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Multiple Interactions of HIV-I Tat Protein with Size-defined Heparin Oligosaccharides*

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Tat protein, a transactivating factor of the human immunodeficiency virus type I, acts also as an extracellular molecule. Heparin affects the bioavailability and biological activity of extracellular Tat (Rusnati, M., Coltrini, D., Oreste, P., Zoppetti, G., Albini, A., Noonan, D., D'Adda di Fagagna, F., Giacca, M., and Presta, M. (1997) *J. Biol. Chem.* 272, 11313–11320). Here, a series of homogeneously sized, ^{3}H-labeled heparin fragments were evaluated for their capacity to bind to free glutathione *S*-transferase (GST)-Tat protein and to immobilized GST-Tat. Hexasaccharides represent the minimum sized heparin fragments able to interact with GST-Tat at physiological ionic strength. Also, the affinity of binding increases with increasing the molecular size of the oligosaccharides, with large fragments (≥18 saccharides) approaching the affinity of full-size heparin. 6-Mer heparin binds GST-Tat with a dissociation constant (K_d) equal to 0.7 ± 0.4 μM and a molar oligosaccharide:GST-Tat ratio of about 1:1. Interaction of GST-Tat with 22-mer or full-size heparin is consistent instead with two-component binding. At subsaturating concentrations, a single molecule of heparin interacts with 4–6 molecules of GST-Tat with high affinity (K_d values in the nanomolar range of concentration); at saturating concentrations, heparin binds GST-Tat with lower affinity (K_d values in the micromolar range of concentration) and a molar oligosaccharide:GST-Tat ratio of about 1:1. In agreement with the binding data, a positive correlation exists between the size of heparin oligosaccharides and their capacity to inhibit cell internalization, long terminal repeat-transactivating activity of extracellular Tat in HL3T1 cells, and its mitogenic activity in murine adenocarcinoma T53 Tat-less cells.

The data demonstrate that the modality of heparin-Tat interaction is strongly affected by the size of the saccharide chain. The possibility of establishing multiple interactions increases the affinity of large heparin fragments for Tat protein and the capacity of the glycosaminoglycan to modulate the biological activity of extracellular Tat.

Tat is a regulatory protein of the human immunodeficiency virus type I (HIV-I)[1] released by HIV-infected cells (1). Extracellular Tat is implicated in the progression of AIDS (2–4) and in the pathogenesis of AIDS-associated pathologies including Kaposi's sarcoma (5, 6), AIDS-dementia (7), and increased tumor incidence (8, 9). Extracellular Tat exerts its pleiotropic effects by acting on different target cells. Three classes of cell-surface receptors have been implicated in the biological activity of extracellular Tat: cell adhesion receptors of the integrin family (10), the vascular endothelial growth factor receptors Flt-1 and Flk-1/KDR (11, 12), and the chemokine receptors CCR2 and CCR3 (13). Interaction of extracellular Tat with these receptors may activate various intracellular signaling pathways responsible for the biological responses elicited by this protein in the different target cells (13–17).

Tat is a heparin-binding protein (18, 19) that interacts with heparan sulfate (HS) proteoglycans (HSPGs) of the cell surface and extracellular matrix (20). This allows the extracellular storage of Tat that can be mobilized in a biologically active form by free heparin (20). HSPG interaction is implicated in cell internalization of Tat (13), and it is required for the HIV-LTR-transactivating activity of extracellular Tat.[2] Both activities are competed by free heparin/HS (19). On the other hand, free heparin potentiates the mitogenic and chemotactic activity exerted by extracellular Tat in cultured endothelial cells and its angiogenic activity *in vivo* (13). Interestingly, polysulfonated heparin-mimicking compounds can interfere with the biological activity of extracellular Tat and may represent potent inhibitors of possible therapeutic value (19).

The molecular bases of heparin-Tat interaction have been investigated. As observed for various heparin binding growth factors and cytokines (21, 22), sulfate groups of heparin are of importance for Tat binding. Indeed, high affinity Tat-heparin interaction requires at least some 2-*O*, 6-*O*, and *N* positions to be sulfated (18). These sulfate groups bind to a stretch of positively charged amino acid residues present in the basic domain of the Tat protein (19). Also, the size of heparin is of

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[1] The abbreviations used are: HIV-I, human immunodeficiency virus type I; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified minimal essential medium; FCS, fetal calf serum; FGF-2, fibroblast growth factor-2; GAG, glycosaminoglycan; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HGF, hepatocyte growth factor; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; TBS, Tris-buffered saline; LTR, long terminal repeat.

[2] M. Giacca, unpublished observations.

importance in mediating Tat interaction, because very low molecular weight heparin shows a poor capacity to bind and antagonize the biological activity of extracellular Tat when compared with conventional heparin (18).

The minimum size of the heparin/HS chain required for protein interaction has been identified for different growth factors. For instance, a hexa-octasaccharide represents the minimum size of HS required for the binding to hepatocyte growth factor (HGF) (23, 24) and platelet-derived growth factor (25), whereas the minimum binding sequence for fibroblast growth factor-2 (FGF-2) has been identify as a pentasaccharide (26, 27). In contrast, larger HS fragments (ranging from 18 to 20 saccharide residues) are required for binding to the platelet factor-4 tetramer (28) and interleukin-8 dimer (29).

The minimum binding sequence of heparin/HS able to interact with the growth factor may be insufficient to promote its biological activity. Indeed, the minimum heparin/HS sequence able to affect the biological activity of FGF-2 is composed of at least 10–12 saccharide residues (30–33). Moreover, a correlation exists between the length of the FGF-2-binding oligosaccharides and their capacity to restore FGF-2-induced proliferation in HSPG-deprived endothelial cells (31). The same correlation exists between the size of HGF-binding sulfated oligosaccharides and their capacity to potentiate the mitogenic activity of the growth factor (34). A possible explanation of these findings is based on the capacity of larger GAG chains to bind several growth factor molecules. For instance, a single molecule of conventional heparin binds up to 10 molecules of FGF-2 (35), thus inducing growth factor oligomerization (30, 36). Also, heparin promotes dimerization of HGF (34, 37) and interleukin-8 (38).

In this present study we investigated the capacity of size-defined, heparin-derived oligosaccharides to bind to Tat protein and to affect the biological activity of extracellular Tat. The results demonstrate that six saccharide residues represent the minimum requirement for Tat interaction. The apparent affinity of binding increases with increasing the molecular size of the heparin chain, with large fragments (≥18 saccharides) approaching the binding capacity and antagonist activity of full-size heparin. Also, depending on its size and relative concentration, one heparin chain can bind up to 4–6 molecules of Tat protein.

