosorbent assay after an additional 24 h. Experiments were performed at least in triplicate; shown are the mean value and S.D. for each measurement. As a control, the mutant recombinant protein GST-Tat R(49–57)A (200 ng/ml) was used. This protein contains arginine to alanine substitutions at positions 49, 52, 53, 55, 56, and 57 and is neither able to enter the cells nor to activate transcription when expressed endogenously (47). *B*, dose-dependent transactivation activity of exogenous recombinant GST-Tat and GST-Tat-GFP. Transactivation of the LTR-CAT construct was analyzed in HL3T1 cells after a 24-h treatment with increasing amounts of GST-Tat and GST-Tat-GFP. *C*, internalization of radiolabeled GST-Tat. HL3T1 cells were incubated in serum-free medium containing 0.15% gelatin and 20 mM Hepes buffer, pH 7.5, in the presence of 20 ng/ml [^{125}I]GST-Tat and 200 ng/ml unlabeled GST-Tat as a carrier. At different time intervals, cells were washed and lysed, and radioactivity of cell lysates was measured. Under these experimental conditions, up to 90% of radioactivity remained associated with the cells after a wash with 2.0 M NaCl in sodium acetate, pH 4.0, thus demonstrating the intracellular localization of cell-associated [^{125}I]GST-Tat (55). *D*, analysis of internalization of GST-Tat-GFP by flow cytometry. HL3T1 cells were incubated for 2, 4, and 24 h with recombinant GST-Tat-GFP (1 μg/ml), washed extensively, and then analyzed for intensity of fluorescence by flow cytometry. Cellular fluorescence was specifically due to Tat-mediated internalization of the recombinant protein, since a GST-GFP fusion (1 μg/ml) was unable to modify cellular fluorescence even after a 24-h treatment (*gray-filled profile*). The rightmost *peak* shows the fluorescence of a CHO K1 cell clone stably expressing the Tat-GFP fusion protein (see Fig. 7*A*). *E*, subcellular localization of internalized GST-Tat. HL3T1 cells were treated with recombinant GST-Tat (1 μg/ml) for the indicated time intervals. After treatment, cells were extensively washed, fixed, and reacted with an anti-Tat antibody followed by recognition with a rhodamine-labeled secondary antibody (*panels* on the right side). In the same preparations, nuclei were also visualized by reactivity to Hoechst 33342 (*panels* on the left side). The two uppermost *panels* show cells treated for 24 h with a recombinant GST protein (1 μg/ml) as a control. *OD*, absorbance.
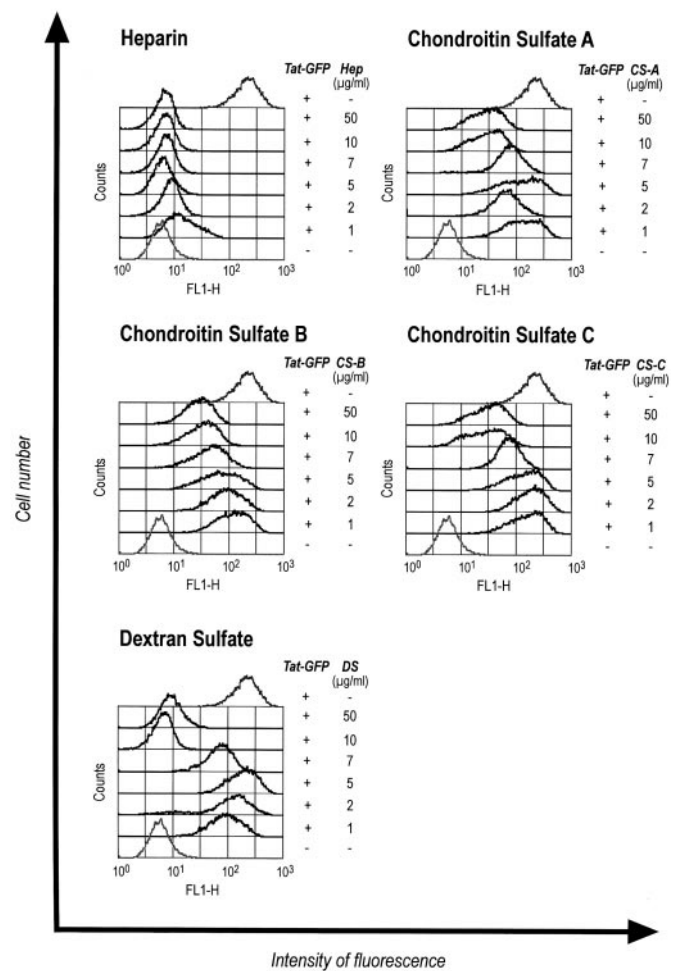
FIG. 2. **Internalization of GST-Tat-GFP in cells treated with soluble GAGs.** CHO K1 cells were incubated with 1 μg/ml recombinant GST-Tat-GFP in the presence of the indicated concentrations of heparin (*Hep*), chondroitin sulfate A, B, and C (*CS-A*, *CS-B*, and *CS-C*, respectively) and dextran sulfate (*DS*). After 14 h, cells were extensively washed and analyzed by flow cytometry.
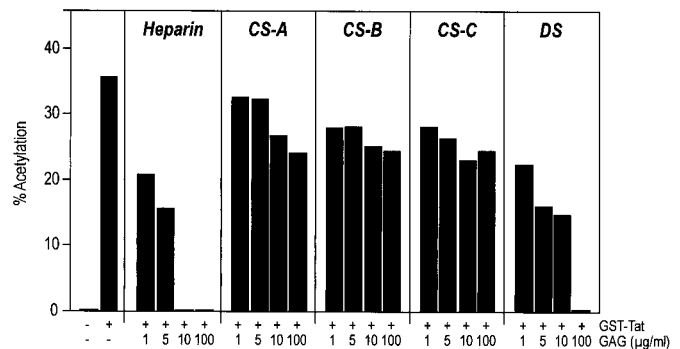
FIG. 3. **Inhibition of Tat transactivation by soluble GAGs.** CHO K1 cells were transfected with an LTR-CAT plasmid and, after 48 h, treated with 1 μg/ml GST-Tat-GFP for 14 h in the presence of the indicated concentrations of soluble GAGs. CAT assays were performed after an additional 36 h.

