THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 25, Issue of June 22, pp. 22420–22425, 2001

Pentosan Polysulfate as an Inhibitor of Extracellular HIV-1 Tat*

Received for publication, November 29, 2000, and in revised form, February 7, 2001
Published, JBC Papers in Press, April 13, 2001, DOI 10.1074/jbc.M010779200

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HIV-1 Tat protein, released from HIV-infected cells, may act as a pleiotropic heparin-binding growth factor. From this observation, extracellular Tat has been implicated in the pathogenesis of AIDS and of AIDS-associated pathologies. Here we demonstrate that the heparin analog pentosan polysulfate (PPS) inhibits the interaction of glutathione *S*-transferase (GST)-Tat protein with heparin immobilized to a BIAcore sensor chip. Competition experiments showed that Tat-PPS interaction occurs with high affinity (K_d = 9.0 nM). Also, GST·Tat prevents the binding of [^{3}H]heparin to GST·Tat immobilized to glutathione-agarose beads. *In vitro*, PPS inhibits GST·Tat internalization and, consequently, HIV-1 long terminal repeat transactivation in HL3T1 cells. Also, PPS inhibits cell surface interaction and mitogenic activity of GST·Tat in murine adenocarcinoma T53 Tat-*less* cells. In all assays, PPS exerts its Tat antagonist activity with an ID_{50} equal to ~1.0 nM. *In vivo*, PPS inhibits the neovascularization induced by GST·Tat or by Tat-overexpressing T53 cells in the chick embryo chorioallantoic membrane. In conclusion, PPS binds Tat protein and inhibits its cell surface interaction, internalization, and biological activity *in vitro* and *in vivo*. PPS may represent a prototypic molecule for the development of novel Tat antagonists with therapeutic implications in AIDS and AIDS-associated pathologies, including Kaposi's sarcoma.

Tat protein, the transactivating factor of the human immunodeficiency virus type 1 (HIV-1),[1] is released from HIV-1 infected cells (1). Extracellular Tat can enter the cell and nucleus to stimulate the transcriptional activity of HIV-LTR (2). Moreover, extracellular Tat induces several biological effects on uninfected target cells (3). Tat has been implicated in pathological processes associated to AIDS, including Kaposi's sarcoma (KS) (4). Indeed, KS spindle cell xenografts grow faster in Tat transgenic mice than in non-transgenic animals (5). Also, Tat-overexpressing T53 cells, derived from an adenocarcinoma of a BKV/*Tat* transgenic mouse (6), are angiogenic and induce highly vascularized tumors in nude mice that resemble KS (7, 8).

Tat binds the polyanionic glycosaminoglycans (GAGs) heparin and heparan sulfate via its basic domain (9, 10). The binding of extracellular Tat to heparan sulfate proteoglycans (HSPGs) mediates cell surface interaction (11) and accumulation in the extracellular matrix (ECM) (12). Also, HSPGs are required for Tat internalization and consequent HIV-LTR transactivation (13). Conversely, the interaction of extracellular Tat with free GAGs inhibits cellular uptake and HIV-LTR transactivating activity in HL3T1 and CHO cells (13, 14), affects its mitogenic and protease-inducing activities in T53 cells (14, 15), and modulates its angiogenic activity *in vivo* (11).

Several polyanionic compounds inhibit HIV-1 replication by preventing the binding of the virus to target cells (16). Some of these compounds, including unmodified heparin, heparan sulfate, and suramin-like distamycin A derivatives, although not dextran sulfate, β-cyclodextrin, chemically desulfated or low molecular weight heparins, also bind extracellular Tat and inhibit its biological activity *in vitro* (9, 10, 14, 17) and *in vivo* (18). Thus, selected polyanionic molecules endowed with the capacity to affect HIV life cycle at different levels can be exploited for the development of novel "multitarget" anti-AIDS drugs.

The heparin analog xylanopolyhydrogensulfate (pentosan polysulfate, PPS) prevents the interaction of HIV-1 with target cells (19) and inhibits its reverse transcriptase activity (20). Also, PPS affects tumorigenicity and angiogenic potential of KS xenografts in nude mice (21). The capacity of PPS to inhibit the biological activity of various heparin-binding angiogenic growth factors, including fibroblast growth factor-2 (FGF-2) (22–25) raises the possibility that PPS may interfere additionally with extracellular Tat protein. To assess this possibility, as a part of a long-term project aimed to identify polyanionic multitarget HIV-Tat antagonists (9, 10, 13–15, 17), we evaluated the capacity of PPS to bind Tat protein and to inhibit the biological activity of extracellular Tat *in vitro* and *in vivo*. The results demonstrate that PPS binds Tat and prevents its inter-

* This work was supported in part by Grant QLK3-CT-1999-00536 from the European Community, Istituto Superiore di Sanità (AIDS Project), Associazione Italiana per la Ricerca sul Cancro, National Research Council (Target Project on Biotechnology) (to M. P.), from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica 2000 (to M. P. and M. R.), and from the Agence Nationale de la recherche sur le SIDA (to H. L-J.). 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.