EXPERIMENTAL PROCEDURES

Preparation and Radiolabeling of Heparin Oligosaccharides—Even-numbered heparin-derived oligosaccharides were prepared from bovine lung heparin and ^{3}H-labeled as described previously (29). Briefly, the polysaccharides were depolymerized with nitrous acid at pH 1.5. Cleavage products were reduced either by N_2BH_4 to prepare nonlabeled fragments or by $NaB[^3H]_4$ to radiolabel them, and size-separated by gel filtration on Bio-Gel P-10 (29). The specific activity of the radiolabeled fragments reached 1×10^6 dpm/μmol fragment.

Recombinant HIV-I Tat—Recombinant HIV-I Tat was expressed in *Escherichia coli* as glutathione *S*-transferase (GST) fusion protein. The corresponding plasmid construct derives from pGST-Tat 2E, originally obtained by cloning the coding region of both exons of HIV-IHXB2 Tat in the commercial vector pGEX2T (39). This construct codes for the 86-amino acid Tat protein. Recombinant GST-Tat was also fused at its C terminus to the green fluorescent protein (GFP). To this purpose, the enhanced GFP coding region was amplified by polymerase chain reactions from the commercial vector pEGFP-N1 (CLONTECH, Palo Alto, CA) using primers 5′ GTGGAATTCATGGTGAGCAAGGGCGAGGAG and 3′ ATTTCGCCGGCGCTGAGATCTGGGCCCGCG, carrying *Eco*RI- and *Sma*I-cleavable sites, respectively. The amplified product was digested with the two restriction enzymes, gel-purified, and ligated in frame to the respective sites in pGST-Tat 2E.

Purification of recombinant GST-Tat and GST-Tat-GFP proteins was performed by glutathione-agarose affinity chromatography as described (39, 40). The purity (>95%) and integrity of the proteins were routinely checked by SDS-polyacrylamide gel electrophoresis and silver staining. Previous studies have shown that the GST and GFP moieties do not interfere with the LTR-transactivating activity of Tat or with its capacity to bind heparin (Ref. 18 and data not shown).

Recombinant GST-Tat was labeled with ^{125}I (17 Ci/mg, NEN Life Science Products) using Iodogen (Pierce) to a specific radioactivity of 400 cpm/fmol as described previously (19).

Immobilization of GST-Tat to Glutathione-Agarose Beads and [^{3}H]-Heparin Binding Assay—Aliquots (400 μl) of glutathione-agarose beads were mixed with 7 nmol of recombinant GST-Tat. After incubation at 4 °C, beads were extensively washed, resuspended in 25 mM Tris-HCl, pH 7.5, containing 150 mM NaCl (TBS), and stored at 4 °C until use. Under these conditions, up to 90% of the protein binds to the resin. The capacity of full-size heparin and oligosaccharides to bind to immobilized Tat was evaluated as described previously (19). Briefly, 110 pmol of ^{3}H-labeled saccharides were loaded onto 80-μl columns containing 1.3 nmol of GST-Tat protein and allowed to recycle inside the column for 15 min at 4 °C at a flow rate of 0.3 ml/min by means of a peristaltic minipump. Preliminary experiments had shown that 15 min of recycling were sufficient to reach equilibrium for the binding of ^{3}H-labeled saccharide to immobilized Tat.[3] Then, the column was extensively washed with TBS, and bound radioactivity was eluted in a single step with a 2.0 M NaCl wash, or it was eluted stepwise with increasing concentrations of NaCl, all in TBS. Radioactivity in the different fractions was measured in a liquid scintillation counter. To evaluate the dissociation constant (K_d) of the interaction of GST-Tat with full-size and 22- and 6-mer heparin, the column was loaded with increasing concentrations of the ^{3}H-labeled saccharides that were allowed to reach equilibrium with immobilized Tat as described above. Bound radioactivity was eluted with a 2.0 M NaCl wash, and binding data were then analyzed according to the procedure originally described by Scatchard (41).

Binding of Heparin Oligosaccharide to Free GST-Tat—To evaluate the capacity of ^{3}H-labeled heparin oligosaccharides to interact with GST-Tat in solution, the method described by Maccarana *et al.* (26) was used with minor modifications. Aliquots of GST-Tat were incubated for 20 min at room temperature with different amounts of ^{3}H-labeled heparin oligosaccharides in a final volume of 200 μl in TBS. Samples were then filtered through 25-mm filters (Polyscreen polyvinylidene difluoride membrane, NEN Life Science Products) equilibrated with TBS and placed onto a Sartorius filtration apparatus. Filters were washed twice with 5 ml of TBS, and retained radioactivity was eluted by washing the filter with 2 ml of 2.0 M NaCl in TBS. The eluate was mixed to 2 ml of water and 16 ml of Ready Safe liquid scintillation mixture (Beckman) and counted in a liquid scintillation counter. Control experiments performed using ^{125}I-GST-Tat as a tracer demonstrated that this procedure allows the recovery of up to 97% of the protein both in the absence or presence of heparin.

Cell Cultures—HL3T1 cells are derived from HeLa cells and contain integrated copies of pL3CAT, a plasmid in which the bacterial gene for chloramphenicol acetyltransferase (CAT) is directed by the HIV-I LTR (42).

T53 Tat-less cells were obtained by subcloning the T53 cell line originally established from adenocarcinoma of skin adnexa of Tat-transgenic mice (9, 43). Dot blot analysis of the conditioned medium of T53 Tat-less cells performed with anti-Tat antibodies revealed that this clone does not produce detectable amounts of extracellular Tat when compared with parental T53 cells (data not shown). Nevertheless, T53 Tat-less cells retain the capacity to proliferate when exposed to extracellular Tat (see below). All cells were grown and maintained in Dulbecco's modified minimal essential medium (DMEM) with 10% fetal calf serum (FCS) (Life Technologies, Inc.).

Cell Internalization of GST-Tat—Cell internalization of GST-Tat was studied by using radiolabeled ^{125}I-GST-Tat and fluorescent GST-Tat-GFP. In the first set of experiments, HL3T1 cells were seeded in 24-well dishes at the density of 45,000 cells/cm^2 in DMEM containing 10% FCS. After 24 h, cell cultures were washed twice with TBS and incubated in binding medium (serum-free medium containing 0.15% gelatin and 20 mM Hepes buffer, pH 7.5) complemented with 20 ng/ml of ^{125}I-GST-Tat plus 200 ng/ml of unlabeled GST-Tat as a carrier in the absence or presence of the different oligosaccharides. 100 μM chloroquine was added to cell cultures to prevent lysosomal degradation of cell-internalized Tat. After 16 h at 37 °C, the cells were washed three times with cold TBS 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.

