THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 26, Issue of June 26, pp. 16027–16037, 1998

Printed in U.S.A.

The Basic Domain in HIV-1 Tat Protein as a Target for Polysulfonated Heparin-mimicking Extracellular Tat Antagonists*

(Received for publication, November 24, 1997, and in revised form, April 17, 1998)

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From the Department of Biomedical Sciences and Biotechnology, University of Brescia, 25123 Brescia, Italy, the ‡International Center for Genetic Engineering and Biotechnology, 34012 Trieste, Italy, §Pharmacia Upjohn, 20014 Nerviano, Milan, Italy, and the ¶Institute of Microbiology and Interdepartment Center for Biotechnology, University of Ferrara, 44100 Ferrara, Italy

Heparin binds extracellular HIV-1 Tat protein and modulates its HIV long terminal repeat (LTR)-transactivating activity (M. Rusnati, D. Coltrini, P. Oreste, G. Zoppetti, A. Albini, D. Noonan, F. d'Adda di Fagagna, M. Giacca, and M. Presta (1997) *J. Biol. Chem.* 272, 11313–11320). On this basis, the glutathione *S*-transferase (GST)-Tat$_{R49/52/53/55/56/57A}$ mutant, in which six arginine residues within the basic domain of Tat were mutagenized to alanine residues, was compared with GST-Tat for its capacity to bind immobilized heparin. Dissociation of the GST-Tat$_{R49/52/53/55/56/57A}$·heparin complex occurred at ionic strength significantly lower than that required to dissociate the GST-Tat·heparin complex. Accordingly, heparin binds immobilized GST-Tat and GST-Tat$_{R49/52/53/55/56/57A}$ with a dissociation constant equal to 0.3 and 1.0 μM, respectively. Also, the synthetic basic domain Tat-(41–60) competes with GST-Tat for heparin binding. Suramin inhibits [^{3}H]heparin/Tat interaction, ^{125}I-GST-Tat internalization, and the LTR-transactivating activity of extracellular Tat in HL3T1 cells and prevents ^{125}I-GST-Tat binding and cell proliferation in Tat-overexpressing T53 cells. The suramin derivative ^{14}C-PNU 145156E binds immobilized GST-Tat with a dissociation constant 5 times higher than heparin and is unable to bind GST-Tat$_{R49/52/53/55/56/57A}$. Although heparin was an antagonist more potent than suramin, modifications of the backbone structure in selected suramin derivatives originated Tat antagonists whose potency was close to that shown by heparin.

In conclusion, suramin derivatives bind the basic domain of Tat, prevent Tat/heparin and Tat/cell surface interactions, and inhibit the biological activity of extracellular Tat. Our data demonstrate that tailored polysulfonated compounds represent potent extracellular Tat inhibitors of possible therapeutic value.

tat is a viral regulatory gene of the human immunodeficiency virus type 1 (HIV-1),[1] the etiologic agent of AIDS (1, 2). *tat* is essential for viral replication, since the Tat protein promotes transcription of the viral genome by interacting with the transactivation responsive element located at the 5′-end of viral mRNAs (3–5). Tat can be released by HIV-infected cells (6), and significant levels of Tat have been detected in HIV-1-seropositive subjects (7). As an extracellular molecule, Tat exerts pleiotropic effects on uninfected cells. Extracellular Tat promotes the production of growth factors and cytokines (8–17) and of cytokine receptors (13, 18–20). Tat stimulates proliferation (10, 21–24), migration (21, 25, 26), and protease production (27–29) in different cell types. Extracellular Tat exerts an angiogenic activity *in vitro* (30) and *in vivo* (21, 28, 31). In the central nervous system, Tat released by HIV-infected macrophages can induce neurotoxicity by acting directly on neurons (32–36) or by stimulating different cell types to produce and release neurotoxic molecules (12, 37, 38). Finally, extracellular Tat transforms and immortalizes keratinocyte in culture (39). Thus, extracellular Tat exerts a wide spectrum of biological effects on several cell types, suggesting its implication in different AIDS-associated pathologies including Kaposi's sarcoma (27, 41), AIDS dementia (42), and the increased incidence of tumors in AIDS patients (28, 43).

The ability of extracellular Tat to induce peripheral blood mononucleated cell apoptosis (7, 44, 45) implicates the protein also in CD4+ cell depletion and in the progression of AIDS. Moreover, extracellular Tat appears to be responsible for the burst of virus replication that takes place at the beginning of HIV infection (46, 47). This possibility is supported by the observation that anti-Tat antibody causes a significant delay in HIV-1 replication in infected peripheral blood mononucleated cells (48–50) and that an inverse correlation exists between the levels of natural anti-Tat antibody and those of p24 antigen in HIV-seropositive subjects (48). Finally, Tat exerts negative effects on the immune system also by repressing the transcription of the major histocompatibility complex class I gene (51), by inhibiting antigen-induced lymphocyte proliferation and generation of functional suppressive CD8+ cells (52, 53), by impairing thymocyte development (54), and by inhibiting interferon-induced iNos activity in macrophages (55). Thus, from the bulk of data it emerges that extracellular Tat may be implicated both in the progression of AIDS and in the pathogenesis of several AIDS-associated diseases. On this basis, Tat protein should be considered as a target for novel anti-HIV strategies.

* This work was supported by "AIDS Project," Istituto Superiore di Sanità, Ministero della Sanità, Rome (to M. P.), by AIRC (Special Project Angiogenesis) (to M. P.), and by the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (60%) (to M. R. and M. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: HIV-1, human immunodeficiency virus type 1; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GAG, glycosaminoglycan; GST, glutathione *S*-transferase; HS, heparan sulfate; FGF, fibroblast growth factor; LTR, long terminal repeat; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin.

Tat protein is a polypeptide of 86–102 amino acids depending on the viral strain, which is encoded by two exons and is translated from multiply spliced 2-kilobase mRNAs (56). The amino acid sequence 1–72 encoded by the first exon is endowed with full transactivating activity (3, 4, 57), while the carboxyl-terminal region encoded by the second exon (amino acids 72–86) is not required either for the transactivating activity of Tat or for the replication of HIV-1 (5, 57). However, this latter region is necessary for different biological activities of Tat (29, 58–60) and contains an Arg-Gly-Asp (RGD) motif responsible for Tat binding to integrin receptors (61, 62).

Within the sequence encoded by the first exon of *tat* is present a so-called basic domain (amino acids 49–57) constituted by a stretch of repeated Arg and Lys residues. This highly immunoreactive region is well conserved among Tat proteins isolated from different strains of HIV-1 (47, 63). The basic domain is implicated in several aspects of Tat biology. Indeed, it is necessary for Tat stability (64), nuclear and nucleolar delivery (65),[2] and interaction with nucleic acids (66). It mediates some of the neurotoxic effects of extracellular Tat (12, 33, 35), and it is necessary for the mitogenic, chemotactic, and angiogenic activities of extracellular Tat (21). The basic domain cooperates with the RGD motif in the interaction of Tat protein with integrins (61), and it is implicated in Tat binding and activation of the Flk-1/KDR receptor (67).

Tat protein binds to cell surface heparan sulfate (HS) and heparin (68, 69). This interaction occurs through the negatively charged sulfate groups of the glycosaminoglycans (GAGs) (68), suggesting that the positively charged Arg and Lys residues present within the basic domain of Tat may be responsible for the interaction. Soluble polysulfated GAGs can inhibit the transactivating activity of extracellular Tat, and their inhibitory action appears to be directly related to their capacity to bind Tat protein (68). These observations point to the basic domain of Tat as a preferential molecular target for polysulfated compounds able to interact with extracellular Tat protein and neutralize its biological activity.

Suramin is a polysulfonated naphthylurea originally developed for the treatment of trypanosomiasis and onchocerciasis. Suramin has been used recently in the treatment of cancer (70). *In vitro*, suramin blocks the activity of several growth factors by inhibiting their binding to cognate receptors (71–73). Suramin inhibits the activity of heparanase (74) and of urokinase-type plasminogen activator (29, 75). Moreover, suramin inhibits cell adhesion and migration (76). All of these *in vitro* activities may explain, at least in part, the capacity of suramin to inhibit tumor growth and metastasis in different experimental models (77, 78). As an angiogenesis inhibitor, suramin has been demonstrated to inhibit the activity exerted by fibroblast growth factors (FGFs) and vascular endothelial growth factor on cultured endothelial cells by preventing their interaction with cell surface HS-proteoglycans and tyrosine-kinase receptors and to block their angiogenic activity in different animal models (see Ref. 79 and references therein). This is due, at least in part, to the capacity of suramin to bind to the heparin-binding region of the growth factor via one or more of its sulfate groups. Accordingly, suramin is able to mimic heparin/HS for the capacity to protect FGF2 from trypsin digestion. Interestingly, the same capacity was observed for the related polysulfonated compound trypan blue (80).

In this work, we have characterized the interaction of heparin, suramin, and suramin-related compounds with Tat protein, focusing on the role played by the basic domain of Tat in this interaction. The data demonstrate the possibility of synthesizing polysulfonated, heparin-mimicking compounds equipotent to natural heparin in interfering with the biological activity of extracellular Tat.