‡ Recipient of a fellowship from the AIDS Project.

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[1] The abbreviations used are: HIV-1, human immunodeficiency virus type 1; CAT, chloramphenicol acetyltransferase; CAM, chick embryo chorioallantoic membrane; 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; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; LTR, long terminal repeats; KS, Kaposi's sarcoma; PPS, pentosan polysulfate; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; RU, resonance units; ELISA, enzyme-linked immunosorbent assay.

action with heparin. Accordingly, PPS inhibits cell surface interaction and internalization of extracellular Tat causing the inhibition of HIV-LTR-transactivating activity, mitogenic capacity, and angiogenic potential of the protein. These observations identify PPS as a prototypic molecule for the design of novel multitarget drugs for the treatment of AIDS and AIDS-associated pathologies.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant HIV-1 Tat (86-amino acid isoform) was expressed in *Escherichia coli* as glutathione *S*-transferase (GST) fusion protein (9). GST·Tat was also fused at its C terminus to the green fluorescent protein (GFP) (14). The GST and GFP moieties do not interfere with LTR-transactivating activity and heparin-binding capacity of Tat (13, 14). Pentosan polysulfate was from Sigma (St Louis, MO). Heparin was from Laboratori Derivati Organici SpA, (Milan, Italy). β-Cyclodextrin tetradecasulfate was from Glycores 2000 (Milan, Italy). Fluorescein-labeled bovine serum albumin (FITC-BSA) was kindly provided by Prof. S. Marchesini (University of Brescia, Italy).

BIAcore Binding Assay—Surface plasmon resonance (SPR) was used to measure changes in refractive index caused by the capacity of free PPS to bind GST·Tat and prevent its interaction with heparin immobilized to a BIAcore sensor chip (26, 27). To this purpose, size-defined heparin (9 kDa) was biotinylated on its reducing end, and a flow cell of an F1 sensor chip was activated with streptavidin. Then, biotinylated heparin was allowed to react with the streptavidin-coated sensor chip. GST·Tat alone or in the presence of increasing concentrations of PPS was then injected over the heparin surface for 5 min to allow the association of the protein with heparin and then washed until dissociation was observed. The SPR signal was expressed in terms of resonance units (RU).

Preparation of ^{3}H-labeled Heparin and GST·Tat Affinity Chromatography—Heparin was ^{3}H-labeled as described (28) with a specific radioactivity of 5,000 cpm/nmol. To assay the capacity of PPS to compete with [^{3}H]heparin for the binding to immobilized GST·Tat, [^{3}H]heparin was loaded onto a GST·Tat-glutathione-agarose column in the presence of increasing concentrations of PPS, β-cyclodextrin, or unlabeled heparin. The column was then washed with phosphate-buffered saline and eluted with 2.0 M NaCl. Radioactivity in the eluate was measured in a liquid scintillation counter.

Cell Cultures—HL3T1 cells are derived from HeLa cells and contain integrated copies of pL3CAT, a plasmid in which the chloramphenicol acetyltransferase (CAT) bacterial gene is driven by HIV-1 LTR (29). The T53 cell line was established from adenocarcinoma of skin adnexa of Tat transgenic mice and secretes biologically active Tat (6, 7). T53 Tat-*less* cells, obtained by subcloning T53 cells, do not produce detectable amounts of extracellular Tat but retain the capacity to proliferate when exposed to exogenous Tat (14). All cell types were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (DMEM/FCS, Life Technologies, Inc., Grand Island, NY).

LTR-CAT Transactivation Assay—HL3T1 cells were seeded in 24-well dishes at 20,000 cells/cm^2 in DMEM/FCS. After 24 h, cells were incubated for another 24 h period in fresh DMEM/FCS and 100 μM chloroquine in the absence or in the presence of 200 ng/ml of GST·Tat and increasing concentrations of the polysulfated compound that was to be tested. Then, conditioned medium was removed, and cells were incubated for an additional 24 h in DMEM/FCS. In some experiments, subconfluent cultures of HL3T1 cells were lipofectin-transfected with 2 μg/ml of pCEP-Tat expression vector harboring the HIV-1 Tat cDNA (kindly provided by Dr. A. Gualandris, University of Torino, Italy). Five hours after transfection, cells were washed and incubated for another 24 h in DMEM/FCS in the absence or presence of PPS (100 nM). At the end of the incubation, the amount of CAT protein present in the cell extracts was determined using the CAT ELISA kit (Roche Molecular Biochemicals).

T53 Cell Proliferation Assays—T53 Tat-*less* cells were seeded in 96-well dishes at 10,000 cells/cm^2 in DMEM/FCS. After 24 h, cells were incubated for another 24 h in fresh DMEM/FCS in the absence or presence of 200 ng/ml of GST·Tat and increasing concentrations of the polysulfated compound being tested. At the end of the incubation, cells were trypsinized and counted in a Burker chamber.