[3] M. Rusnati, unpublished observations.

Under these experimental conditions, up to 90% of the radioactivity remains associated to the cell after a wash with 2.0 M NaCl in sodium acetate, pH 4.0 (45), thus demonstrating the intracellular localization of cell-associated ^{125}I.

In a second set of experiments, glass coverslips (10 mm in diameter) were immersed in 65% HNO_3 for 1 h, washed with distilled water, immersed in 7% NaOH for one more hour, washed with distilled water again, and dried. Coverslips were then placed within 24-well tissue culture plates, and HL3T1 cells were seeded at 20,000 cells/cm^2 and allowed to adhere onto glass coverslips. Adherent cells were then incubated for 6 h at 37 °C in DMEM containing 10% FCS in the presence of 400 ng/ml of GST-Tat-GFP in the absence or presence of the different oligosaccharides. 100 μM chloroquine was added to cell cultures to prevent lysosomal degradation of internalized Tat. At the end of incubation, the excess of GST-Tat-GFP was removed, and cell cultures were washed twice with 2.0 M NaCl in phosphate-buffered saline to remove GST-Tat-GFP adsorbed onto the cell surface. Then cells were fixed by a 5-min incubation with 3% paraformaldehyde in phosphate-buffered saline containing 2% sucrose. Observations were carried out under a Nikon photomicroscope equipped for epifluorescence.

LTR-CAT-transactivating Activity Assay—HL3T1 cells were seeded in 24-well dishes at the density of 20,000 cells/cm^2 in DMEM containing 10% FCS. After 24 h, cell cultures were washed twice with TBS and incubated for another 24 h in fresh medium containing 10% FCS and 100 μM chloroquine in the absence or presence of recombinant GST-Tat (200 ng/ml) and of increasing concentrations of the oligosaccharide under test. After 24 h the medium was changed to DMEM with 10% FCS, and cells were incubated for another 24 h. At the end of the 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.

T53 Tat-less Cell Proliferation Assay—T53 Tat-less cells were seeded in 96-well dishes at 15,000 cells/cm^2 in DMEM containing 10% FCS. After 24 h, subconfluent cultures were washed twice with DMEM and treated with fresh medium containing 10% FCS and 100 ng/ml of GST-Tat in the absence or presence of different heparin-derived oligosaccharides or full-length heparin. After 24 h of incubation at 37 °C, cells were trypsinized and counted in a Burker chamber.

RESULTS

Heparin Oligosaccharide Binding to Immobilized Tat—To identify the minimum size of heparin required for Tat interaction, heparin-derived ^3H-labeled oligosaccharides of defined size were evaluated for their capacity to bind to GST-Tat immobilized onto glutathione-agarose beads. This experimental model had already been used to characterize the binding of Tat to heparin and different polysulfated/polysulfonated compounds (18, 19). To this purpose, 110 pmol of the different even-numbered ^3H-labeled heparin fragments were loaded onto 80-μl columns containing a molar excess (1.3 nmol) of immobilized GST-Tat. As described under "Experimental Procedures," the samples were allowed to reach equilibrium within the column at 4 °C and then, after extensive washing under physiological buffer conditions, bound radioactivity was eluted with a 2.0 M NaCl wash and measured in a liquid scintillation counter. Full-size [^{3}H]heparin (average M_r = 11,000, corresponding to approximately 32 saccharide units) was used as a control (18). Under these experimental conditions, more than 80% of the total amount of loaded 6-mer heparin and larger oligosaccharides bind to immobilized Tat (Fig. 1). In contrast, 4-mer heparin shows a very limited specific interaction with the protein. The specificity of the binding was demonstrated by the capacity of a 10-fold molar excess of unlabeled full-size heparin to inhibit the binding of ^3H oligomers (data not shown) and of full-size [^{3}H]heparin (Fig. 1) to immobilized GST-Tat and by the incapacity of full-size [^{3}H]heparin to interact with immobilized GST devoid of the Tat moiety (18).

We then evaluated the relative affinity of the different heparin oligosaccharides for immobilized GST-Tat. To this purpose, subsaturating amounts of ^3H-labeled full-size and 22-, 12-, 6-, and 4-mer heparin (all at 110 pmol per sample) were loaded onto 80-μl columns containing 1.3 nmol of immobilized GST-Tat protein and were allowed to reach equilibrium at 4 °C. After extensive washing with TBS, the columns were stepwise eluted with increasing concentrations of NaCl in TBS, and radioactivity was measured in the different fractions. Again, all the heparin oligosaccharides fully bind to immobilized Tat with the only exception being 4-mer heparin, which is mostly recovered in the flow-through of the column and in the first 0.2 M NaCl wash (Fig. 2). The ionic strength necessary to disrupt Tat-heparin interaction increases with increasing the size of the oligosaccharide and 6-, 12-, 22-mer-, and full-size heparin eluting from the column at 0.4, 0.6, 1.0, and 1.2 M NaCl, respectively (Fig. 2).

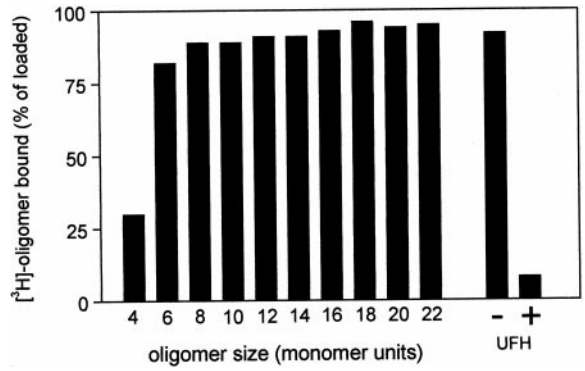

FIG. 1. **Binding of [^{3}H]heparin oligosaccharides to immobilized GST-Tat.** ^3H-Labeled even-numbered heparin fragments were loaded at 110 pmol/sample onto 80-μl glutathione-agarose columns containing 1.3 nmol of immobilized GST-Tat and were allowed to reach equilibrium at 4 °C. After extensive washing under physiological buffer conditions, the radioactivity bound to the column was eluted with a 2.0 M NaCl wash and measured in a liquid scintillation counter. Full-size [^{3}H]heparin (*UFH*) in the absence (−) or presence (+) of a 10-fold molar excess of unlabeled heparin was used as positive and negative control, respectively. Data are expressed as percents of the radioactivity bound to the column in respect to the radioactivity originally loaded. The experiment is representative of two independent experiments with similar results.