MATERIALS AND METHODS

Reagents—Suramin was from Bayer AG (Leverkusen, Germany). Trypan blue was from Sigma. Heparin (13.6 kDa) was from Laboratori Derivati Organici SpA (Milan, Italy). Chondroitin sulfate C was a gift of M. Del Rosso (University of Florence, Italy). β-Cyclodextrin tetradecasulfate was from Consultants on Glycosaminoglycans (Milan, Italy). The PNU compounds are suramin-related distamycin A derivatives (81) (see Fig. 9 for structural details). Anti-Tat polyclonal antibody were from American Biotechnologies/Intracel (London). This antibody recognizes with the same efficiency all of the Tat mutants utilized in this study (data not shown). The synthetic peptides representing the amino acid sequences 1–20, 41–60, and 71–85 of HIV-1 Tat (virus strain HIV-1 LAI) were obtained from the Medical Research Council AIDS Reagent Project (National Institute for Biological Standards and Control, Potters Bar, Herts, UK). The synthetic peptides representing the sequences 103–120 and 103–146 of basic fibroblast growth factor (FGF-2) were kind gifts from A. Baird (Prizm Pharmaceuticals, San Diego, CA).

Preparation of Recombinant Wild Type HIV-1 Tat and of the Different Tat Mutants—Recombinant wild type HIV-1 Tat and the different Tat mutants were expressed in *Escherichia coli* as glutathione *S*-transferase (GST) fusion proteins. The respective plasmid constructs are derivatives of plasmid pGST-Tat 2E, which was originally obtained by cloning the coding region of both exons of HIV-1HXB2 Tat in the commercial vector pGEX2T, as already described (82). This construct codes for the wild type 86-amino acid Tat protein. The mutated derivatives include GST-Tat-1e (containing one-exon Tat and comprising the first 72 amino acids of the protein), GST-$Tat_{\Delta 1-21}$ (containing a deletion of the amino acid sequence 1–21), GST-Tat_{H13E} (containing a mutation of histidine 13 to glutamine), and GST-$Tat_{K49/52/53/55/56/57A}$ (in which the arginine residues at positions 49, 52, 53, 55, 56, and 57 in the basic domain were mutated to alanine residues). These constructs were obtained by a recombinant polymerase chain reaction procedure using overlapping oligonucleotides corresponding to the mutated sequences; a detailed description of the construction of these mutants as well as of their transcriptional properties will be presented elsewhere.[3] Recombinant fusion proteins were purified to homogeneity from bacterial lysates by glutathione-Sepharose affinity chromatography (Amersham Pharmacia Biotech) according to the manufacturer's instructions, with minor modifications. Briefly, lysates were mixed with 1 ml of a 50% (v/v) slurry of glutathione cross-linked agarose beads (Sigma). The fusion protein was allowed to bind to the beads at 4 °C on a rotating wheel for 1 h. The suspension was then loaded on an empty plastic column, letting the unbound proteins pass through, and the beads were submitted to a high salt wash (0.8 M NaCl) to free the fusion protein from contaminating bacterial nucleic acids. The fusion protein was eluted in 1 ml of 100 mM Tris, pH 8.0, containing 2 mM dithiothreitol and 20 mM free glutathione (Sigma). The purity and integrity of the protein was routinely checked by SDS-polyacrylamide gel electrophoresis and silver staining. Usually, this purification procedure leads to >95% purification of the recombinant proteins. The purified proteins were stored in aliquots at −80 °C until use.

Cell Cultures—The T53 cell line was established from adenocarcinoma of skin adnexa of BKV/*Tat* transgenic mice, and it expresses and secretes high levels of Tat protein (24, 28). HL3T1 cells are derived from HeLa cells and contain integrated copies of pL3CAT, a plasmid where the bacterial gene for chloramphenicol acetyltransferase (CAT) is directed by the HIV-1 LTR (83). All cells were grown and maintained in Dulbecco's modified minimal essential medium (DMEM) with 10% fetal calf serum (FCS) (Life Technologies, Inc.).

LTR/CAT-transactivating Assay—HL3T1 cells were seeded in 24-well dishes at a density of 20,000 cells/cm^2 in DMEM containing 10% FCS. After 24 h, cell cultures were washed twice with PBS and incubated for a further 24 h in fresh medium containing 10% FCS and 100 μM chloroquine in the absence or in the presence of recombinant GST-Tat (200 ng/ml) and of increasing concentrations of the molecule under test. At the end of incubation, conditioned medium was removed, and cell cultures were incubated for a further 24 h in DMEM containing 10% FCS. At the end of incubation, cells were extracted, and the amount of CAT present in the cell extracts was determined by the CAT ELISA kit (Boehringer Mannheim) according to manufacturer's instructions.

[2] M. Giacca, unpublished observations.

[3] M. Giacca, manuscript in preparation.

Labeling of GST-Tat—Recombinant GST-Tat was labeled with ^{125}I (17 Ci/mg, NEN Life Science Products) using IODO-GEN (Pierce). Iodogen was dissolved in chloroform to a concentration of 10 μg/ml. 200 μl of this solution were added to glass tube and evaporated to dryness under a steam of nitrogen. Recombinant GST-Tat (4 μg) was resuspended in 80 μl of 0.2 M sodium phosphate, pH 7.2, and added to the glass tube together with 1 mCi of sodium ^{125}I. The mix was then incubated at room temperature for 20 min. At the end of incubation, the reaction was stopped by the addition of 30 μl of the dipeptide Gly-Tyr (0.1 M in H_2O). Free ^{125}I was separated from ^{125}I-GST-Tat by affinity chromatography onto a heparin-Sepharose column (100 μl) equilibrated in 25 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl (TBS) and added with 0.1% BSA (TBS/BSA). To this purpose, the reaction mixture was loaded onto the column, which was then extensively washed with TBS/BSA. ^{125}I-GST-Tat was then eluted by the column with TBS/BSA containing 2 M NaCl. The radioactivity recovered in the 2 M NaCl eluate was routinely 80% trichloroacetic acid-precipitable. The specific radioactivity of ^{125}I-GST-Tat was 400 cpm/fmol.

Cell Internalization of ^{125}I-GST-Tat—HL3T1 cells were seeded in 24-well dishes at a density of 45,000 cells/cm^2 in DMEM containing 10% FCS. After 24 h, cell cultures were washed twice with TBS and incubated at 37 °C for different periods of time in binding medium (serum-free medium containing 0.15% gelatin and 20 mM Hepes buffer, pH 7.5) added with the compound under test in the presence of 20 ng/ml of ^{125}I-GST-Tat and 200 ng/ml of unlabeled GST-Tat used as a carrier. At the end of incubation, the medium was removed, and cells were washed three times with cold TBS and lysed by incubation with 0.5% Triton X-100 in 0.1 sodium phosphate, pH 8.1. Radioactivity was then measured in the cell extract. Nonspecific radioactivity was evaluated by incubating the cells at 4 °C with 20 ng of ^{125}I-GST-Tat and was subtracted from each experimental point.

T53 Cell Proliferation Assay and Binding of ^{125}I-GST-Tat—T53 cells were seeded in 24-well dishes at 25,000 cells/cm^2, in DMEM containing 10% FCS. After 24 h, subconfluent cultures were washed twice with DMEM and incubated for 24 h in fresh medium containing 10% FCS in the absence or in the presence of the compounds under test. At the end of incubation, cells were trypsinized and counted in a Burker chamber. The extent of cell proliferation dependent on the autocrine activity of endogenous extracellular Tat was assessed by incubating cells with a 1:25 dilution of anti-Tat antiserum during the assay.

For the binding assay, T53 cells were seeded in 24-well dishes at 40,000 cells/cm^2, in DMEM containing 10% FCS. After 24 h, subconfluent cultures were washed twice with 20 mM sodium acetate buffer, pH 4.0, containing 2 M NaCl. This procedure allows the removal of endogenous Tat from T53 cell surface. Cells were then washed twice with DMEM and incubated for 2 h at 4 °C in binding medium containing 20 ng/ml of ^{125}I-GST-Tat in the absence or in the presence of the compounds under test. At the end of the incubation, radioactivity was extracted by incubating the cells for 30 min at 37 °C with 2% SDS in H_2O. Nonspecific binding was measured in cell cultures treated with 40 ng/ml of ^{125}I-GST-Tat in the presence of a 200-fold molar excess (4 μg/ml) of unlabeled GST-Tat and was subtracted from each experimental point.

Preparation of Heparin-conjugated Sepharose Gel and Heparin-Sepharose Affinity Chromatography—1,6-Diaminoexyl-derivatized Sepharose gel was prepared from CNBr-activated Sepharose 6B gel (Amersham Pharmacia Biotech) according to the manufacturer's instructions. 1-ml aliquots of the gel were then suspended in 3 ml of distilled water, pH 4.5, containing 1 mg of heparin and 10 mg of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide HCl. The suspension was kept overnight at 4 °C under gentle mixing. Gels were then sequentially washed with 10 ml of H_2O, pH 4.5, 10 ml of 2.0 M NaCl, and 20 ml of H_2O. The resin was then used to evaluate the capacity of the different GST-Tat mutants to bind to immobilized heparin. In detail, 3-μg aliquots of the different recombinant GST-Tat mutants were loaded onto heparin-Sepharose columns (5 × 60 mm) connected to a fast protein liquid chromatography apparatus (Amersham Pharmacia Biotech). The column was eluted with a 0.15–2.0 M NaCl gradient. Tat immunoreactivity in the various fractions was evaluated by immuno-dot blot analysis with the anti-Tat antiserum and quantified by soft laser scanning of the nitrocellulose membrane.