nalization after removal of different cell surface GAGs by specific lyases. CHO K1 cells were treated with the enzymes indicated in Fig. 4, incubated with the Tat-GFP protein, and analyzed 5 h later by flow cytometry to assess the amount of intracellular fluorescence. Cell treatment with heparitinase (heparinase-III), an enzyme mostly active on HS (64), impaired Tat internalization in a dose-dependent manner. In contrast, treatment with heparinase I, known to be active on heparin but at a much lesser extent on HS (64), showed detectable inhibi-
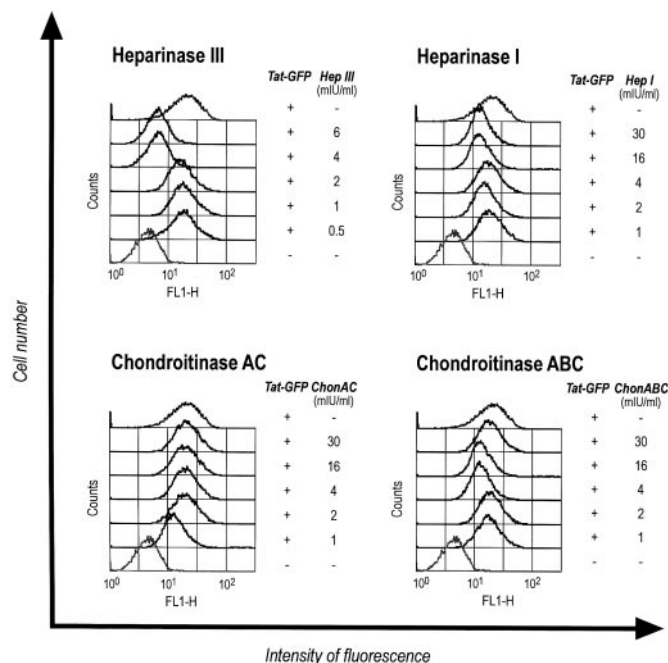
FIG. 4. **Effects of GAG lyases on the uptake of extracellular Tat.** CHO K1 cells were incubated with the indicated GAG lyases for 40 min and then incubated with 1 μg/ml GST-Tat-GFP for 5 h; this short incubation time was chosen to minimize synthesis of novel proteoglycans. After this time period, cells were extensively washed and analyzed by flow cytometry. *Hep I*, heparinase I; *Hep III*, heparinase III; *ChonAC*, chondroitinase AC; *ChonABC*, chondroitinase ABC.