Internalization of GST·Tat·GFP in HL3T1 Cells—HL3T1 cells adherent to glass coverslips were incubated for 6 h at 37 °C in DMEM/FCS containing 400 ng/ml of GST·Tat·GFP in the absence or presence of increasing concentrations of the polysulfated compound being tested. At the end of the incubation, the cells were washed and fixed. GST·Tat·GFP internalization was quantified by computerized image analysis as described (14).

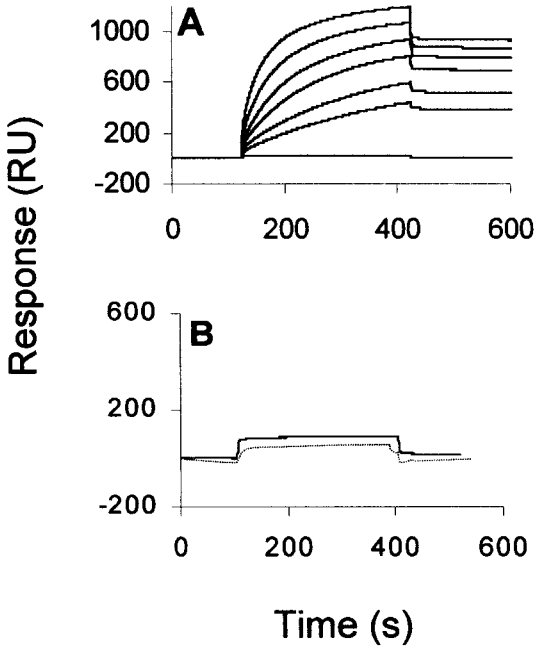

FIG. 1. **Interaction of GST·Tat with biotinylated heparin immobilized onto BIAcore sensor chip:** *A,* GST·Tat was injected at (*from top curve to bottom*) 20, 13.3, 8.9, 5.9, 3.9, 2.6, and 0 μg/ml for 5 min over a BIAcore sensor chip containing streptavidin plus 120 resonance units (*RU*) of immobilized biotinylated heparin. *B,* GST·Tat (*solid line*) or GST alone (*dotted line*) (both at 5 μg/ml) were injected for 5 min over a streptavidin surface or a streptavidin-heparin surface, respectively. In both experiments, the responses (in RU) were recorded as a function of time.

Binding of GST·Tat·GFP to T53 Tat-less Cells—T53 Tat-*less* cells adherent to glass coverslips (20,000/cm^2) were incubated for 6 h at 37 °C in DMEM/FCS and 400 ng/ml of GST·Tat·GFP in the absence or presence of PPS (1 μM). At the end of the incubation, the medium was removed, and cells were washed and fixed as described (8). Observations were carried out in a Nikon photomicroscope equipped for epifluorescence.

Chick Embryo Chorioallantoic Membrane (CAM) Assay—The CAM assay was performed as described (30). Briefly, a window was opened in the egg shell of 3-day-old fertilized chicken eggs. At day 8, gelatin sponges (Gelfoam; Upjohn Co., Kalamazoo, MI) were implanted on the CAMs and adsorbed with 10 μl of phosphate-buffered saline alone or containing GST or GST·Tat (both at 400 ng/sponge) in the absence or presence of PPS (25 μg/sponge; 5–6 embryos per group). In parallel experiments, the sponges were adsorbed with 10 μl of DMEM/FCS alone or containing T53 cells (6×10^4 cells/sponge) in the absence or presence of 25 μg of PPS (5–6 embryos per group). After 4 days, CAMs were observed *in ovo* under a Zeiss SR stereomicroscope, and the angiogenic response was scored by two investigators without knowledge of the samples tested and graded on an arbitrary scale of 0–4+, with 0 representing no angiogenic response and 4+ representing the strongest activity.

RESULTS

PPS Binds Tat Protein—To assess its ability to interact with Tat protein, PPS was evaluated for the capacity to prevent GST·Tat binding to biotinylated heparin immobilized onto a streptavidin-activated BIAcore sensor chip. Preliminary experiments were performed to assess the ability of GST·Tat to bind immobilized heparin. In a typical experiment, increasing concentrations of GST·Tat were injected over the heparin surface, and a set of sensograms was obtained (Fig. 1*A*). An association rate constant (k_{on}) equal to 4.2×10^4 M^{-1} s^{-1} and a slow dissociation rate constant (k_{off}) equal to 2.7×10^{-3} s^{-1} characterized the interaction of GST·Tat with immobilized heparin. Thus, GST·Tat-heparin interaction occurs with high affinity (dissociation constant (K_d) = k_{off}/k_{on} = 64 nM) and results in the