Preparation of ^3H-Labeled Heparin—Heparin was ^3H-labeled as described previously (84) with minor modifications. Briefly, 3 mg of heparin were dissolved in 600 μl of 0.1 M Tris-HCl, pH 8.0, containing 2.5 mCi of NaB^3H_4 (1 Ci/mmol) (NEN Life Science Products) and incubated for 3 h at room temperature under gentle shaking. 18 mg of glucose were then added to the suspension, and it was incubated for a further 3 h. At the end of incubation, the sample was extensively dialyzed (cut-off, 1000 Da) against distilled water and kept at −20 °C until use. The specific radioactivity of the ^3H-labeled heparin was approximately 5000 cpm/nmol.

Immobilization of GST-Tat to Glutathione-Agarose Beads—Aliquots (400 μl) of glutathione-agarose beads were mixed with 250-μg aliquots of recombinant GST-Tat, of GST-Tat$_{R49/52/53/55/56/57A}$ mutant or of GST protein devoid of the Tat moiety. After 6 h of incubation at 4 °C, the resin beads were extensively washed, resuspended in TBS, and stored at 4 °C until use. Under these conditions, up to 90% of the proteins bound to the resin. To evaluate the capacity of heparin to bind to wild type and mutagenized Tat, 50 μg of [^3H]heparin were loaded onto 80 μl columns containing the different immobilized GST-Tat proteins. After extensive washing with TBS, the columns were eluted stepwise with TBS containing increasing concentrations of NaCl. Radioactivity in the different fractions was measured in a liquid scintillation counter. To evaluate the dissociation constant (K_d) of the interaction of heparin with immobilized wild type Tat or Tat mutant, the columns were loaded with increasing concentrations of [^3H]heparin and eluted with 3.0 M NaCl. Binding data were then analyzed according to the procedure originally described by Scatchard (85).

[^3H]Heparin Competition Binding Assay to Immobilized GST-Tat—To evaluate their relative affinity for Tat protein, the different compounds were tested for their capacity to compete for the binding of ^3H-labeled heparin to immobilized GST-Tat. For this purpose, a series of 80-μl GST-Tat-glutathione-agarose columns were loaded with samples containing 25 μg of ^3H-labeled heparin and increasing concentrations of the molecule under test. Columns were then washed extensively with TBS and eluted with a 3.0 M NaCl wash. Radioactivity in the eluate was measured in a liquid scintillation counter.

Coating of GST-Tat Fusion Proteins to Plastic and Binding Assay—100-μl aliquots of 100 mM $NaHCO_3$, pH 9.6 (carbonate buffer), containing 40 μg/ml of GST-Tat or of GST-Tat$_{R49/52/53/55/56/57A}$ were added to polystyrene non-tissue culture microtiter plates. After 16 h of incubation at 4 °C, the solution was removed, and wells were washed three times with TBS. The amount of GST, GST-Tat, and GST-Tat$_{R49/52/53/55/56/57A}$ bound to plastic was evaluated by protein determination using the Quantigold reagent (Diversified Biotech, Boston, MA). The results demonstrated that 12.8 ± 0.2, 10 ± 0.9, and 11.5 ± 0.5% of original added GST, GST-Tat, and GST-Tat$_{R49/52/53/55/56/57A}$ bind to plastic, respectively, indicating that there were no significant differences in coating efficiency among the molecules tested. Plastic-bound Tat resists extraction with 6 M urea or 95% ethanol and can be solubilized only by drastic treatment with detergent (*i.e.* 1-h incubation at 37 °C with 0.5% Triton X-100 or 30-min incubation at 50 °C with 2% SDS). For the competition binding assay, 4 nmol of ^{14}C-PNU 145156E (12,500 cpm/nmol) (Pharmacia Upjohn, Nerviano, Italy) were incubated for 2 h at 4 °C in wells coated with 40 μg/ml GST-Tat in the absence or in the presence of the competitor under test. At the end of incubation, wells were washed three times with cold TBS, and Tat-associated radioactivity was solubilized by incubating the wells for 30 min at 50 °C with 2% SDS in H_2O, collected, and measured in a liquid scintillation counter.

For the determination of the K_d of interaction of ^{14}C-PNU 145156E with Tat molecules, 100-μl aliquots of TBS containing different concentrations of ^{14}C-PNU 145156E were added into wells coated with 40 μg/ml GST-Tat or GST-Tat$_{K49/52/53/55/56/57A}$ mutant. Then samples were processed exactly as described above. Binding data were analyzed by the Scatchard plot procedure. Nonspecific ^{14}C-PNU 145156E binding was evaluated by using wells coated with a 40 μg/ml concentration of the GST protein devoid of the Tat moiety. Similar results were obtained when binding experiments were performed in the absence or in the presence of 1% BSA overcoating of the wells (30 min at room temperature).

RESULTS

Role of the Basic Domain of Tat Protein in Heparin Interaction—To investigate the role of the basic domain of Tat in heparin binding, four recombinant Tat mutants were produced as GST fusion proteins in *E. coli*: GST-Tat$_{R49/52/53/55/56/57A}$, obtained by the substitution of six arginine residues within the basic domain with alanine residues; Tat-1e, characterized by the deletion of the amino acid sequence encoded by the second exon of the *tat* gene and containing the RGD motif; GST-Tat$_{\Delta1–21}$, obtained by deletion of the first 21 amino-terminal residues required for transactivating activity (3, 4, 57); and GST-Tat$_{H13E}$, characterized by the substitution of histidine at position 13 with a glutamic acid residue. These mutants were

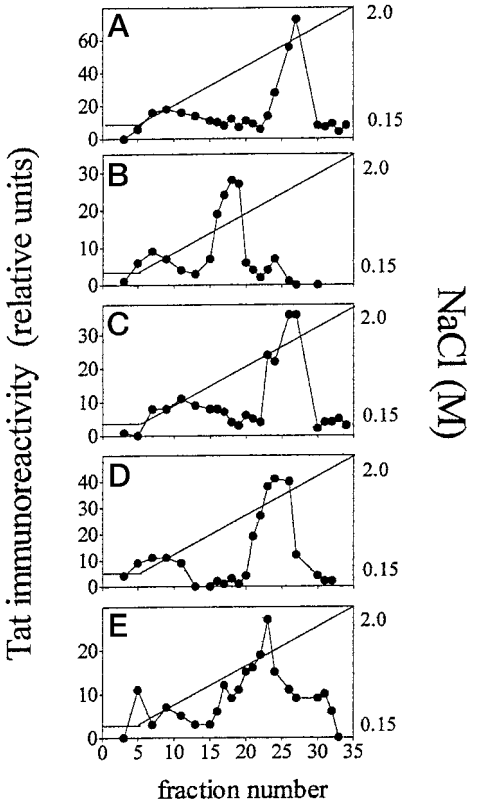

FIG. 1. **Binding of different GST-Tat mutants to heparin-Sepharose.** Heparin was conjugated to Sepharose gel. 3-μg aliquots of GST-Tat (*A*), GST-Tat$_{R49/52/53/55/56/57A}$ (*B*), GST-Tat$_{H13E}$ (*C*), GST-Tat-1e (*D*), and GST-Tat$_{\Delta1-21}$ (*E*) were then loaded onto heparin-Sepharose columns and eluted with a 0.15–2.0 M NaCl gradient. Tat immunoreactivity in the various fractions was evaluated by immuno-dot blot analysis with anti-Tat antiserum and quantified by soft laser scanning of the nitrocellulose membrane.

compared with wild type Tat (amino acid residues 1–86) expressed as GST fusion protein for the capacity to bind to heparin-Sepharose and to be eluted from the resin by a linear gradient of NaCl concentration. Previous experiments had demonstrated that the GST moiety does not interfere in Tat/heparin interaction (68).

As shown in Fig. 1, all of the mutants tested bind to heparin-Sepharose but are eluted from the column at different ionic strength. As anticipated, both wild type GST-Tat and GST-Tat$_{H13E}$ elute at 1.6 M NaCl. Likewise, the two deletion mutants GST-Tat-1e and GST-Tat$_{\Delta1-21}$ elute from heparin-Sepharose at high ionic strength (1.3 M NaCl). In contrast, GST-Tat$_{R49/52/53/55/56/57A}$ elutes from the column at a NaCl concentration significantly lower (0.6 M) than the other GST-Tat forms, thus indicating that neutralization of the positive charges in the basic domain of Tat significantly reduces its interaction with the GAG.