Taken together, the data indicate that 5–6 saccharide residues represent the minimum requirement for a significant Tat interaction under physiological buffer conditions. Also, they demonstrate that the affinity of heparin-Tat interaction strictly depends upon the size of the heparin oligomer and raise the possibility that, in the presence of a molar excess of GST-Tat, a saccharide chain of appropriate length can set up multiple interactions with more than one molecule of Tat, thus increasing the strength of the interaction.

To further investigate this possibility, we evaluated the ionic strength required to disrupt heparin-Tat interaction when subsaturating and saturating amounts of full-size [^{3}H]heparin (0.04 and 3.10 nmol, respectively) were loaded onto 80-μl glutathione-agarose columns containing 1.3 nmol of immobilized GST-Tat. In agreement with the data shown in Fig. 2*A*, under subsaturating conditions heparin elutes from immobilized GST-Tat at high ionic strength (1.2 M NaCl, Fig. 3*A*). In contrast, when heparin binds to GST-Tat under saturating conditions, it elutes from the column at a much lower ionic strength (0.4–0.6 M NaCl, Fig. 3*B*). These data are in keeping with the hypothesis that subsaturating concentrations of heparin may favor the binding of a single saccharide chain with several Tat molecules, leading to a strong interaction. In contrast, a weaker interaction occurs when an excess of heparin chains compete for the binding to a limited number of GST-Tat molecules, possibly leading to the formation of equimolar heparin-GST-Tat complexes.

To directly demonstrate the possibility that a single heparin chain of appropriate size can establish multiple interactions

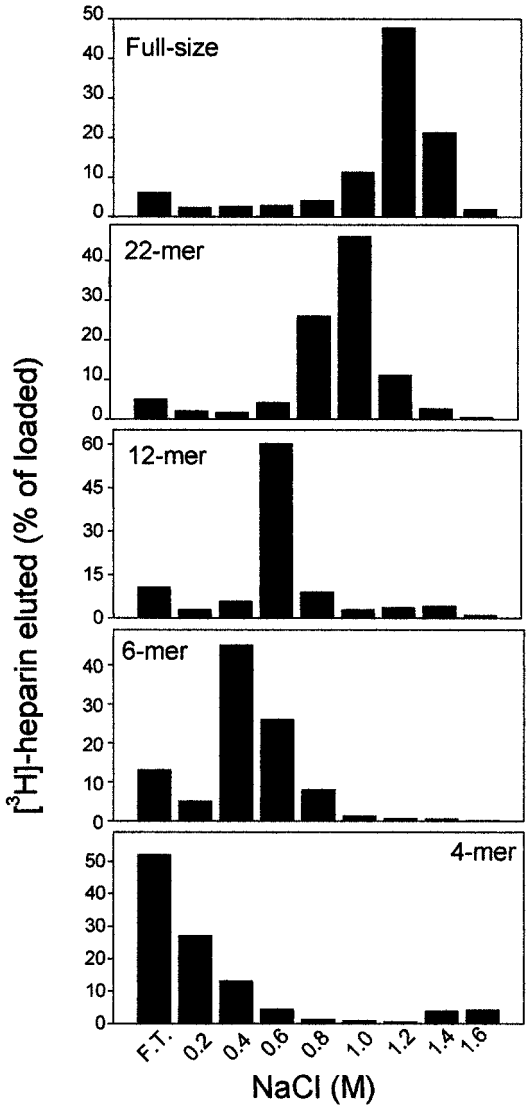

FIG. 2. **Affinity chromatography of [^{3}H]heparin oligosaccharides on immobilized GST-Tat.** Full-size and 22-, 12-, 6-, and 4-mer [^{3}H]heparin fragments were loaded at 110 pmol/sample onto 80-μl glutathione-agarose columns containing 1.3 nmol of immobilized GST-Tat and were allowed to reach equilibrium at 4 °C. After extensive washing under physiological buffer conditions, columns were stepwise eluted with increasing concentrations of NaCl. Radioactivity in the eluted fractions was measured in a liquid scintillation counter. The experiments shown are representative of two to three independent experiments with similar results. *F.T.*, flow-through of the column.

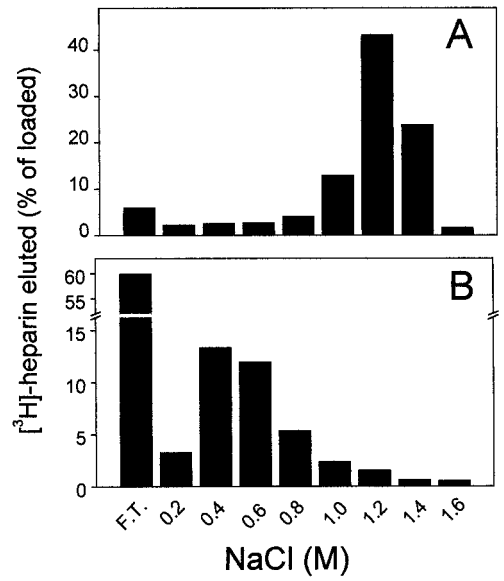

FIG. 3. **Effect of the concentration of heparin on its interaction with immobilized GST-Tat.** Full-size [^{3}H]heparin was loaded at 0.04 nmol/sample (*A*) or at 3.1 nmol/sample (*B*) onto 80-μl glutathione-agarose columns containing 1.3 nmol of immobilized GST-Tat and was allowed to reach equilibrium at 4 °C. After extensive washing under physiological buffer conditions, columns were stepwise eluted with increasing concentrations of NaCl. Radioactivity in the eluted fractions was measured in a liquid scintillation counter. The experiments were repeated twice with similar results. *F.T.*, flow-through of the column.