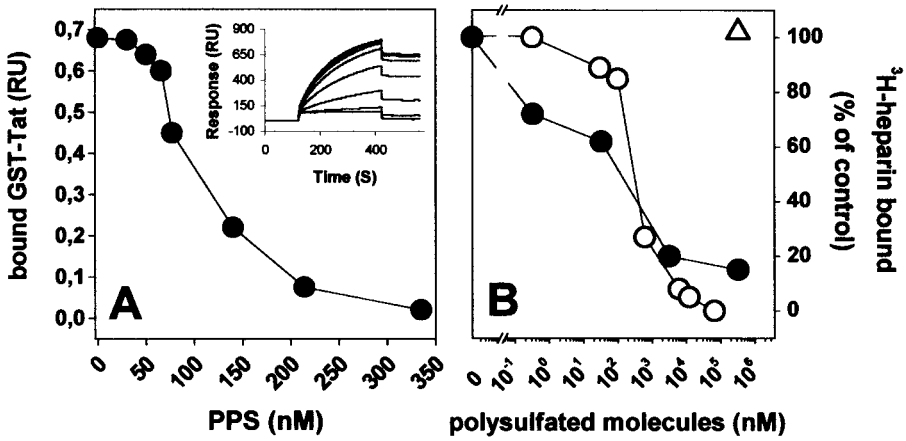

FIG. 2. **Interaction of PPS with GST·Tat.** Competition binding assays: *A,* surface plasmon resonance, GST·Tat (5 μg/ml) alone or in the presence of increasing concentrations of PPS was injected over a flow cell of a BIAcore sensor chip containing streptavidin plus 120 RU of immobilized biotinylated heparin. The responses (in RU) were plotted as a function of PPS concentration. *Inset*, overlay of sensograms showing the binding of GST·Tat to immobilized heparin in the presence of increasing concentrations of PPS (0, 0.085, 0.13, 0.2, 0.3, 0.45, 0.66, 1.0 μg/ml; *from top curve* to *bottom*). *B,* GST·Tat glutathione-agarose affinity chromatography, GST·Tat-glutathione-agarose columns (80 μl) were loaded with 300 μl samples containing 25 μg of [^{3}H]heparin alone or added with increasing concentrations of unlabeled heparin (○), PPS (●), or polysulfated β-cyclodextrin (△). Radioactivity in the 2.0 M NaCl eluate was measured in a liquid scintillation counter. Nonspecific binding, measured in the presence of an excess of unlabeled heparin (1.0 mM) was subtracted from all the values. Each point is the mean of 2–4 determinations performed in duplicate. The S.E. never exceeded 6% of the mean value.

formation of highly stable complexes. This interaction is specific because GST protein devoid of the Tat moiety does not bind to immobilized heparin, and GST·Tat does not bind to a streptavidin surface in the absence of immobilized heparin (Fig. 1*B*). In some experiments, the association phase of GST·Tat-heparin interaction was allowed to proceed to equilibrium, and the data were used to calculate an affinity value independent of the kinetics of binding. This analysis demonstrates that GST·Tat-heparin interaction occurs with a K_d equal to 58 nM, a value consistent with our kinetics analysis and with previous determinations obtained with different experimental approaches (14). On this basis, we evaluated the capacity of PPS to compete with immobilized heparin for the binding to GST·Tat. To this purpose, increasing concentrations of PPS were preincubated with GST·Tat and then injected onto the heparin-coated sensor chip. As shown in Fig. 2*A*, PPS causes a dose-dependent inhibition of GST·Tat-heparin interaction, with an ID_{50} equal to 120 nM. The binding data allowed the calculation of the relative affinity of PPS-GST·Tat interaction (K_d is equal to 9.0 nM).

PPS was also evaluated for its capacity to compete with free [^{3}H]heparin for the binding to GST·Tat immobilized onto glutathione-agarose beads. As shown in Fig. 2*B*, PPS inhibits the binding of [^{3}H]heparin to GST·Tat in a dose-dependent manner with a potency similar to unlabeled heparin (ID_{50} equal to 100 and 300 nM, respectively). Under the same experimental conditions, the polysulfated β-cyclodextrin, here used as a negative control (10), did not prevent the binding of [^{3}H]heparin to immobilized GST·Tat even when tested at doses as high as 300 μM (Fig. 2*B*). Taken together, the data demonstrate that PPS binds Tat in a specific manner with an affinity comparable with that of Tat-heparin interaction.