To confirm this observation, GST-Tat and GST-Tat$_{R49/52/53/55/56/57A}$ were immobilized onto glutathione-agarose columns and evaluated for the capacity to bind soluble [^{3}H]heparin. Under the same experimental conditions [^{3}H]heparin does not bind to immobilized GST devoid of the Tat moiety (68). As shown in Fig. 2, *A* and *B*, [^{3}H]heparin binds to immobilized GST-Tat and GST-Tat$_{R49/52/53/55/56/57A}$, but it elutes from the mutant at an ionic strength significantly lower (0.8 M NaCl) than that required to elute from the wild type molecule (1.4 M NaCl).

In order to estimate the affinity of heparin for GST-Tat and GST-Tat$_{R49/52/53/55/56/57A}$, the two proteins were immobilized onto glutathione-agarose beads that were then incubated with increasing concentrations of soluble [^{3}H]heparin. Analysis of the binding data (Fig. 2*C*) by the Scatchard plot procedure (Fig. 2*D*) demonstrates that [^{3}H]heparin binds to GST-Tat$_{R49/52/53/55/56/57A}$ with an affinity significantly lower than to GST-Tat (K_d = 1.0 and 0.3 μM for the two proteins, respectively).

The involvement of the basic domain of HIV-1 Tat in heparin interaction was also investigated by a different experimental approach in which synthetic peptides representing different regions of the HIV-1 Tat molecule were evaluated for their capacity to interact with heparin. For this purpose, increasing amounts of the peptide Tat-(41–60), representing the basic domain neutralized in the GST-Tat$_{R49/52/53/55/56/57A}$ mutant, and of the peptides Tat-(1–20) and Tat-(71–85), representing the amino and carboxyl terminus of the protein deleted in the mutants GST-Tat$_{\Delta1-21}$ and GST-Tat-1e, respectively, were evaluated for their capacity to compete with GST-Tat immobilized onto glutathione-agarose beads for the binding to [^{3}H]heparin. As shown in Fig. 3, peptide Tat-(41–60) prevents the binding of [^{3}H]heparin to immobilized GST-Tat in a dose-dependent manner, whereas peptides Tat-(1–20) and Tat-(71–85) are ineffective. Under the same experimental conditions, the heparin-binding synthetic peptide FGF-2-(103–120) that carries six basic amino acid residues (86) competes with immobilized GST-Tat for the binding to [^{3}H]heparin with a potency that is approximately 5 times lower than that of peptide Tat-(41–60). Interestingly, the heparin-binding synthetic peptide FGF-2-(103–146) also competes poorly (approximately 30% inhibition of [^{3}H]heparin binding at 25 μg/sample) despite the fact that it carries 11 basic residues (86) (data not shown), thus supporting the specificity of the interaction of the peptide Tat-(41–60) with the GAG.

In conclusion, the data indicate that the arginine residues within the basic domain of Tat protein play an important role in the interaction with the sulfate groups of the heparin molecule.

Effect of Polyanionic Molecules on Heparin Binding and HIV LTR-transactivating Activity of Extracellular Tat—The above data indicate that the basic domain of Tat may represent a target for compounds able to inhibit Tat/heparin interaction and, possibly, the biological activity of extracellular Tat. On this basis, the capacity of non-GAG polyanions to inhibit the heparin binding and HIV LTR-transactivating activity of extracellular Tat was evaluated.

To evaluate this inhibition, [^{3}H]heparin was allowed to bind to GST-Tat immobilized onto a glutathione-agarose column. Then different concentrations of suramin, trypan blue, or polysulfated β-cyclodextrin were loaded onto the column and evaluated for their capacity to displace [^{3}H]heparin from immobilized Tat. As shown in Fig. 4*A*, suramin and trypan blue displace [^{3}H]heparin from GST-Tat with a similar potency (ED_{50} equal to approximately 5 μM for both compounds), significantly lower than that of unlabeled heparin (ED_{50} equal to 0.2 μM). In contrast, polysulfated β-cyclodextrin does not displace [^{3}H]heparin from GST-Tat although this compound has the highest degree of sulfation among the molecules tested (14 sulfate groups per molecule *versus* 6 and 4 sulfonate groups per molecule for polysulfated β-cyclodextrin, suramin, and trypan blue, respectively). These data underline the importance of the backbone structure of the molecule in presenting its sulfate/sulfonate group(s) to Tat protein.

Polysulfonates were evaluated also for their capacity to inhibit the HIV LTR-transactivating activity of extracellular Tat. For this purpose, subconfluent cultures of HL3T1 cells, containing the CAT gene under the control of HIV-1 LTR, were incubated with 200 ng/ml GST-Tat in the presence of increasing concentrations of the compound under test. At the end of

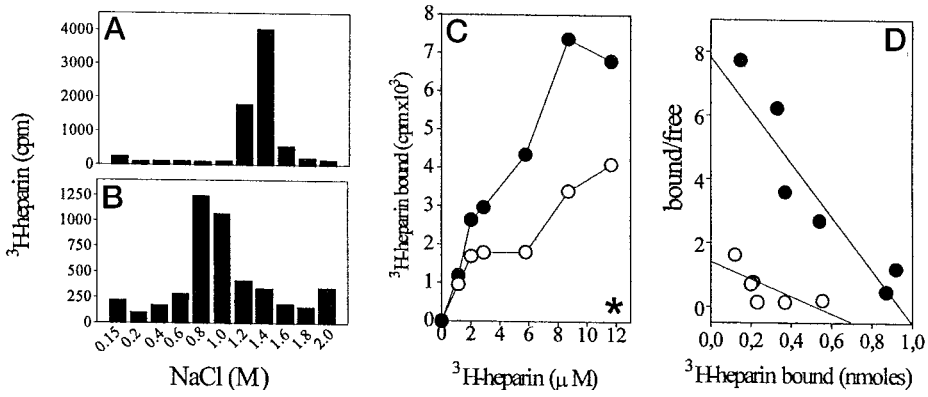

FIG. 2. **Binding of [^{3}H]heparin to GST-Tat and GST-Tat$_{R49/52/53/55/56/57A}$ immobilized onto glutathione-agarose beads.** 100 μg of recombinant GST-Tat (*A* and *solid symbols* in *C* and *D*), of GST-Tat$_{R49/52/53/55/56/57A}$ (*B* and *open symbols* in *C* and *D*), or of recombinant GST protein devoid of the Tat moiety (*asterisk* in *C*) were immobilized onto 200-μl glutathione-agarose columns. *A* and *B*, columns were loaded with 300-μl samples containing 12 μM of ^{3}H-labeled heparin. After extensive washing, columns were eluted step-wise with increasing concentrations of NaCl. Radioactivity in the eluted fractions was measured in a liquid scintillation counter. *C*, columns were loaded with increasing concentrations of [^{3}H]heparin and washed with 500 μl of TBS, and Tat-bound [^{3}H]heparin was eluted with the same volume of 3.0 M NaCl in TBS. Unbound and 3.0 M NaCl-eluted radioactivity was then measured in a liquid scintillation counter, and binding data were analyzed by the Scatchard plot procedure (*D*). The experiment shown is representative of three independent experiments with similar results.

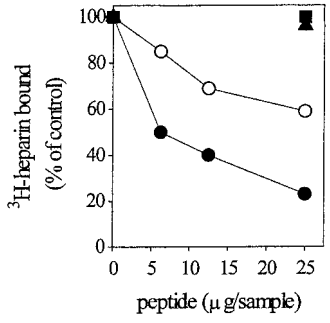

FIG. 3. **Heparin-binding capacity of synthetic peptides representing different fragments of HIV-1 Tat.** The indicated amounts of peptide HIV-1 Tat-(1–20) (▲), HIV-1 Tat-(41–60) (●), and HIV-1 Tat-(72–86) (■) were incubated for 20 min at room temperature with 5 μg of [^{3}H]heparin in 200 μl of TBS. At the end of incubation, samples were loaded onto GST-Tat-glutathione-agarose columns (80 μl) that were then washed extensively with TBS. [^{3}H]heparin bound to the column was eluted with 500 μl of 3.0 M NaCl in TBS, and eluted radioactivity was measured in a liquid scintillation counter. The heparin-binding peptide FGF-2-(103–121) (○) was used as control (86). Each point is the mean of two determinations in duplicate. S.E. never exceeded 10% of the mean value.

incubation, the amount of CAT produced by the cells in the different experimental conditions, proportional to the LTR-transactivating activity exerted by GST-Tat, was measured by a CAT ELISA. As shown in Fig. 4*B*, suramin, trypan blue, and heparin abolish the transactivating activity exerted by GST-Tat, although with different potency (ID_{50} = 2 nM for heparin and 2 μM for suramin and trypan blue). In agreement with its incapacity to affect Tat/heparin interaction, polysulfated β-cyclodextrin does not inhibit the transactivating activity of GST-Tat (Fig. 4*B*).

In conclusion, polyanionic compounds prevent Tat/heparin interaction and inhibit the LTR-transactivating activity of extracellular Tat. Their Tat-antagonist activity, less potent than that of heparin, appears to depend not only on the sulfate/sulfonate group density of the molecule but also on its backbone structure.