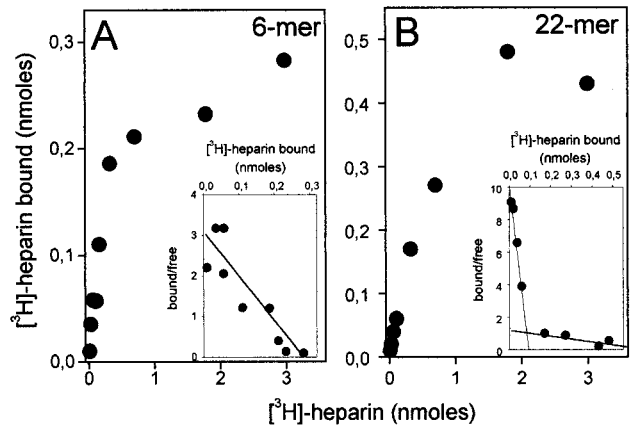

FIG. 4. **Scatchard plot analysis of the binding of 6- and 22-mer [^{3}H]heparin to immobilized GST-Tat.** Increasing concentrations of 6-mer [^{3}H]heparin (*A*) or of 22-mer [^{3}H]heparin (*B*) were loaded onto 80-μl glutathione-agarose columns containing 0.4 nmol of immobilized GST-Tat and were allowed to reach equilibrium at 4 °C. After extensive washing under physiological buffer conditions, radioactivity bound to the column was eluted with a 2.0 M NaCl wash, measured in a liquid scintillation counter, and expressed as nanomoles of heparin molecules bound to immobilized GST-Tat. The *insets* show the Scatchard plot interpolations of the binding data. The experiments were repeated two to three times with similar results.

with numerous GST-Tat molecules, we calculated the molar ratio and K_d of the interaction of immobilized GST-Tat with 6-mer heparin and 22-mer heparin (19). For this purpose, increasing amounts (from subsaturating to saturating concentrations) of the two ^{3}H-labeled oligomers were loaded onto glutathione-agarose columns containing 0.4 nmol of immobilized GST-Tat and were allowed to reach binding equilibrium with immobilized Tat. After extensive washing, bound radioactivity was eluted with a 2.0 M NaCl wash. As shown in Fig. 4, the binding of 6-mer [^{3}H]heparin fragments and of 22-mer [^{3}H]heparin fragments to immobilized GST-Tat is dose-dependent and saturable. Scatchard plot analysis of the binding data reveals a single component binding for 6-mer [^{3}H]heparin-Tat interaction with a K_d equal to 0.7 ± 0.4 μM and a molar oligosaccharide:GST-Tat ratio of about 1:1 (Fig. 4*A*, *inset*). In contrast, the binding of 22-mer [^{3}H]heparin to immobilized GST-Tat is consistent with a two-component binding curve (Fig. 4*B*, *inset*). A high affinity binding (K_d = 30 ± 14 nM) occurs at subsaturating concentrations of 22-mer [^{3}H]heparin with a molar oligosaccharide:GST-Tat ratio equal to approximately 1:4. A low affinity binding (K_d = 1.6 ± 0.4 μM) occurs instead at saturating concentrations of 22-mer [^{3}H]heparin with a molar ratio equal to approximately 1:1. A biphasic interaction was also obtained for the binding of full-size [^{3}H]heparin to immobilized GST-Tat. At subsaturating concentrations, full-size heparin binds to immobilized GST-Tat with a high affinity interaction (K_d = 73 ± 14 nM) and a molar ratio equal to approximately 1:3. Full-size heparin binds instead with a low affinity interaction (K_d = 0.9 ± 0.4 μM) and a 1:1

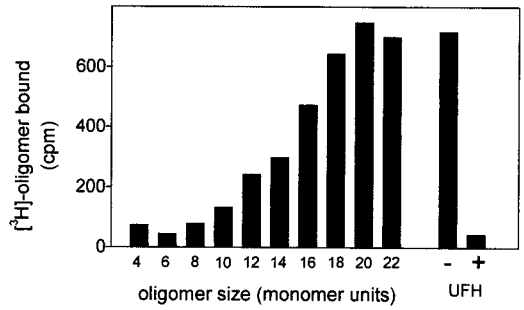

FIG. 5. **Heparin oligosaccharide binding to GST-Tat in solution.** ^{3}H-Labeled even-numbered heparin fragments (110 pmol/sample) were incubated for 20 min at room temperature with 110 pmol of GST-Tat in a final volume of 200 μl of phosphate-buffered saline. At the end of incubation, samples were applied to polyvinylidene difluoride membrane under suction. The protein, together with any retained radioactivity, was recovered from the membrane and measured in a liquid scintillation counter. Full-size [^{3}H]heparin (*UFH*) in the absence (-) or presence (+) of a 10-fold molar excess of unlabeled heparin was used as positive and negative control, respectively. The experiment is representative of three independent experiments with similar results.

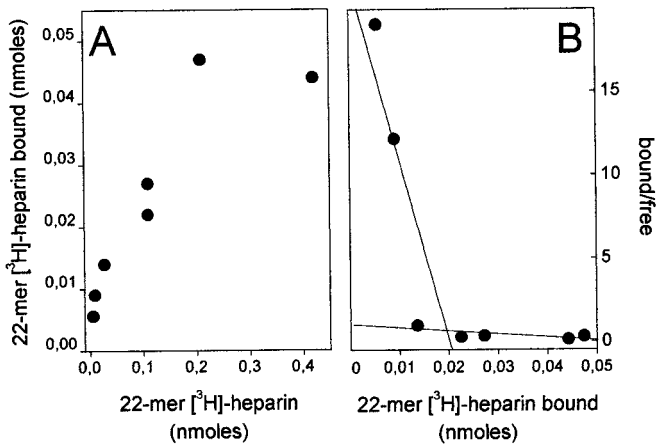

FIG. 6. **Scatchard plot analysis of the interaction between 22-mer [^{3}H]heparin and free GST-Tat.** *A*, the binding of increasing concentrations of 22-mer [^{3}H]heparin to 110 pmol of free GST-Tat was evaluated as described in the legend to Fig. 5. *B*, Scatchard plot analysis of the binding data. The experiments were repeated twice with similar results.

molar ratio at saturating concentrations (data not shown). Therefore, at low ratios of 22-mer or full-size heparin to Tat, approximately 3–4 molecules of the protein bind per GAG molecule with high affinity, whereas at higher heparin concentrations only 1 Tat molecule binds heparin at a much lower affinity. Accordingly, short heparin oligosaccharides (6-mer) can establish only a low affinity, equimolar interaction with Tat protein.

Heparin Oligosaccharide Binding to Free Tat—When tested for biological activity, GST-Tat is administered as a free molecule (see below). On this basis, the capacity of the different heparin oligosaccharides to interact with GST-Tat was also investigated in solution by using a filter binding assay (26). Experiments were performed in the presence of equimolar amounts of heparin fragments in respect to free Tat to emphasize their different affinity for the protein. To this purpose, 110 pmol of free GST-Tat were incubated for 20 min at room temperature with 110 pmol of heparin ^{3}H-oligomers in a final volume of 200 μl of TBS. At the end of incubation, ^{3}H-oligomer-GST-Tat complexes were recovered by a passage through a nitrocellulose membrane, and protein-bound radioactivity was measured (Fig. 5). Under these experimental conditions, 18-mer heparin represents the smallest oligosaccharide able to retain a Tat-binding capacity similar to that shown by full-size heparin. A decrease of the size of the oligosaccharide results in a proportional decrease of its Tat-binding capacity, with no significant binding being observed for ≤8-mer oligosaccharides. In agreement with the data obtained with immobilized GST-Tat (see Fig. 2), these findings demonstrate that the affinity of heparin fragment-GST-Tat interaction increases with increasing the size of the oligosaccharide.