tion of Tat internalization only at high concentrations. Treatments with chondroitinase ABC, cleaving at a linkage found in all chondroitin sulfates including dermatan sulfate (chondroitin sulfate B), or with chondroitinase AC, digesting specifically chondroitin sulfates A and C, were completely ineffective in inhibiting Tat internalization.

To provide a final genetic proof that cell surface HS proteoglycans act as major receptors for Tat internalization, we analyzed different mutant cell lines originated from CHO K1 cells and defective in GAG biosynthesis (57, 65, 66). Wild type and mutant cells were treated by the addition of the recombinant Tat-GFP protein to the cell culture medium, and cellular fluorescence was analyzed at different time intervals by flow cytometry (Fig. 5). In agreement with the data shown in Figs. 2 and 4, internalization of the protein was clearly detectable in the wild type CHO K1 cells, and it was proportional to the time of treatment.

In contrast, Tat entry was undetectable in pgs A-745 mutant cells, which are defective in the enzyme xylosyltransferase, which initiates GAG synthesis, and it was very reduced in pgs B-618 cells, which lack galactosyltransferase-I activity catalyzing the second step in GAG synthesis and produce about 15% of the amount of GAGs synthesized by wild type cells (65, 66). Cell line pgs C-605, which is deficient in sulfate transport but retains its