PPS Inhibits Cell Internalization and HIV-LTR Transactivating Activity of Extracellular Tat—The capacity of PPS to abolish the interaction of Tat with immobilized heparin raised the possibility that PPS may prevent the binding of extracellular Tat to cell-associated heparin-like HSPGs that are required for its internalization (13). To assess this hypothesis, we evaluated the capacity of PPS to inhibit cell internalization of GST·Tat conjugated to fluorescent GFP. As shown in Fig. 3, PPS inhibits the internalization of GST·Tat·GFP in HL3T1 cells in a dose-dependent manner with an ID_{50} equal to 1.0 nM, close to that observed for heparin (ID_{50} = 3.0 nM). When tested under the same experimental conditions, PPS does not affect the uptake of fluorescein-labeled BSA, as assessed by image analysis of the total fluorescence internalized by HL3T1 cells incubated with 400 ng/ml of FITC-BSA in the absence or in the presence of 100 nM PPS (1.21 ± 0.48 and 1.92 ± 0.67 arbitrary units, respectively). It must be pointed out that ~90% of GST·Tat·GFP or FITC-BSA remained associated to the cells after a 2.0 M NaCl wash of the cell monolayer, thus confirming the intracellular localization of the fluorescent proteins (data not shown). The specificity of PPS-Tat interaction was demonstrated further by the inability of polysulfated β-cyclodextrin to affect Tat internalization (Fig. 3*A*).

In agreement with its capacity to inhibit Tat internalization, PPS abolished the LTR-CAT transactivating activity exerted by GST·Tat in HL3T1 cells whereas β-cyclodextrin was ineffective (Fig. 4*A*). It should be noted that 100 nM PPS did not affect the basal levels of LTR-CAT transactivation measured in HL3T1 cells incubated in the absence of GST·Tat (0.166 ± 0.036 and 0.170 ± 0.034 $A_{405\ nm}$ units for PPS-untreated and -treated cells, respectively). Also, no inhibition of LTR-CAT transactivation was observed when Tat was expressed as an intracellular protein in PPS-treated HL3T1 cells following transient transfection with an expression vector harboring the HIV-1 Tat cDNA (Fig. 4*B*). These data demonstrate that the antagonist effect of PPS on GST·Tat-mediated LTR-CAT transactivation is specific and depends on an extracellular interaction with Tat protein.

To investigate further the mechanism(s) of action of PPS on extracellular Tat activity, PPS was administered to HL3T1 cells together with GST·Tat or at different times after the transactivating factor. As shown in Fig. 4*C*, the LTR-transactivating activity of Tat is completely abolished when PPS was administered within the first 2 h following the beginning of GST·Tat treatment. Addition of PPS to the cell culture medium at later time points instead causes a progressive decrease of its antagonist activity that depends on an early interaction with extracellular Tat. Similar results were obtained when PPS was replaced by heparin as Tat antagonist (Fig. 4*C*).

PPS Inhibits Cell Surface Binding and Mitogenic Activity of Extracellular Tat—Endogenous Tat induces cell proliferation in T53 cells derived from an adenocarcinoma of the skin adnexa

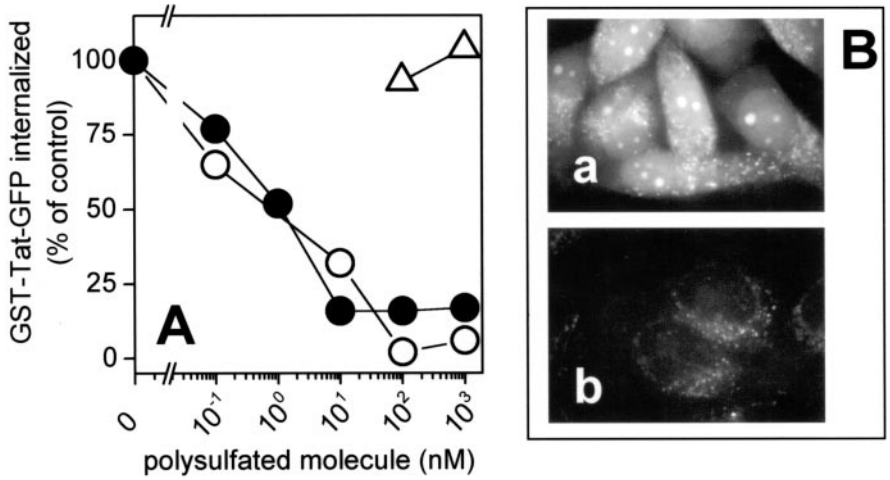

FIG. 3. **Effect of PPS on GST·Tat·GFP internalization in HL3T1 cells.** *A,* subconfluent cell cultures were treated with GST·Tat·GFP (400 ng/ml) in the absence or presence of increasing concentrations of PPS (●), heparin (○), or polysulfated β-cyclodextrin (△). Then, the amount of GST·Tat·GFP internalized was evaluated by image analysis. Data are expressed as percent of GST·Tat·GFP internalized in the absence of polysulfated molecules. Each point is the mean of 3–6 determinations performed in duplicate. The S.E. never exceeded 9–16% of the mean value. *B,* microphotographs (original magnification × 600) of HL3T1 cells treated with GST·Tat·GFP alone (*a*) or in the presence (*b*) of PPS (100 nM).