To assess whether large heparin fragments retain the capacity to complex numerous Tat molecules when the protein is presented in a free form to the GAG, increasing amounts of 22-mer [^{3}H]heparin were incubated for 20 min at room temperature with 110 pmol of free GST-Tat in a final volume of 200 μl of TBS. At the end of incubation, 22-mer [^{3}H]heparin-GST-Tat complexes were recovered by a passage through a nitrocellulose membrane, and radioactivity was measured. As shown in Fig. 6*A*, 22-mer [^{3}H]heparin binds free GST-Tat in a dose-dependent, saturable manner. Also in this case, Scatchard plot analysis of the binding data reveals a biphasic binding (Fig. 6*B*). As observed for immobilized GST-Tat, a high affinity binding (K_d = 5 ± 3 nM) occurs at subsaturating concentrations of 22-mer [^{3}H]heparin with a molar oligosaccharide:GST-Tat ratio equal to approximately 1:6. In contrast, a low affinity binding (K_d = 0.3 ± 0.1 μM) with a molar ratio equal to approximately 1:1 occurs at saturating concentrations of 22-mer [^{3}H]heparin.

Effect of Heparin Oligosaccharides on the Biological Activity of Extracellular Tat—Heparin oligosaccharides were evaluated for their capacity to inhibit the cellular uptake and the HIV-I-LTR-transactivating activity of extracellular Tat in HL3T1 cells. These cells are derived from HeLa cells and contain integrated copies of pL3CAT, a plasmid in which the bacterial *cat* gene is directed by the HIV-I LTR (42). Full-size heparin has been demonstrated to inhibit both cell internalization and LTR-transactivating activity of extracellular Tat in this experimental model (19). In a first series of experiments, subconfluent cultures of HL3T1 were incubated for 16 h at 37 °C with 20 ng/ml of ^{125}I-GST-Tat complemented with 200 ng/ml of unlabeled GST-Tat as a carrier, in the absence or presence of increasing concentrations of 6-mer heparin or of full-size heparin. At the end of incubation, free and cell surface-associated ^{125}I-GST-Tat was removed, cells were lysed, and the amount of intracellular radioactivity was measured. As shown in Fig. 7*A*, full-size heparin inhibits the internalization of ^{125}I-GST-Tat in a dose-dependent manner (ID_{50} ≥ 10 nM), whereas 6-mer heparin is barely effective (ID_{50} ≥ 10 μM). When all the oligosaccharides available were tested in the same assay at the dose of 1.8 μM, the results shown in Fig. 7*B* were obtained. Clearly, the ability of the different oligosaccharides to inhibit ^{125}I-GST-Tat internalization in HL3T1 cells is directly related to their size, with the smallest oligosaccharides (≤8-mer) being almost ineffective.

To confirm these observations, the effect of heparin oligosaccharides on the internalization of fluorescent GST-Tat-GFP was evaluated. Preliminary experiments had shown that the GFP moiety does not significantly affect the heparin-binding capacity, cellular uptake, or LTR-transactivating activity of Tat.[3] On this basis, subconfluent cultures of HL3T1 cells were incubated for 6 h at 37 °C with 400 ng/ml of GST-Tat-GFP in the absence or presence of 1.8 μM 4- or 22-mer heparin. At the end of incubation, free and cell surface-associated GST-Tat-GFP were removed, and cells were photographed under a microscope equipped for epifluorescence. In control cells, internalized GST-Tat-GFP accumulates in punctuated structures corresponding to cell lysosomes (Fig. 7*C*, *panel a*). Addition of 22-mer heparin completely inhibits the internalization of GST-

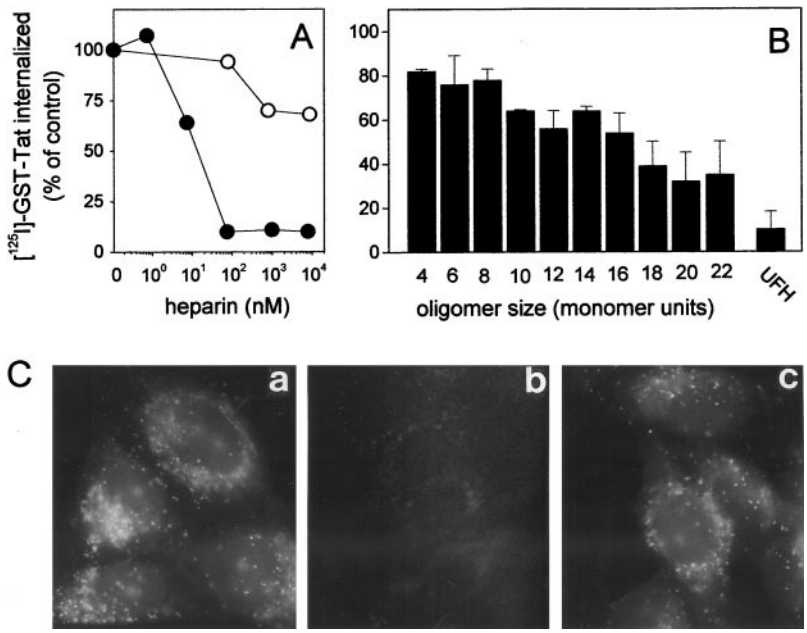

FIG. 7. **Effect of heparin oligosaccharides on cell internalization of extracellular GST-Tat.** HL3T1 cells were treated with 20 ng/ml of ^{125}I-GST-Tat plus 200 ng/ml of unlabeled protein as a carrier in the absence or presence of increasing concentrations of 6-mer- (○) or full-size (●) heparin (*A*) or with 1.8 μM amounts of the various even-numbered heparin fragments (*B*). After 16 h of incubation at 37 °C, the medium was removed, cells were lysed, and radioactivity was measured in the cell extract. Nonspecific radioactivity was evaluated by incubating the cells at 4 °C in the presence of 20 ng/ml ^{125}I-GST-Tat and a 200-fold molar excess of unlabeled GST-Tat (4 μg/ml) and was subtracted from each experimental point. In *A*, each point is the mean of one to six determinations in duplicate, and S.E. never exceeded 11% of the mean value. In *B*, each point is the mean ± S.E. of one to three determinations in duplicate. *C,* HL3T1 cells were treated with 400 ng/ml GST-Tat-GFP alone (*panel a*) or added with 1.8 μM 22-mer- (*panel b*) or 6-mer heparin (*panel c*). After 6 h of incubation at 37 °C, free and cell surface-associated GST-Tat-GFP was removed, and cells were fixed and photographed under a microscope equipped for epifluorescence. *UFH*, full-size [^{3}H]heparin.