sulfated GAG synthetic capacity (58), behaved as wild type cells. In contrast, very little Tat-GFP internalization was observed in the HS-deficient pgs D-677 cells, bearing a single mutation that affects both *N*-acetylglucosaminyltransferase and glucuronosyltransferase activities, which are necessary for the polymerization of HS disaccharide chains (66). Interestingly, in these cells the defect in HS production is paralleled by an increase in chondroitin sulfate synthesis that is, however, unable to support Tat internalization. Finally, Tat entry was severely impaired also in the mutant pgs E-606 cell line (66), which produces an *N*-undersulfated form of HS due to a defect in the HS *N*-sulfotransferase enzyme. This observation
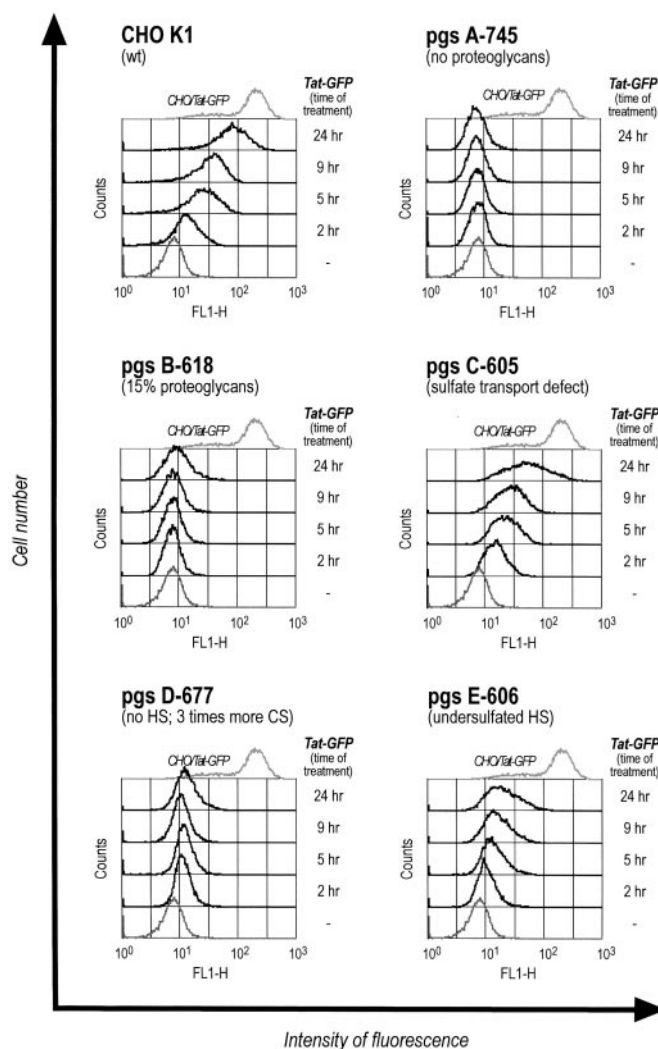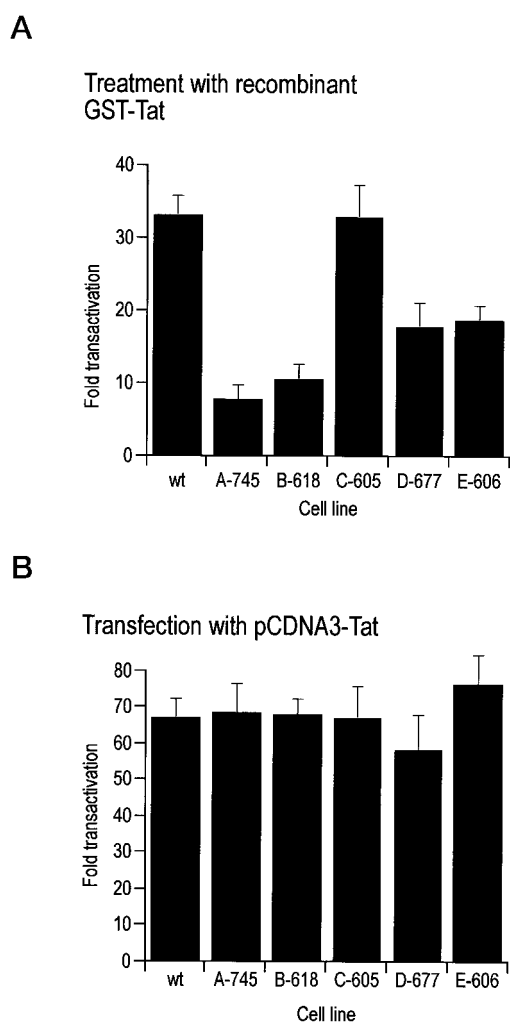

FIG. 5. **Tat internalization into cells genetically defective in GAG biosynthesis.** Wild type (*wt*) CHO K1 cells and the indicated cell clones mutated at different steps in GAG biosynthesis were treated with 1 μg/ml GST-Tat-GFP and analyzed by flow cytometry after 2, 5, 9, and 24 h of treatment. In each *panel*, the uppermost FACS profile shows fluorescence of CHO K1 cells clone stably expressing Tat-GPF (see Fig. 7*A*). The main recognized defect of each cell line is indicated.

further supports the notion that selective sulfation of HS is an important determinant for Tat/HS recognition (55).