Suramin and Heparin Exert Their Tat Antagonist Activity by a Similar Mechanism of Action—On the basis of the results described above, suramin was utilized to study the mechanism of action of polysulfonated compounds as extracellular Tat antagonists. In a first set of experiments, suramin and heparin were administered to HL3T1 together with GST-Tat or at different times after GST-Tat administration. As shown in Fig. 5, the transactivating activity of Tat is completely abolished when the two molecules are administered within the first 2–3 h following the beginning of GST-Tat treatment. The addition of heparin or suramin to the cell culture medium 5 h after the beginning of Tat treatment is instead ineffective. These data suggest that the inhibitory activity exerted by heparin and suramin depends on an early interaction with GST-Tat. Also, they are in agreement with previous observations indicating that Tat-mediated LTR transactivation is rapid and transient with maximal stimulation 5 h after the beginning of Tat treatment (82).

The effect of suramin and heparin on the kinetics of internalization of GST-Tat into HL3T1 cells was therefore investigated. To this purpose, subconfluent cultures of HL3T1 cells were incubated with 20 ng/ml of ^{125}I-GST-Tat added with 200 ng/ml of unlabeled GST-Tat as a carrier. At different times of incubation at 37 °C, free ^{125}I-GST-Tat was removed, cell were lysed, and the amount of intracellular radioactivity was measured. As shown in Fig. 6*A*, ^{125}I-GST-Tat internalization is time-dependent, reaching a plateau at approximately 12 h. When cells were incubated with ^{125}I-GST-Tat in the presence of 100 μg/ml of suramin or heparin, a complete inhibition of GST-Tat internalization was observed both at early and late times of incubation. The effect of the two antagonists on ^{125}I-GST-Tat internalization was dose-dependent, with ID_{50} = 10 nM and 0.7 μM for heparin and suramin, respectively (Fig. 6*B*), close to the potency shown by the two compounds in inhibiting the LTR-transactivating activity exerted by GST-Tat on the same cells (2 nM and 2 μM for heparin and suramin, respectively; see Fig. 4*B*). Taken together, the data strongly suggest that the inhibitory action exerted by heparin and suramin on the transactivating activity of GST-Tat depends, at least in part, on their capacity to bind to the extracellular protein, thus preventing its interaction and internalization within the cell. It must be pointed out that heparin and suramin-related compounds are not internalized within the cell when incubated alone or in the presence of Tat protein.[4]

Murine adenocarcinoma T53 cells were originated from *tat*-transgenic mice (28). They produce and secrete high amounts of Tat protein, which, in turn, stimulates them to proliferate. This extracellular autocrine loop of stimulation can be interrupted

[4] A. Corallini, manuscript in preparation.

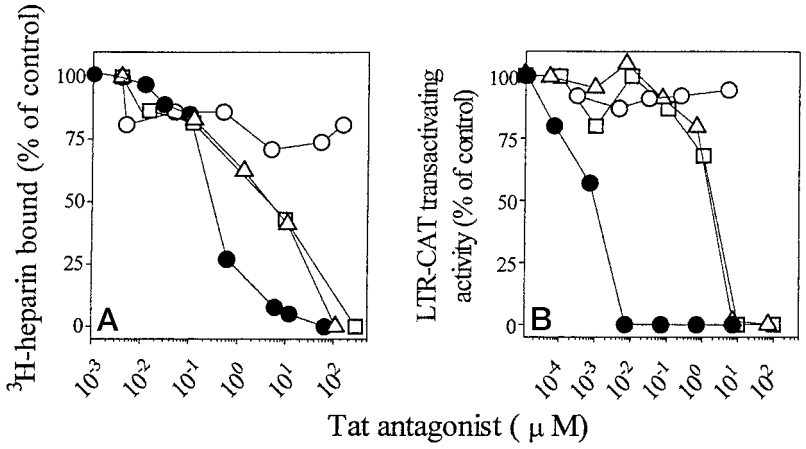

FIG. 4. **Interaction of polyanionic compounds with GST-Tat: competition binding assay and inhibition of transactivating activity.** *A*, GST-Tat-glutathione-agarose columns (80 μl) were loaded with 300-μl samples containing 25 μg of [^{3}H]heparin with no addition or supplemented with increasing concentrations of unlabeled heparin (●), suramin (△), trypan blue (□), and polysulfated β-cyclodextrin (○). Columns were washed extensively with TBS, and radioactivity in the 3.0 M NaCl eluate was measured in a liquid scintillation counter. Nonspecific binding, measured in the presence of a large excess of unlabeled heparin (1 mM) was subtracted from all the values. Each point is the mean of 2–4 determinations in duplicate. S.E. never exceeded 6% of the mean value. *B*, HL3T1 cells were treated with 200 ng/ml GST-Tat in the absence or in the presence of increasing concentrations of polyanionic compounds (*symbols* as in *A*). After 48 h, cell extracts were assayed for the levels of CAT antigen by ELISA, and data were expressed as percentage of the transactivating activity measured in control cultures treated with GST-Tat alone. Each point is the mean of 4–8 determinations in duplicate. S.E. never exceeded 12% of the mean value.

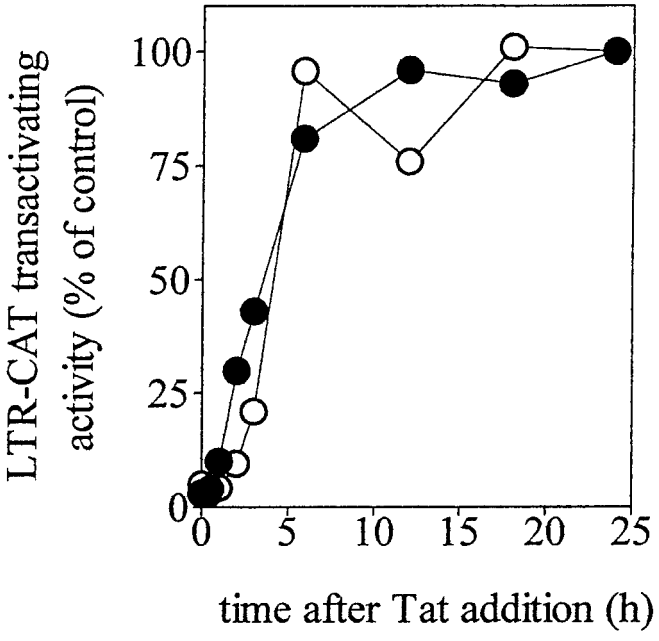

FIG. 5. **Time-dependent inhibition of the transactivating activity of extracellular Tat by heparin and suramin.** Subconfluent cultures of HL3T1 cells were treated with 200 ng/ml of GST-Tat. Heparin (●) or suramin (○) was added to cell cultures at 100 μg/ml together with GST-Tat or after the indicated periods of time. 48 h after the beginning of GST-Tat treatment, cell extracts were assayed for the levels of CAT antigen by ELISA, and data were expressed as percentage of the transactivating activity measured in control cultures treated with GST-Tat alone. Each point is the mean of two or three determinations in duplicate. S.E. never exceeded 12% of the mean value.

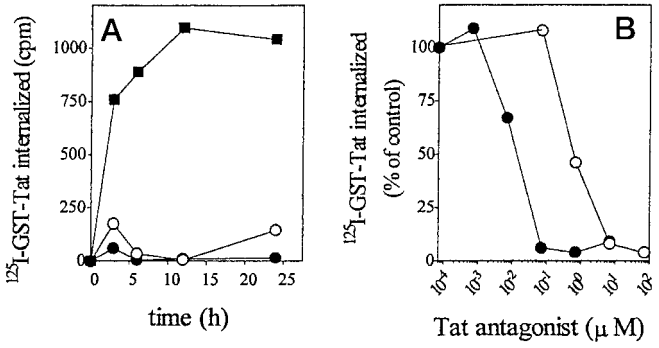

FIG. 6. **Effect of heparin and suramin on the internalization of ^{125}I-GST-Tat in HL3T1 cells.** *A*, confluent cultures of HL3T1 cells were incubated at 37 °C in binding medium for the indicated periods of time with 20 ng/ml of ^{125}I-GST-Tat added with 200 ng/ml of unlabeled GST-Tat in the absence (■) or in the presence of heparin (●) or suramin (○) (both at 100 μg/ml). At the end of incubation, internalized radioactivity was measured in the cell extracts. Nonspecific radioactivity was evaluated by incubating the cells at 4 °C with 20 ng of ^{125}I-GST-Tat and was subtracted from each experimental point. *B*, HL3T1 cells were incubated for 24 h at 37 °C with 20 ng/ml of ^{125}I-GST-Tat added with 200 ng/ml of unlabeled GST-Tat in the presence of increasing concentrations of heparin or suramin (*symbols* as in *A*). At the end of the incubation, cell internalized radioactivity was measured. Data are expressed as percentage of ^{125}I-GST-Tat internalized in the absence of antagonist. Each point is the mean of two determinations in duplicate. S.E. never exceeded 16% of the mean value.

by neutralizing anti-Tat antibodies added to the cell culture medium, thus suppressing T53 cell proliferation (24). Indeed, in our experimental conditions, control cultures incubated in 10% FCS with no addition or with a 1:25 dilution of neutralizing anti-Tat antiserum undergo 1.5 and 0.3 cell population doublings, respectively. When subconfluent cultures of T53 cells were incubated with heparin or suramin, a significant inhibition of cell proliferation was observed with a maximal inhibitory effect at approximately 20 and 200 μM for the two molecules, respectively (Fig. 7*A*). The specificity of the inhibitory activity of the two compounds is demonstrated by the ineffectiveness in the same assay of chondroitin sulfate (data not shown), a sulfated GAG that does not interact with Tat protein (68). Heparin and suramin also inhibit the binding of ^{125}I-GST-Tat to T53 cell surface (Fig. 7*B*), supporting the hypothesis that the inhibitory activity on T53 cell proliferation depends, at least in part, on their capacity to bind endogenous extracellular Tat.