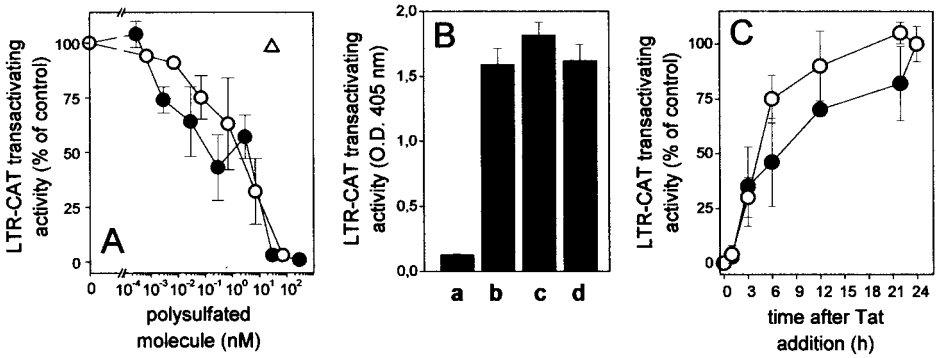

FIG. 4. **Effect of PPS on the transactivating activity of GST·Tat in HL3T1 cells.** *A,* subconfluent cell cultures were treated with GST·Tat (200 ng/ml) in the presence of increasing concentrations of PPS (●), heparin (○), or polysulfated β-cyclodextrin (△). Then, cell extracts were assayed for levels of CAT antigen by ELISA, and data were expressed as percent of the transactivating activity measured in cultures treated with GST·Tat alone. Each point is the mean ± S.E. of 2–8 determinations in duplicate. *B,* subconfluent cell cultures were transiently transfected with the pCEP-Tat expression vector devoid (*a*) or harboring the HIV-1 Tat cDNA (*b–d*). After transfection, cells were incubated in fresh medium in the absence (*a* and *b*) or in the presence of PPS (*c*) or heparin (*d*), both at 100 nM. Then, cell extracts were assayed for the levels of CAT antigen by ELISA. Each point is the mean ± S.E. of three determinations in duplicate. *C,* subconfluent cell cultures were treated with 200 ng/ml of GST·Tat. At the indicated periods of time after the beginning of GST·Tat treatment, cells were administered with PPS (●) or heparin (○) (both at 100 nM). Forty-eight hours after the beginning of GST·Tat treatment, cell extracts were assayed for levels of CAT antigen by ELISA, and data were expressed as percent of the transactivating activity measured in cultures treated with GST·Tat alone. Each point is the mean ± S.E. of three determinations in duplicate.

of a *tat* transgenic mouse (6). We have characterized a subclone of T53 cells, named T53 Tat-*less* cells, that does not produce significant amounts of endogenous Tat but that retains the capacity to proliferate when exposed to exogenous Tat (14). Also, the mitogenic activity exerted by Tat in T53 Tat-*less* cells is inhibited by heparin (14). On this basis, the Tat antagonist activity of PPS was evaluated in this model. As shown in Fig. 5*A*, PPS inhibits the binding of GST·Tat·GFP to the surface of T53 Tat-*less* cells. Approximately 80% of cell-associated fluorescence was removed by a 2.0 M NaCl wash of the cell monolayer, thus confirming the extracellular nature of GST·Tat·GFP (data not shown). In agreement with these observations, PPS inhibits the mitogenic activity exerted by GST·Tat with a potency similar to that shown by heparin (ID_{50} equal to 1.0 nM for both compounds) (Fig. 5*B*). On the contrary, PPS does not affect T53 Tat-*less* cell proliferation induced by 10% FCS (Fig. 5*B*).

PPS Inhibits the Angiogenic Activity of Extracellular Tat—Extracellular Tat is endowed with angiogenic potential *in vivo* (7, 8, 11, 17). Accordingly, GST·Tat induces neovascularization in the chick embryo chorioallantoic membrane (CAM) (Fig. 6*A*). This activity is specific because GST devoid of the Tat moiety does not exert any effect when tested under the same experimental conditions (Fig. 6*A*). In agreement with its *in vitro* antagonist activity, PPS caused a significant inhibition of GST·Tat-induced neovascularization when tested *in vivo* at the dose of 25 μg/implant (Fig. 6*A*). PPS also inhibited the unstimulated, physiological vascularization of the CAM that is mediated by endogenous FGF-2 produced by the chick embryo (31).

Tat-overexpressing parental T53 cells exert a significant angiogenic response when implanted on the top of the CAM (Fig. 6*B*), a biological effect because of the release of bioactive Tat protein (7, 8, 17). Again, PPS was able to suppress neovascularization induced by T53 cells when tested at the dose of 25 μg/implant (Fig. 6, *B* and *C*).