Next, the ability of CHO K1 mutants to support Tat-mediated LTR transactivation was tested by transient transfection of an LTR reporter gene construct followed either by treatment with recombinant Tat or by co-transfection of a Tat-expressing plasmid. All cells efficiently supported Tat functions when this was expressed as an endogenous protein, indicating that the intracellular mechanisms mediating Tat transactivation were intact in all these cell lines (Fig. 6*B*). In contrast, mutants pgs A-745 and pgs B-618 were markedly impaired in LTR transactivation by exogenously added Tat (Fig. 6*A*). The residual LTR transactivation activity in the former mutant is most likely attributable to the low level of xylosyltransferase activity ($\frac{1}{15}$ normal), which is still present in these cells and might be sufficient for the production of limited amounts of proteoglycans (66). Consistent with the GST-Tat-GFP internalization data, the specific role of HS proteoglycans in Tat entry was indicated by the results obtained with pgs D-677 cells, overproducing chondroitin sulfate, and pgs E-606 cells, producing an undersulfated form of HS. In both cell lines, LTR transactivation by recombinant Tat was clearly impaired. In contrast, LTR

A

Treatment with recombinant
GST-Tat

B

Transfection with pCDNA3-Tat

FIG. 6. **HIV-1 LTR transactivation in cells genetically defective in GAG biosynthesis.** Wild type (*wt*) CHO K1 cells and the indicated cell clones mutated at different steps in GAG biosynthesis were transfected with an LTR-CAT reporter plasmid (10 μg) and either treated with recombinant GST-Tat (200 ng/ml; *panel A*) or co-transfected with a Tat-expressing plasmid (1 μg; *panel B*). CAT assays were performed after 48 h. The results shown represent average values and S.D. obtained in several (at least three) independent transfections.

transactivation by exogenous Tat was normal in pgs C-605 cells.

Overall, these data indicate that cell surface HS proteoglycans are the major cellular receptors for Tat internalization. In addition, the reduced ability of Tat entry in pgs E-606 cells suggests that the density and distribution of sulfate groups in HS are important determinants for the efficiency of this process.

Transcellular Transactivation—Tat is released from expressing cells and enters neighboring cells through an endosome-mediated pathway. To assess the role of cell surface proteoglycans in this physiological process, we obtained wild type CHO K1 and GAG-deficient pgs A-745 cell clones constitutively expressing Tat-GFP. The two cell lines were undistinguishable by fluorescent microscopic examination and showed nucleoplasmic localization of the expressed protein (Fig. 7*A*). Both cell lines were co-cultured for 72 h with wild type hamster CHO K1 or human HeLa cells previously transfected with an LTR-CAT reporter gene construct. In both cases, transactivation of the LTR promoter contained in the latter cells could be readily detected (Fig. 7*B*). In contrast, transactivation did not occur when the reporter cells were the GAG-deficient pgs A-745