In conclusion, our data indicate that heparin and suramin act on exogenously added recombinant GST-Tat fusion protein and on endogenously produced native Tat protein by binding them in the extracellular environment, thus preventing their interaction with the cell surface and/or their internalization into the cell.

Suramin Derivative Binds Tat Protein: Role of the Basic

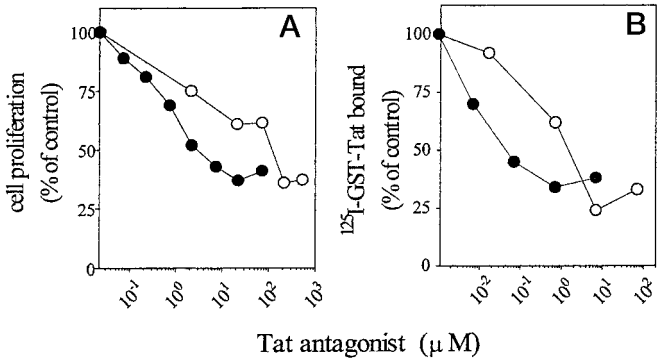

FIG. 7. **Effect of heparin and suramin on cell proliferation and cell surface ^{125}I-GST-Tat binding to T53 cells.** *A*, subconfluent cultures of T53 cells were incubated for 24 h with DMEM containing 10% FCS in the absence or in the presence of increasing concentrations of heparin (●) or suramin (○). At the end of incubation, cells were trypsinized and counted in a Burker chamber. The extent of T53 cell proliferation dependent on endogenous Tat was evaluated by incubating the cells with a 1:25 dilution of neutralizing anti-Tat antiserum. In our experimental conditions, control cultures incubated in 10% FCS with no addition or with anti-Tat antiserum undergo 1.5 and 0.3 cell population doublings, respectively. Each point is the mean of 2–4 determinations in duplicate. S.E. never exceeded 13% of the mean value. *B*, confluent cultures of T53 cells were washed twice with sodium 20 mM acetate buffer, pH 4, containing 2.0 M NaCl to remove endogenous Tat from the cell surface. Cell cultures were then washed twice with DMEM and incubated for 2 h at 4 °C with 40 ng/ml of ^{125}I-GST-Tat in the absence or in the presence of increasing concentrations of heparin or suramin (*symbols* as in *A*). At the end of incubation, cell-associated radioactivity was extracted. Nonspecific binding was measured in cell cultures treated with 40 ng/ml ^{125}I-GST-Tat in the presence of 4 μg/ml unlabeled GST-Tat and was subtracted from each experimental point. Values are the mean of two or three determinations in duplicate. S.E. never exceeded 10% of the mean value.

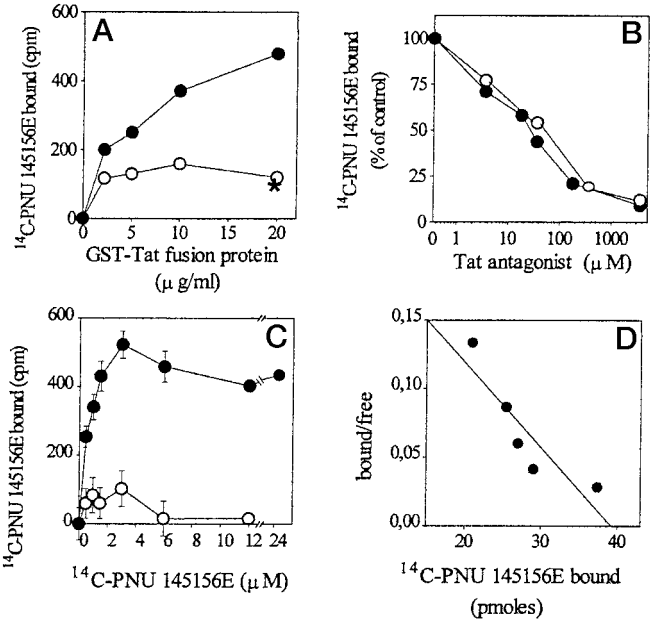

FIG. 8. **Binding of ^{14}C-PNU 145156E to immobilized GST-Tat and GST-Tat$_{R49/52/53/55/56/57A}$.** *A*, GST-Tat (●), GST devoid of the Tat moiety (○), or BSA (*) was incubated in carbonate buffer at the indicated concentrations on 96-well non-tissue culture plates for 16 h at 4 °C. Then plates were washed and incubated for a further 30 min at room temperature with 1% BSA in carbonate buffer. At the end of incubation, plates were washed and incubated for 2 h at 4 °C with 100-μl aliquots containing 4 nmol (50,000 cpm) of ^{14}C-PNU 145156E. After extensive washing, the amount of radioactivity associated to the immobilized protein was extracted with SDS and measured. The experiment shown is representative of two experiments that gave similar results. *B*, 100-μl aliquots containing 4 nmol of ^{14}C-PNU 145156E were incubated for 2 h at 4 °C onto GST-Tat-coated wells in the absence or in the presence of increasing concentrations of unlabeled suramin (●) or of unlabeled PNU 145156E (○). At the end of incubation, the amount of GST-Tat-bound radioactivity was measured. Each point is the mean of two determinations in duplicate. S.E. never exceeded 5% of the mean value. *C*, increasing concentrations of ^{14}C-PNU 145156E were incubated for 2 h at 4 °C onto wells coated with 40 μg/ml of GST-Tat (●) or GST-Tat$_{R49/52/53/55/56/57A}$ (○). At the end of incubation, the amount of radioactivity associated to the immobilized proteins was measured. Radioactivity bound to plastic coated with 40 μg/ml GST devoid of the Tat moiety was subtracted from all values. Each point is the mean ± S.E. of two or three determinations in duplicate. At variance with its binding to wild type GST-Tat, the binding of ^{14}C-PNU 145156E to GST-Tat$_{R49/52/53/55/56/57A}$ was not statistically different from that to GST alone (Student's *t* test). *D*, Scatchard plot analysis of the ^{14}C-PNU 145156E binding data to GST-Tat.

Domain of Tat—Our results demonstrate that arginine residues in the basic domain of Tat protein play an important role in heparin interaction (see above). The similar antagonist activity exerted by heparin and suramin on the biological activity of extracellular Tat prompted us to assess the possible role of the basic domain of Tat in its interaction with polysulfonated compounds. For this purpose, the fusion protein GST-Tat was coated onto non-tissue culture plastic and evaluated for its capacity to bind ^{14}C-PNU 145156E, a polysulfonated distamycin A derivative structurally related to suramin (formerly named FCE 26644 (87)). As shown in Fig. 8*A*, the binding of ^{14}C-PNU 145156E to GST-Tat-coated plastic is dose-dependent, with ED_{50} = 5 μg/ml. Under the same experimental conditions, ^{14}C-PNU 145156E binds poorly and to a similar extent to GST protein devoid of the Tat moiety and to BSA, here used as a negative control, thus demonstrating the specificity of Tat interaction. In agreement with their structural similarities, unlabeled PNU 145156E and suramin inhibit the binding of ^{14}C-PNU 145156E to GST-Tat-coated plastic with the same potency, half-maximal inhibition being observed at concentrations of the unlabeled compounds equimolar to the radiolabeled molecule (Fig. 8*B*).