DISCUSSION

Tat protein has been considered as a target for anti-AIDS therapies aimed to block its intracellular interaction with RNA target structures and various intracellular cofactors required for transactivation of the viral genome (32). More recently, an increasing body of evidence point to a role for extracellular Tat in the progression of AIDS and AIDS-associated pathologies (3). This suggests that the use of extracellular Tat antagonists may be of therapeutic benefit for AIDS patients. Accordingly, the development of anti-Tat vaccines has been undertaken (33, 34). Also, *in vitro* and *in vivo* studies have underscored the possibility of using polysulfated/polysulfonated compounds as extracellular Tat antagonists (10, 14, 17, 18, 35). Here, we provide experimental evidence that PPS binds extracellular Tat with high affinity (K_d = 9.0 nM), similar to the physiological

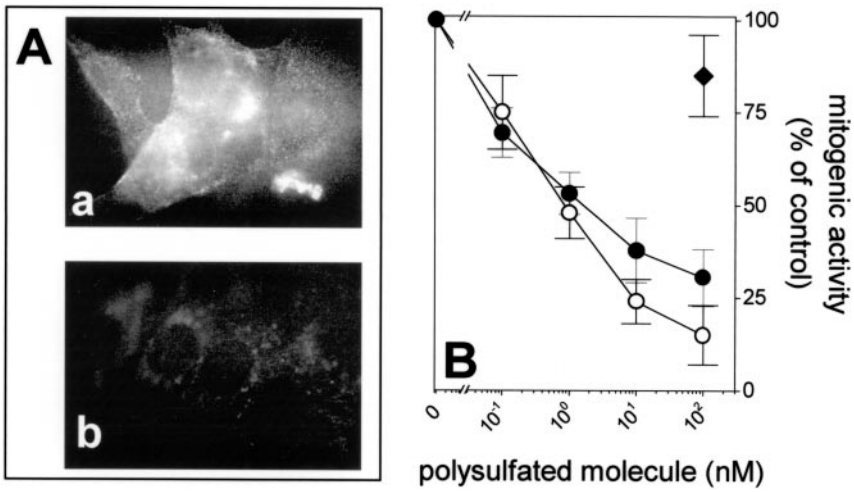

FIG. 5. **Effect of PPS on cell surface binding and mitogenic activity of Tat in T53 Tat-*less* cells.** *A,* subconfluent cell cultures were treated with GST·Tat·GFP (400 ng/ml) in the absence (*a*) or in the presence (*b*) of 1 μM PPS. Then, cells were fixed and microphotographed (original magnification × 600). *B,* subconfluent cell cultures were treated with GST·Tat (200 ng/ml) in the presence of increasing concentrations of PPS (●) or heparin (○). Parallel cultures were treated with DMEM/FCS alone in the absence or presence (♦) of 100 nM PPS. Then, cells were trypsinized and counted in a Burker chamber. In a typical experiment, cells incubated with DMEM/FCS alone or added with GST·Tat undergo 0.8 and 1.6 cell population doublings, respectively. Cell proliferation was expressed as percent of the cell number measured in PPS-untreated cell cultures. Each point is the mean ± S.E. of three determinations in duplicate.

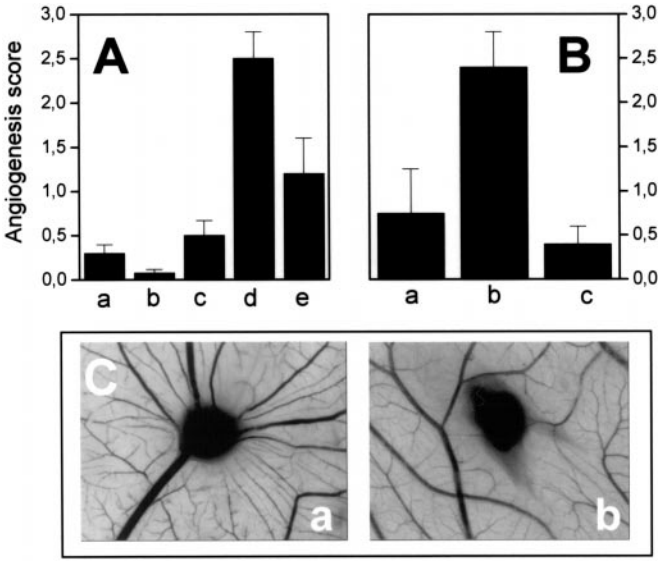

FIG. 6. **PPS inhibits Tat-induced angiogenesis in the chick embryo CAM.** *A,* CAMs were implanted with gelatin sponges adsorbed with vehicle in the absence (*a*) or presence (*b*) of PPS (25 μg), with 400 ng of GST protein (*c*), or with 400 ng of GST·Tat in the absence (*d*) or presence (*e*) of PPS (25 μg). *B,* CAMs were implanted with gelatin sponges adsorbed with vehicle (*a*) or with 6×10^4 T53 cells in the absence (*b*) or presence (*c*) of PPS (25 μg). Angiogenic response was graded on an arbitrary scale of 0–4+, with 0 representing no angiogenic response and 4+ representing the strongest activity. Each *bar* represents the mean ± S.E. of two independent experiments. *C,* representative CAMs implanted with gelatin sponges adsorbed with 6×10^4 parental T53 cells in the absence (*a*) or presence (*b*) of PPS were fixed by a 2-h incubation with Bouin fluid, removed from the egg, and placed on a diascope. The images were input via TV camera mounted onto the stereomicroscope and digitalized by the Image Pro-Plus analysis system (original magnification × 5).