A

B
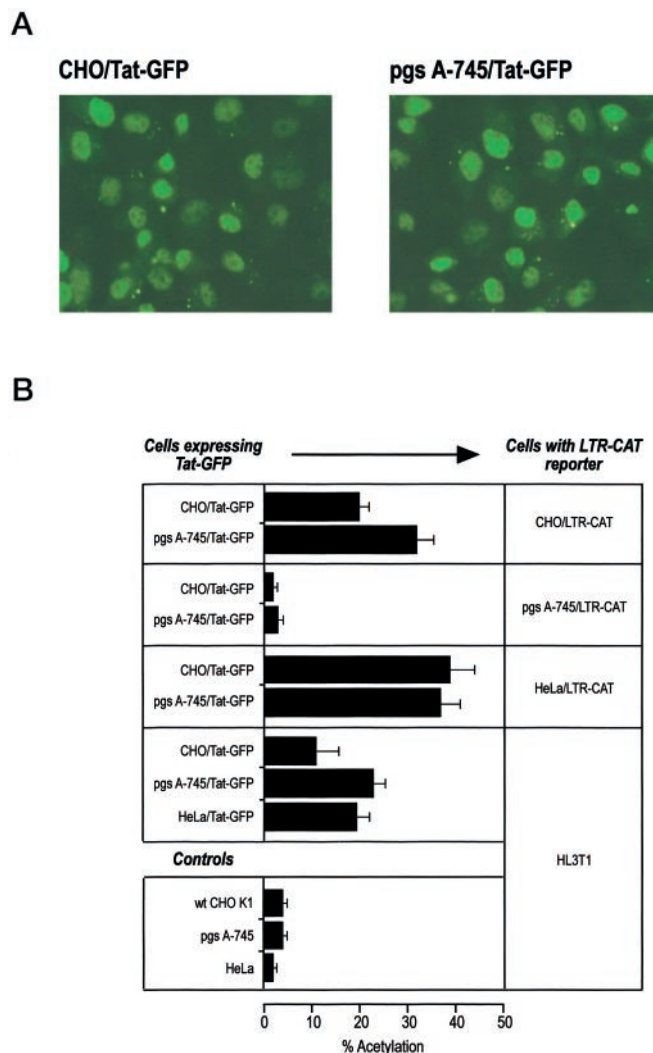
FIG. 7. **Transcellular transactivation.** *A*, fluorescence pattern of CHO clones producing Tat-GFP. Cellular clones stably expressing Tat-GFP were obtained for wild type CHO K1 and pgs A-745 cells impaired in proteoglycan production (left and right sides, respectively). In both cell lines, the protein showed predominant nuclear localization with exclusion of the nucleoli. , results of co-culture experiments. Wild type (*wt*) CHO/Tat-GFP and mutated pgs A-745/Tat-GFP cells were co-cultured (1:1 ratio) for 72 h with wild type CHO K1, pgs A-745, and HeLa cells that had been previously transfected with an LTR-CAT reporter plasmid. The histograms indicate the average levels and S.D. of LTR-CAT transactivation obtained in three independent experiments. As a further control of the transcellular transactivation of the Tat-GFP protein produced by CHO/Tat-GFP and pgs A-745/Tat-GFP cells, these were also co-cultured with HL3T1 cells, a HeLa derivative containing an integrated LTR-CAT construct.

mutants (Fig. 7*B*). Thus, in keeping with the results obtained with exogenously added recombinant Tat protein, cell surface proteoglycans mediate internalization of endogenously expressed Tat released from producing cells. It is worth noting that the capacity of both CHO/Tat-GFP and pgs A-745/Tat-GFP transfectants to release significant amounts of biologically active Tat indicates that cell surface proteoglycans are not involved in the process of Tat export from the cell.

DISCUSSION

The results presented here provide genetic and biochemical evidence that cell-associated HS proteoglycans function as cell surface receptors for extracellular Tat internalization. This conclusion is supported by the findings that Tat uptake is inhibited by heparin but not by other soluble GAGs, that cell treatment with GAG lyases specific for HS, but not for chon-

droitin sulfates, blocks Tat internalization, and that cell lines with genetic defects in the cellular pathway involved in the production of sulfated HS proteoglycans fail to internalize Tat. The identification of HS proteoglycans as cell surface receptors for Tat internalization is consistent with the notion that Tat is able to enter into a wide variety of human, rodent, and simian cell lines, indicating that it utilizes a ubiquitous cell surface molecule for cell entry. This conclusion is also in agreement with the observation that extracellular Tat enters most of the exposed cells, as concluded by the narrow FACS peaks observed after treatment with recombinant Tat-GFP and by the immuno-staining results shown in Fig. 1*E*.

A common peptidic motif for heparin/HS binding consists of a region rich in basic amino acids flanked by hydrophobic residues (61–63). The arginine-rich domain of Tat (amino acids 49–57) conforms to these characteristics. Consistently, this domain is sufficient to promote cell internalization of Tat and of other tat-fusion proteins (46, 52, 67). We have recently observed that mutation of the arginines in this domain or its occupancy by polysulfonated compounds prevents heparin binding and cell internalization of GST-Tat, respectively (55).