Tat-GFP (Fig. 7*C*, *panel b*), whereas 4-mer heparin is ineffective (Fig. 7*C*, *panel c*).

Heparin-derived oligosaccharides were then evaluated for their capacity to inhibit the LTR-transactivating activity of extracellular Tat. To this purpose, subconfluent cultures of HL3T1 cells were incubated with 200 ng/ml of GST-Tat in the presence of increasing concentrations of 6- and 14-mer- or full-size heparin. At the end of the incubation, the amount of intracellular CAT protein, proportional to the LTR-transactivating activity exerted by GST-Tat, was measured by a CAT enzyme-linked immunosorbent assay kit. As shown in Fig. 8*A*, the different heparin molecules inhibit the LTR-transactivating activity of GST-Tat in a dose-dependent manner with ID_{50} equal to approximately 100, 1.0, and 0.1 nM for 6- and 14-mer- and full-size heparin, respectively. In agreement with these observations, when all the oligosaccharides available were tested at 70 nM, the capacity of the different heparin oligomers to inhibit the LTR-transactivating activity of Tat was directly related to their size, with 4-mer heparin being ineffective and 18-mer heparin being the smallest fragment that retains an inhibitory potency similar to that shown by full-size heparin (Fig. 8*B*).

Heparin-derived oligosaccharides were also evaluated for their capacity to inhibit the mitogenic activity exerted by extracellular Tat on T53 Tat-less cells. These cells were obtained by subcloning the Tat-producing T53 cell line originally established from adenocarcinoma of skin adnexa of Tat-transgenic mice (43, 9). T53 Tat-less cells do not produce significant amounts of extracellular Tat when compared with parental T53 cells (see "Experimental Procedures"). When incubated for 24 h in 10% FCS in the absence or presence of 100 ng/ml of GST-Tat, subconfluent cultures of T53 Tat-less cells undergo 0.8 and 1.8 cell population doublings, respectively, thus indicating that extracellular Tat is able to induce proliferation of these cells. Full-size heparin inhibits the mitogenic activity of GST-Tat in a dose-dependent manner with an ID_{50} equal to 0.1 nM. In contrast, 6-mer heparin does not affect the mitogenic activity of GST-Tat when tested at 1 μM (Fig. 9*A*). Also in this experimental model, the antagonist activity of the heparin oligosaccharides was directly related to their size, with 20–22 saccharide residues being the minimum size required to elicit an inhibitory activity similar to that exerted by full-size heparin (Fig. 9*B*).

DISCUSSION

HIV-I Tat, originally viewed as a viral transactivating factor with an intracellular mechanism of action, can also act as an extracellular growth factor endowed with heparin-binding capacity. The interaction of Tat with heparin modulates various biological effects exerted by extracellular Tat on target cells (13, 18, 19).

Here, by using a series of size-defined heparin fragments, we have observed that 5–6 saccharide residues represent the minimum size required for a significant interaction with Tat protein under physiological buffer conditions. Affinity chromatography on immobilized GST-Tat followed by Scatchard plot analysis of the binding data has shown that 1 molecule of 6-mer heparin binds 1 molecule of immobilized GST-Tat with low affinity (K_d equal to approximately 0.7 μM). Accordingly, the interaction is disrupted at relatively low ionic strength (0.4 M NaCl). It must be pointed out that all the hexasaccharide fragments of the 6-mer heparin population bind Tat when the protein is present in a molar excess in respect to the heparin oligosaccharide. This indicates that all the fragments contain Tat-binding sequence(s). Previous studies had shown that Tat-heparin interaction requires at least some 2-*O*-, 6-*O*-, and *N*-positions to be sulfated (18). Disaccharide units containing 2-*O*-, 6-*O*-, and *N*-sulfate groups are predominantly present in heparin (21), even though specific factor binding sequences, possibly present in HS, may be hidden in heparin because of its

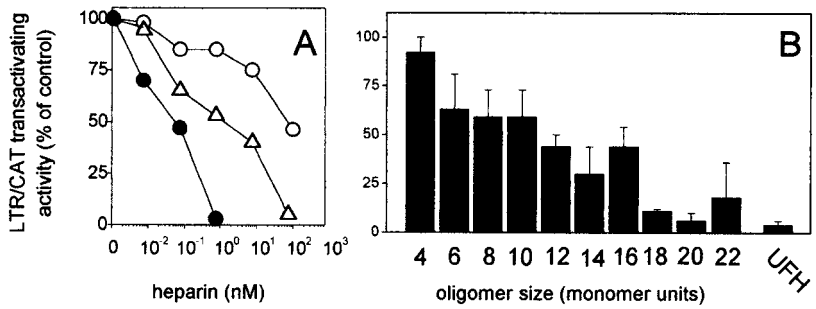

FIG. 8. **Effect of heparin oligosaccharides on LTR-transactivating activity of extracellular GST-Tat.** HL3T1 cells were treated with 200 ng/ml GST-Tat in the absence or presence of increasing concentrations of 6-mer (○), 14-mer (△), or full-size heparin (●) (*A*) or with 70 nM amounts of the various even-numbered heparin fragments (*B*). After 48 h of incubation at 37 °C, cell extracts were assayed for the levels of CAT antigen by enzyme-linked immunosorbent assay. Data are expressed as percents of the LTR-transactivating activity measured in control cultures treated with GST-Tat alone. In *A*, each point is the mean of two to three determinations in duplicate, and S.E. never exceeded 15% of the mean value. In *B*, each point is the mean ± S.E. of three determinations in duplicate. *UFH*, full-size heparin.