To investigate the role of the basic domain of Tat protein in polysulfate interaction, 40 μg/ml wild-type GST-Tat or GST-Tat$_{R49/52/53/55/56/57A}$ mutant were adsorbed onto plastic and incubated with increasing concentrations of ^{14}C-PNU 145156E. At the end of incubation, free ^{14}C-PNU 145156E was removed, and the amount of radioactivity bound to plastic was measured. Radioactivity nonspecifically bound to plastic coated with 40 μg/ml GST devoid of the Tat moiety was subtracted from all of the values. As shown in Fig. 8*C*, the specific binding of ^{14}C-PNU 145156E to GST-Tat was dose-dependent and saturable, while no significant specific binding to the GST-Tat$_{R49/52/53/55/56/57A}$ mutant was observed. It is interesting to note that the K_d value for the interaction of immobilized wild type GST-Tat with ^{14}C-PNU 145156E (1.5 μM, Fig. 8*D*) is approximately 5 times higher than that calculated for [^{3}H]heparin interaction (0.3 μM, see Fig. 2*D*). This is agreement with the reduced capacity of suramin to displace [^{3}H]heparin from immobilized GST-Tat when compared with the unlabeled GAG (see Fig. 4*A*). Relevant to this point is also the observation that unlabeled PNU 145156E displaces [^{3}H]heparin from GST-Tat immobilized to glutathione-agarose with an ID_{50} similar to suramin.[4]

Tat Antagonist Activity of Suramin Derivatives—Suramin shows a limited potency in inhibiting the biological activity of extracellular Tat when compared with heparin. We have demonstrated previously the possibility to modulate the Tat-binding capacity and antagonist activity of heparin by structural modifications of the polysaccharide molecule (68). These data, together with the observation that sulfated β-cyclodextrin does not exert a significant Tat antagonist activity (see above), indicate that the backbone structure of the molecule is of importance in presenting its sulfate groups to Tat protein in an optimal spatial conformation. To assess whether structural

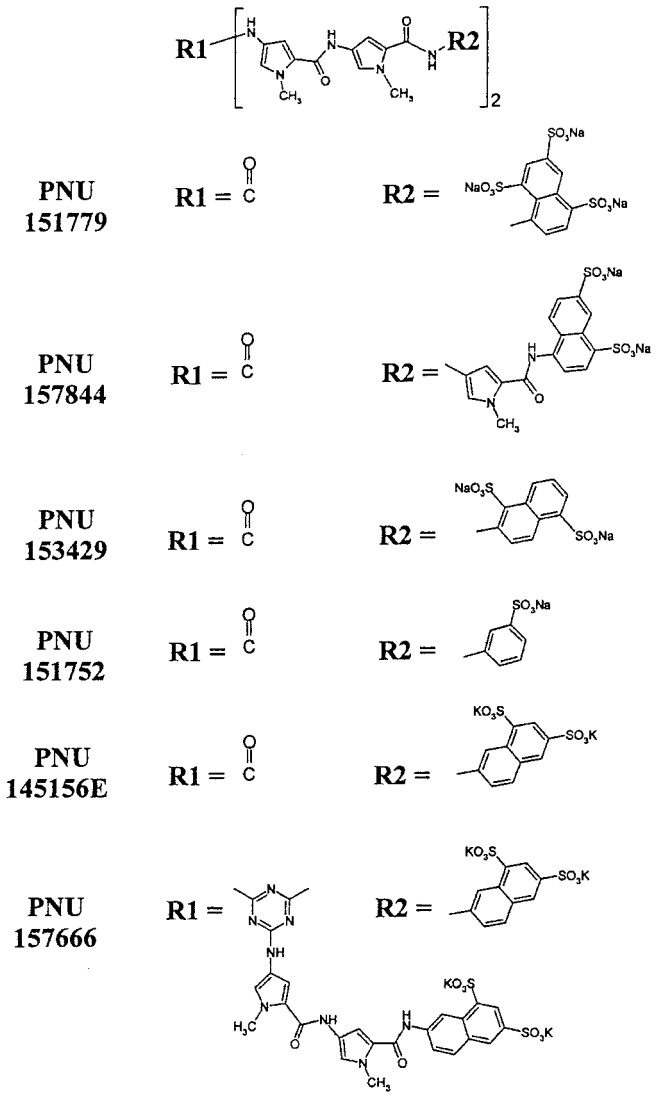

FIG. 9. **Chemical structures of the suramin-related, distamycin A derivatives utilized in the present study.**

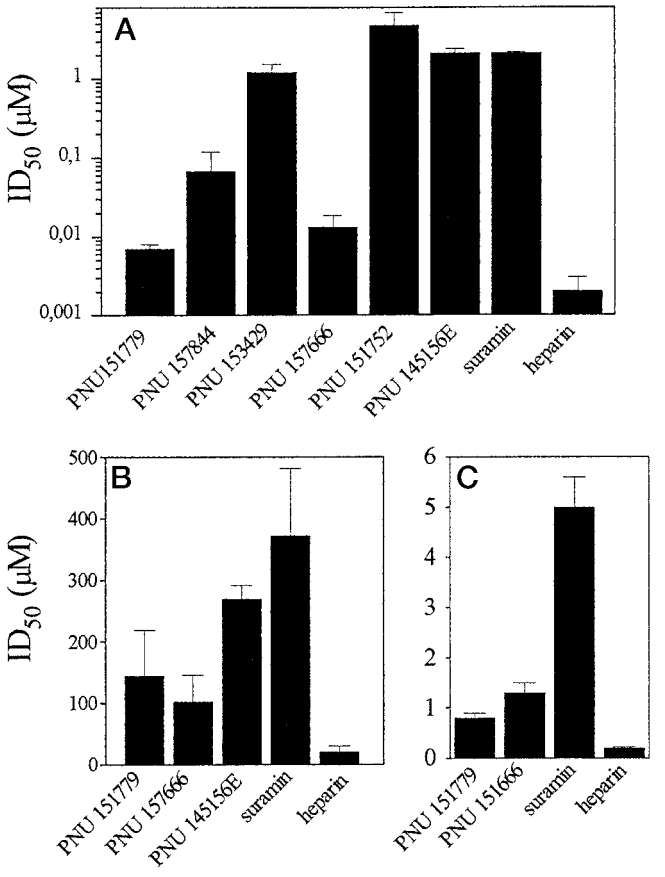

FIG. 10. **Effect of suramin-related, distamycin A derivatives on selected biological activities of Tat.** *A*, subconfluent cultures of HL3T1 cells were treated with 200 ng/ml of GST-Tat in the absence or in the presence of increasing concentrations of the indicated distamycin A derivatives, suramin, or heparin. At the end of incubation, cell extracts were assayed for the levels of CAT antigen by ELISA. *B*, subconfluent cultures of T53 cells were incubated for 24 h in the absence or in the presence of increasing concentrations of the indicated molecules. At the end of incubation, cells were trypsinized and counted in a Burker chamber as described in the legend to Fig. 7. *C*, GST-Tat-glutathione-agarose columns (80 μl) were loaded with 300-μl samples containing 50 μg of [^{3}H]heparin with no addition or added with increasing concentrations of the indicated molecules. Columns were washed extensively with TBS, and radioactivity in the 3.0 M NaCl eluate was measured in a liquid scintillation counter. In each *panel*, data are expressed as the concentration of the molecule required to elicit half-maximal inhibition of the corresponding Tat activity (ID_{50}). Each point is the mean ± S.E. of 2–6 determinations in duplicate.

modifications of a polysulfonated compound might mimic the structural requirements for optimal Tat interaction, a series of distamycin A derivatives structurally related to suramin were tested for their capacity to inhibit the LTR-transactivating activity exerted by GST-Tat on H3T1 cells. These molecules are dimeric ureido compounds incorporating the bisamido-*N*-methylpyrrolenaphthalene-sulfonic acid group with differences in the number and position of the sulfonic acids in the naphthalene ring (see Fig. 9) (81). For each molecule, dose-dependent experiments were performed, and ID_{50} values were calculated and compared with those obtained for suramin and heparin. Among the molecules tested, PNU 151779 and PNU 157666 showed a Tat-inhibitory activity that is approximately 100 times more potent than that of suramin and similar to that exerted by heparin (Fig. 10*A*). Accordingly, when the distamycin A derivatives were tested for their capacity to inhibit T53 cell proliferation, PNU 151779 and PNU 157666 exerted an inhibitory activity more potent than suramin (Fig. 10*B*).

To assess the possibility that the high potency shown by some distamycin A derivatives in antagonizing the biological activity of Tat was due, at least in part, to a higher affinity for the protein, the compounds PNU 151779 and PNU 151666 were compared with unlabeled heparin and suramin for their capacity to displace [^{3}H]heparin from GST-Tat immobilized onto a glutathione-agarose column. As shown in Fig. 10*C*, PNU 151779 and PNU 151666 displace [^{3}H]heparin from immobilized GST-Tat with a potency close to that of unlabeled heparin and significantly higher than that of suramin. The ID_{50} values of the different compounds for Tat/heparin interaction correlate to their ID_{50} values for the inhibition of the transactivating activity ($p < 0.02$) and mitogenic capacity ($p < 0.03$) of extracellular Tat in a linear regression analysis (data not shown), supporting the hypothesis that the inhibitory activity of polysulfated compound is related to their Tat-binding capacity.

DISCUSSION

In the present paper, we have shown that polysulfonated compounds interact with HIV-1 Tat protein. This interaction affects the capacity of Tat to bind heparin and inhibits the biological activity of extracellular Tat both when administered exogenously as a recombinant GST-Tat protein and when produced endogenously by Tat-overexpressing cells.

Results obtained in our laboratory had demonstrated that Tat protein interacts with specific region(s) of heparin/HS and

that a high affinity interaction requires at least some 2-*O*-, 6-*O*-, and *N*-positions to be sulfated (68). These data indicate that defined structural features of the sulfated GAG (size, backbone structure, degree of sulfation) are required to allow its optimal interaction with Tat protein. Moreover, the Tat binding activities of the sulfated GAGs tested correlate with their capacities to affect the transactivating activity of extracellular Tat (68). On this basis, we concluded that the design of specifically tailored, polysulfated saccharide analogs with high affinity for Tat protein might allow the development of potent extracellular Tat antagonists of possible therapeutic value.