ligand heparin (14). This interaction inhibits the binding of Tat to free and surface-immobilized heparin. Accordingly, PPS inhibits cell internalization of extracellular Tat and its biological activity in different experimental models. However, PPS does not inhibit LTR-CAT transactivation exerted by endogenous intracellular Tat and does not affect FITC-BSA internalization. Also, PPS inhibits the mitogenic activity exerted by extracellular Tat in T53 cells without affecting cell proliferation induced by serum. Taken together these data support the hypothesis that the antagonist activity of PPS is mainly caused by a specific interaction with extracellular Tat protein.

The interaction of extracellular Tat with cell-associated HSPGs allows the accumulation of the bioactive protein in the ECM and mediates its interaction with the cell surface (11, 12), leading to cell internalization of the protein and LTR-transactivation (13). The capacity of PPS to prevent the binding of GST·Tat to immobilized heparin indicates that PPS may hamper HSPG-mediated interaction of extracellular Tat with target cells. Accordingly, PPS inhibits the internalization of extracellular Tat in HL3T1 cells and its association with the surface of T53 Tat-*less* cells. Consequently, PPS antagonizes the transactivating and mitogenic activity exerted by extracellular Tat in the two cell lines, respectively. Association with free heparin is required for the chemotactic and angiogenic activity of extracellular Tat (11). Together with its ability to inhibit FGF-2-mediated angiogenesis, the capacity of PPS to prevent the binding of Tat to free heparin may contribute to the angiostatic activity exerted by PPS *in vivo*.

PPS retains its Tat antagonist activity when added to HL3T1 cells 2 h after the administration of the transactivating factor, when extracellular Tat is already bound to the cell surface (10). This appears to be of particular importance when considering that significant amounts of Tat can be avidly sequestered by ECM HSPGs (12), as indicated by the slow dissociation rate of the Tat-immobilized heparin complex. It should be pointed out that, at variance with PPS, thrombospondin-1 retains its Tat antagonist activity only when the transactivating factor is present in the extracellular environment as a free protein, being ineffective when Tat is bound to the cell surface (8). It is also interesting to note that the high stability of the Tat-heparin complex appears to be a unique feature of this transactivating factor. Indeed, different heparin-binding growth factors and cytokines, including FGF-2 (36), stromal cell-derived factor 1α (SDF-1α) (27), and RANTES,[2] dissociate rapidly from the GAG.

The capacity of sulfated GAGs and polysulfonated compounds to bind extracellular Tat and to inhibit its biological activity depends on the backbone structure and, at least in

[2] H. Lortat-Jacob, unpublished observations.

part, on the degree of sulfation (9, 10). Indeed, a series of distamycin A derivatives structurally related to suramin but characterized by different chemical structure and degree of sulfation differ significantly for their Tat antagonist activity (10). Here, we have demonstrated that PPS binds Tat and inhibits its biological activity with a potency similar to that shown by heparin and 1,000 times higher than that of suramin (10, 14). These data indicate that the backbone structure of PPS presents its sulfate group(s) to Tat protein under optimal steric conditions, resembling more closely heparin interaction.

PPS inhibits the receptor binding and mitogenic activity of FGF-1 and FGF-2, being ineffective on cell proliferation mediated by IGF-1 or TGF-α. In contrast, suramin exerts a nonselective inhibitory effect at doses 100 times higher than that of PPS (23). Previous observations had shown that PPS binds also HIV gp120, thus preventing its interaction with cellular CD4 (19), and inhibits HIV-induced syncytium formation (37) and lymphocyte-to-epithelial transmission of HIV-1 (38). Moreover, PPS inhibits reverse transcriptase activity (20). Here we demonstrate that PPS is a potent extracellular Tat antagonist at doses significantly lower than those required to inhibit blood coagulation (21). Accordingly, phase I and II clinical trials have shown that PPS is well tolerated and may cause stabilization of the disease in AIDS-related KS patients (39–41). Taken together, the data indicate that PPS may represent a prototypic molecule for the design of novel multitarget extracellular Tat antagonists to be used for the treatment of AIDS and AIDS-associated pathologies. In particular, the potency and specificity shown by PPS in inhibiting the biological activity of Tat and FGF-2 may be an advantage for the treatment of AIDS-related KS where both proteins appear to contribute to the progression of the disease (42).

Acknowledgments—We thank A. Corallini for parental T53 cells and B. Musulin and A. Bugatti for expert technical assistance.

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