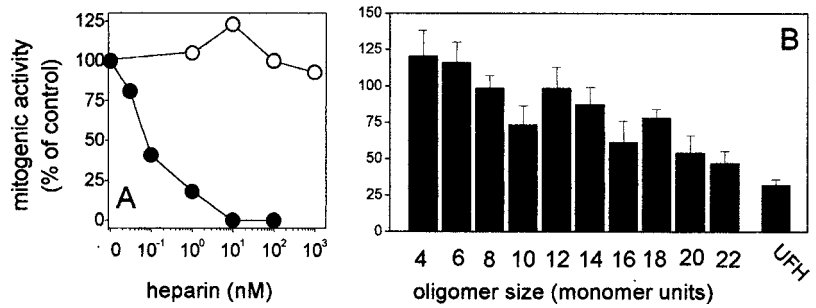

FIG. 9. **Effect of heparin oligosaccharides on the mitogenic activity of extracellular GST-Tat.** T53 Tat-less cells were incubated in DMEM complemented with 10% FCS and treated with 100 ng/ml GST-Tat in the absence or presence of increasing concentrations of 6-mer (○) or full-size heparin (●) (*A*) or with 1.0 nM amounts of the various even-numbered heparin fragments (*B*). After 24 h of incubation at 37 °C, cell were trypsinized and counted. Data are expressed as percents of the increase of cell proliferation observed in cell cultures treated with GST-Tat alone in respect to untreated control cultures. When incubated for 24 h in 10% FCS in the absence or presence of 100 ng/ml of GST-Tat, subconfluent cultures of T53 Tat-less cells undergo 0.8 and 1.8 cell population doublings, respectively. *UFH*, full-size heparin.

high degree of sulfation.

A low affinity interaction was also observed when longer heparin saccharide chains (22-mer and full-size heparin) were allowed to interact with Tat at a high ratio of heparin to immobilized protein. Indeed, when only one molecule of immobilized Tat binds one GAG chain, the interaction has a K_d equal to approximately 0.9–1.6 μM and is disrupted at 0.4 M NaCl. However, a significant increase of the affinity of the binding was observed when subsaturating concentrations of 22-mer or full-size heparin are presented to an excess of Tat protein. Under these experimental conditions, 3–4 molecules of immobilized Tat can bind a single 22-mer or full-size heparin chain. This causes a significant increase of the affinity of Tat-heparin interaction (with K_d values ranging from 30 to 70 nM), which is disrupted at NaCl concentrations ≥1.0 M. These data were confirmed by studying the interaction of 22-mer heparin with GST-Tat in solution. Also in this case, an increase from 1 to 6 of the number of GST-Tat molecules that bind to a single heparin chain causes a dramatic increase of the affinity of the interaction (K_d equal to 0.3 μM and 5 nM, respectively). These data suggest the possibility that Tat may establish a cooperative interaction with heparin in which the binding of the first Tat molecule facilitates the interaction of more Tat molecules to adjacent Tat-binding regions of the GAG chain. This may cause the formation of highly packed multimeric Tat-heparin complexes, as already observed for FGF-2-heparin complexes in which a single heparin chain (M_r = 15,000) can bind up to 6–7 molecules of the growth factor (46).

The possibility that Tat molecules can establish mutual interactions leading to Tat oligomerization has been advanced (47, 48). The heparin chain may therefore provide the appropriate scaffold to facilitate and stabilize this interaction, as already observed for different growth factors and cytokines, including FGF-2 (30, 36), HGF (37), platelet-derived growth factor (28), and midkine (49). All of these growth factors share the characteristic to bind to cognate tyrosine kinase receptors that require dimerization to signal inside the cell. On this basis, growth factor oligomerization mediated by cell-surface HSPGs may promote receptor dimerization, signaling, and biological activity (34, 50). Interestingly, Tat also binds to tyrosine kinase receptors, including Flt-1, responsible for the chemotactic activity exerted by Tat on monocyte/macrophages (11), and Flk-1/KDR, responsible for the chemotactic, mitogenic, and angiogenic activity exerted by Tat on endothelial cells (12). These observations raise the possibility that cell surface HSPGs may facilitate Tat oligomerization and that this may be of importance for the biological activity of extracellular Tat. Experiments are in progress in our laboratory to assess this hypothesis.

Our data show that heparin oligosaccharides exert a Tat-antagonist activity in different biological assays. As observed for polysulfated/polysulfonated compounds (19), heparin fragments inhibit cell internalization and LTR-transactivating activity of extracellular Tat in H3T1 cells and its mitogenic activity in T53 Tat-less cells. In agreement with the *in vitro* binding studies, the relative capacity of heparin oligomers to inhibit the activity of extracellular Tat in the different assays is a function of their size, with at least 18–20 saccharide residues being required to confer to the oligosaccharide chain an antagonist potency similar to full-size heparin. Interestingly, 6-mer heparin is unable to exert a significant antagonist activity when compared with full-size heparin. Indeed, the ID_{50} values for 6-mer heparin are at least 1,000 times higher than that of full-size heparin in all the biological assays. This lack of activity cannot be entirely explained by the lower affinity of 6-mer heparin for Tat (K_d = 0.7 μM) when compared with full-size

heparin (K_d = 70 nM), suggesting that different structural requirements and/or oligomerization-inducing ability are implicated in Tat interaction and antagonist activity of heparin. A dissociation between binding capacity and antagonist activity of heparin oligosaccharides had been already demonstrated for heparin-FGF-2 interaction in which a pentasaccharide represents the minimum FGF-2-binding sequence (26, 27), whereas at least 10 saccharide residues are required to modulate the biological activity of the growth factor (30–33).

Taken together, the data suggest that *in vivo* cell surface interaction and biological activity of extracellular Tat may depend strictly on the GAG milieu of the extracellular environment where free and cell-associated HSPGs, depending upon their relative concentration and size, compete for the binding to extracellular Tat, thus regulating its bioavailability and biological activity. Relevant to this point, it is interesting to note that the expression and release of HSPGs undergo significant changes during AIDS progression. For instance, a novel proteoglycan has been isolated in the urine from AIDS patients (51), and HSPGs are selectively expressed during the development of AIDS-associated Kaposi's sarcoma (52), a pathological condition in which Tat plays a key role by inducing neovascularization as well as cell proliferation and chemotaxis of Kaposi's sarcoma spindle cells (5, 53).

Extracellular Tat has been proposed as a target for pharmacological and immunological approaches in the therapy of AIDS and AIDS-associated pathologies (54). Besides their Tat-antagonist activity, heparin and polysulfated compounds can prevent HIV infection and cell-killing *in vitro* (55), suggesting the potential use of heparin-like molecules as "multi-target" compounds able to affect different aspects of HIV infection and AIDS-related disorders. A pilot clinical trial in patients with advanced AIDS has demonstrated the possibility of treating patients with low molecular weight heparin on a long term basis with no evidence of drug toxicity or bleeding episodes (44). Our results may help to design tailored Tat-antagonist oligosaccharides with a favorable therapeutic window.

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Multiple Interactions of HIV-I Tat Protein with Size-defined Heparin Oligosaccharides

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