These conclusions were based on increasing evidence of the involvement of extracellular Tat in different AIDS-associated disorders including Kaposi's sarcoma (27, 41), neurological disorders (42), and increased tumor incidence (28, 43). Also, the capacity of anti-Tat antibody to delay HIV-1 replication in infected peripheral blood mononucleated cells (48–50) and the inverse correlation between the levels of natural anti-Tat antibody and those of p24 antigen in HIV-seropositive subjects (48) suggest that extracellular Tat may be involved in the progression of the disease. On this basis, Tat has been recently proposed as a specific target for AIDS vaccine (80), thus supporting the potential use of Tat antagonists in HIV infection.

Our data indicate that suramin and its derivatives act as Tat antagonists by mimicking heparin/HS for its capacity to bind extracellular Tat via its basic domain. We have shown that neutralization of arginine residues 49, 52, 53, 55, 56, and 57 results in a Tat mutant characterized by a reduced affinity for heparin when compared with the wild type molecule. This was observed both when free GST-Tat$_{R49/52/53/55/56/57A}$ was evaluated for its capacity to interact with immobilized heparin or when the immobilized mutant was assessed for its interaction with free heparin. Accordingly, Scatchard plot analysis of the binding data demonstrated a 3-fold decrease in the affinity of [^{3}H]heparin for GST-Tat$_{R49/52/53/55/56/57A}$ when compared with GST-Tat. The possibility that the reduced heparin-binding capacity of GST-Tat$_{R49/52/53/55/56/57A}$ is the mere consequence of conformational changes of the mutagenized protein appears to be ruled out by the following observations. (i) Different mutations in the Tat molecule, including the deletion of 21 amino acid residues at the N terminus of the protein or of the whole sequence encoded by the second exon, do not affect significantly Tat/heparin interaction; (ii) a synthetic peptide representing the basic domain of Tat specifically prevents the interaction of free heparin with immobilized Tat; and (iii) the GST-Tat$_{R49/52/53/55/56/57A}$ mutant shows a capacity to bind thrombospondin-1 similar to that of the wild type molecule.[5] However, the neutralization of six arginine residues within the basic domain is not sufficient to abolish the heparin-binding capacity of Tat. This indicates that the remaining lysine residues in the basic region and/or other basic amino acids scattered onto the surface of the Tat protein may also contribute to heparin binding by interacting with various sulfate groups distributed along the GAG molecule. This should not be the case for the suramin derivative ^{14}C-PNU 145156E, whose smaller size would allow the interaction of its sulfonate group(s) mainly with the basic domain of Tat, as shown by its incapacity to bind the Tat$_{R49/52/53/55/56/57A}$ mutant. This observation, together with the ability of suramin, trypan blue, and various suramin-like distamycin A derivatives to displace [^{3}H]heparin from immobilized GST-Tat, supports the hypothesis that low molecular weight polysulfonated compounds compete with heparin/HS for the binding to the basic domain of the Tat molecule.

Interestingly, the basic domain mediates several biological activities of extracellular Tat. It is necessary for the mitogenic, chemotactic, and angiogenic activity exerted by Tat (21); it cooperates with the RGD motif in integrin interaction (61); and it is involved in the binding and activation of the Flk-1/KDR receptor (67). The basic domain is necessary also for Tat stability (64), nuclear and nucleolar delivery (65),[2] and interaction with nucleic acids (66). It mediates some of the neurotoxic effects of extracellular Tat (12, 33, 35). Again, some of the biological activities described for synthetic peptides mimicking the basic domain of Tat may reflect their capacity to bind ECM and cell surface HS-proteoglycans. Indeed, cell-associated HS molecules may act as receptors for extracellular Tat (21, 69).

Our results demonstrate that heparin and suramin are able to prevent cell surface binding and internalization of extracellular Tat with a consequent inhibition of its mitogenic and LTR-transactivating activity. Of particular interest is the capacity of polyanionic compounds also to inhibit cell proliferation in Tat-overexpressing T53 cells. In these cells, neutralizing anti-Tat antibodies cause approximately an 80% decrease in cell proliferation (see above), thus indicating that T53 cell proliferation depends mainly on an extracellular loop of stimulation triggered by endogenously produced Tat. Although we cannot rule out the possibility that polyanionic compounds may also interact with T53 cell-produced growth factors other than Tat, our data suggest these compounds can bind native Tat, thus interfering with its autocrine loop of stimulation. This hypothesis is supported by the capacity of polyanionic compounds to inhibit the binding of ^{125}I-GST-Tat to T53 cell surface. Also, a linear correlation exists among the ID_{50} values of heparin, suramin, and distamycin A derivatives for the competition of Tat/heparin interaction and the inhibition of T53 cell proliferation (see above). Finally, it is important to recall that suramin and heparin do not enter the cells either in the absence or in the presence of Tat (see above), ruling out the possibility that the two compounds interfere with intracellular transduction signals for mitogenesis.

The absolute potency shown by the various polyanionic compounds tested in inhibiting the different biological activities of free Tat is significantly different from that required to displace heparin from the immobilized protein. This apparent discrepancy may be related to the different experimental conditions utilized in the various biological and biochemical assays. They include, for instance, the different amount of GST-Tat present in the assays (200 ng/ml free Tat for the LTR transactivation assay *versus* a 600 μg/ml concentration of the immobilized protein in the [^{3}H]heparin/GST-Tat competition binding assay). Also, Tat is added exogenously as a GST chimera in the LTR transactivation and ^{125}I-GST-Tat binding assays, while it is produced endogenously in the native form during the T53 proliferation assay. Finally, it is important to note that heparin represents an optimal ligand for Tat when compared with other sulfated GAGs, including HS (68). This appears to be of importance when the data obtained in the [^{3}H]heparin/GST-Tat competition binding assay are compared with those obtained in the LTR transactivation and T53 cell proliferation assays. Indeed, while in the former assay polyanionic compounds must displace heparin already bound to immobilized GST-Tat, they compete with cell surface HS proteoglycans for the binding to free Tat in the latter assays.

As observed previously for sulfated GAG/Tat interaction (68), the capacity of non-GAG polysulfonated compounds to bind extracellular Tat and to inhibit its biological activity depends on the backbone structure of the molecule and, at least in part, on its degree of sulfation. We have found that polysulfated β-cyclodextrin is unable to bind Tat and to inhibit its LTR-transactivating activity although this molecule carries 14 sul-

[5] M. Rusnati, manuscript in preparation.

fated groups. On the contrary, suramin and trypan blue exert a significant Tat antagonist activity despite the fact that they carry only 6 or 4 sulfonate groups per molecule, respectively. Relevant to this point is the observation that a series of distamycin A derivatives structurally related to suramin (81) differ significantly for their Tat-binding capacity and antagonist activity. In particular, PNU 151779 and PNU 157666 are more potent than suramin in inhibiting the LTR-transactivating activity and mitogenic capacity of extracellular Tat. Like suramin, both compounds carry six sulfonate groups per molecule (Fig. 9), while PNU 151752, which carries only two sulfonate groups, is the least active among the derivatives tested. Clearly, these data point to the importance of the backbone structure of the molecule in presenting its sulfonate group(s) to Tat protein under optimal steric conditions. A detailed structure-activity relationship study will be required to define the molecular features of polysulfonated compounds suitable for the design of potent extracellular Tat antagonists. In this respect, our preliminary observations indicate the possibility of synthesizing compounds almost equipotent to natural heparin.

Previous observations had shown that polysulfated compounds, including sulfated GAGs, might act as inhibitors of HIV. For instance, heparin and dextran sulfate prevent HIV adsorption and internalization in $CD4^+$-sensitive cells (88) as a consequence of their capacity to disrupt gp120 interaction with CD4 (89, 90) and/or with cell-associated HS (91, 92). Also, polysulfated polysaccharides prevent syncytium formation between HIV-infected and uninfected cells (89, 90). This may explain the capacity of polysulfated polysaccharides to inhibit HIV-1 replication and cytopathogenicity in different experimental models (reviewed in Ref. 93). Accordingly, suramin and suramin-related compounds have been demonstrated to inhibit reverse transcriptase activity of several retroviruses and to suppress the replication and cytopathic effect of HIV at concentrations that are nontoxic for the host cells and attainable in humans (94, 95). In particular, several PNU compounds prevent virus attachment to $CD4^+$ cells and inhibit HIV-induced cell killing and viral replication (81). Interestingly, preliminary observations from our laboratory indicate that the potency of the different PNU compounds in inhibiting HIV-induced cell killing parallels their potency as extracellular Tat antagonists.[6] This correlation may be of importance for the design of "multitarget" polysulfated compounds able to affect different aspects of HIV infection and AIDS-related disorders.

The "multitargeting" activity of polysulfated compounds, synergistic with other anti-HIV drugs like AZT (93), together with their capacity to interact with the highly conserved basic region of Tat, may overcome the problems related to the heterogeneity, high rate of mutation, and drug resistance development of HIV that represent major impediments to conventional vaccination and chemotherapy against AIDS.

Acknowledgments—We thank Dr. P. Oreste and Dr. G. Zoppetti for polysulfated β-cyclodextrin, the Medical Research Council AIDS Reagent Project (Potters Bar, Herts, UK) for the synthetic peptides representing different fragments of HIV-1 Tat, and L. Pizzi and M. Stramezzi for expert technical assistance.

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