Similar to the interaction of heparin with other cellular macromolecules (68–70), binding of Tat to heparin/HS is most likely determined by both ionic interactions and specific structure recognition. Other small basic proteins (having isoelectric points and sizes comparable with those of Tat) such as histone H1 and basic fibroblast growth factor, although binding to heparan sulfate, cannot enter the cells through this interaction nor mediate protein transduction (71, 72). Additionally, other proteins with the same characteristics such as cytochrome *c* do not even bind with high affinity to heparan sulfates (41).

Tat/HS binding affinity is proportional to the size of heparin oligosaccharides, with at least six saccharide residues required for this interaction to occur (54, 56). Biochemical data also indicate that selective 2-*O*-, 6-*O*-, or *N*-desulfation/*N*-acetylation dramatically reduce the capacity of heparin to bind Tat (55). This observation agrees with the results obtained with pgs E-606 cells, which produce *N*-undersulfated HS and are severely impaired in Tat internalization. Finally, the requirement of structure recognition for the Tat-HS interaction to occur is further supported by the observation that several highly negatively charged molecules such as chondroitin sulfates, dextran sulfate (see Fig. 2), and sulfated β-cyclodextrin (55) are poor Tat antagonists. This observation is in agreement with the inability of pgs D-677 cells, which do not produce HS, to support Tat internalization despite their overproduction of cell surface chondroitin sulfates. Altogether, from these considerations, it may be concluded that the interaction between Tat and heparin/HS is specific and is determined by size, saccharide composition, and extent and distribution of sulfation of the GAG backbone.

The identification of cell membrane HS proteoglycans as receptors for extracellular Tat uptake and the observation that Tat can drive internalization of larger protein cargos inside the cells with a relatively rapid kinetics further support the use of Tat as a biotechnological tool for protein transduction. This property of Tat can be exploited either by obtaining recombinant proteins of pharmacological interest fused to Tat or Tat peptides (52, 53) or by expressing these fusions *in vivo* after appropriate gene transfer. In this respect, it should be observed that the molecular mechanisms that promote escape of internalized Tat from the endosomal vesicles are still largely unexplored. The immunofluorescence data shown in Fig. 1*E* indicate that at 4 h after the addition to the cell culture medium the protein has a clear nuclear localization. Previous experiments in which we measured the levels of LTR-driven reporter gene mRNA in cells treated with extracellular Tat indicated that LTR transactivation is maximal after ~5 h, whereas it progressively decreases at later time points to become undetectable after 12 h (48). These data are consistent with the pattern of residual Tat localization at 24 h, when immunoreactive Tat protein is still appreciable inside the cells but with a distribution that is no longer nuclear and might be compatible with its localization within late endosomal-lysosomal vescicles. Similar to the exit of internalized Tat from endosomes, the molecular mechanisms responsible for Tat release from expressing cells are also poorly understood. The results presented in Fig. 7 on the capacity of GAG-deficient pgs A-745 cells to sustain Tat release clearly indicate that cell surface proteoglycans are not involved in this process.

Finally, the results presented in Fig. 7 as well as other consistent results reported in the literature (37–40) clearly indicated that the process of Tat-mediated transcellular transactivation is not limited to the experimental use of high doses of recombinant Tat added to the extracellular medium but is easily observable for endogenously produced Tat released by expressing cells. This event is even more significant when considering that even very limited amounts of Tat are sufficient to produce a significant level of viral LTR transactivation (45). What is the functional significance of transcellular transactivation? It has been suggested that under some conditions Tat might act as a viral growth factor to stimulate viral replication in latently infected cells (45). Alternatively, it might be envisaged that the pleiotropic functions of Tat on the expression of several cellular genes could stimulate the generation of a cellular environment more permissible for viral infection. Should additional experiments indicate that this process has pathogenetic significance during the course of HIV infection, the interaction between Tat and cell surface HS proteoglycans would also constitute a novel important target for therapeutic intervention.

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Internalization of HIV-1 Tat Requires Cell Surface Heparan Sulfate Proteoglycans

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