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Heparin-Mimicking Sulfonic Acid Polymers as Multitarget Inhibitors of Human Immunodeficiency Virus Type 1 Tat and gp120 Proteins[▽]

Antonella Bugatti,[1] Chiara Urbinati,[1] Cosetta Ravelli,[1] Erik De Clercq,[2] Sandra Liekens,[2] and Marco Rusnati[1]*

Unit of General Pathology and Immunology, Department of Biomedical Sciences and Biotechnology, School of Medicine, University of Brescia, Brescia, Italy,[1] and Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium[2]

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Human immunodeficiency virus (HIV) Tat and gp120 intriguingly share the feature of being basic peptides that, once released by HIV$^+$ cells, bind to polyanionic heparan sulfate proteoglycans (HSPGs) on target uninfected cells, contributing to the onset of AIDS-associated pathologies. To identify multitarget anti-HIV prodrugs, we investigated the gp120 and Tat antagonist potentials of a series of polyanionic synthetic sulfonic acid polymers (SSAPs). Surface plasmon resonance revealed that SSAPs inhibit with a competitive mechanism of action the binding of Tat and gp120 to surface-immobilized heparin, an experimental condition that resembles binding to cellular HSPGs. Accordingly, SSAPs inhibited HSPG-dependent cell internalization and the transactivating activity of Tat. Little is known about the binding of free gp120 to target cells. Here, we identified two classes of gp120 receptors expressed on endothelial cells, one of which was consistent with an HSPG-binding, low-affinity/high-capacity receptor that is inhibited by free heparin. SSAPs inhibited the binding of free gp120 to endothelial cells, as well as its capacity to induce apoptosis in the same cells. In all the assays, poly(4-styrenesulfonic acid) (PSS) proved to be the most potent antagonist of Tat and gp120. Accordingly, PSS bound both proteins with high affinity. In conclusion, SSAPs represent an interesting class of compounds that bind both gp120 and Tat and inhibit their HSPG-dependent cell surface binding and pathological effects. As these activities contribute to both AIDS progression and associated pathologies, SSAPs can be considered prototypic molecules for the development of multitarget drugs for the treatment of HIV infection and AIDS-associated pathologies.

The clinical features that characterize AIDS cannot be ascribed to simple CD4$^+$ cell infection by human immunodeficiency virus (HIV). Indeed, several distinct pathological diseases arise in HIV-infected individuals (references 44 and 45 and references therein), caused by the actions of viral products (such as the HIV type 1 [HIV-1] transactivating factor [Tat] and the envelope gp120 protein) that, once released by HIV-infected cells into the extracellular environment, target HIV-nonpermissive cell types, altering their functions and thus contributing to the rise of AIDS-associated pathologies.

In the last few years, highly active antiretroviral therapy has significantly impacted the health of AIDS patients, prolonging their life expectancy mainly by delaying a drop in CD4$^+$ cells. A drawback, however, is the fact that the improved survival has increased the incidence of AIDS-associated diseases. This, together with the fact that highly active antiretroviral therapy suffers from cost, patient compliance, deleterious side effects, and the development of drug resistance, calls for novel complementary therapeutical targets/drugs.

Tat and gp120 act as the main transactivator of the viral genome (20) and the main determinant of viral infectivity (11), respectively. Also, they are both released by HIV-infected cells (25, 63). In their extracellular forms, Tat and gp120 target different non-HIV-infected/HIV-nonpermissive cells, contributing to the development of several AIDS-associated pathologies. In particular, Tat and gp120 cooperate in inducing oxidative stress in blood-brain barrier endothelial cells (ECs) (38) and apoptosis in cardiomyocytes (18), neurons (53), and keratinocytes (1). Taken together, these observations provide the opportunity to develop multitargeted therapies aimed at blocking gp120 and Tat simultaneously. These therapies, dealing at one time with both AIDS progression and AIDS-associated pathologies, would provide improved therapeutical benefits to AIDS patients.

Tat is a cationic 86- to 101-amino-acid polypeptide that, once released, can enter latently HIV-infected cells, activating the transcription of the viral genome and contributing to the "burst" of replication associated with the early phases of HIV infection, where synchronized virion replication takes place (35). Also, extracellular Tat promotes HIV-1 coreceptor expression (51), inducing a self-perpetuating permissivity for HIV-1 infection. Finally, extracellular Tat targets different HIV-nonpermissive cells, causing a variety of biological effects related to AIDS-associated pathologies, such as central and peripheral neuropathies, immune suppression, and tumorigenesis (14, 34). Tat interacts with at least four classes of receptors present on the surfaces of different target cells: cell adhesion receptors of the integrin family (58); the vascular endothelial growth factor receptors Flt-1 and Flk-1/KDR (4); the chemokine receptors CCR2, CCR3, and CXCR4 (3, 65); and heparan sulfate proteoglycans (HSPGs) that, by binding Tat, increase

* Corresponding author. Mailing address: General Pathology and Immunology, Department of Biomedical Sciences and Biotechnology, School of Medicine, University of Brescia, viale Europa 11, 25123 Brescia, Italy. Phone: 39-0303717315. Fax: 39-0303701157. E-mail: rusnati@med.unibs.it.

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its local concentration at the extracellular matrix (ECM) and mediate its internalization, transactivating activity, and several other pathological effects (reference 45 and references therein).

HIV-embedded gp120 binds CD4, chemokine receptors (CCR5 and CXCR4), and HSPGs (11, 33, 40) present on the surfaces of HIV-permissive cells. gp120-mediated interaction of HIV with HSPGs leads to the adsorption of the virus to the cell surface, with a consequent increase in infectivity (6). Along with Tat, gp120 is also released by HIV-infected cells (reference 25 and references therein). In its extracellular free form, gp120 targets several HIV-nonpermissive cell types. Acting on neurons, it contributes to AIDS dementia (12). It also induces EC apoptosis (23) and alters the permeability of the blood-brain barrier (9) and the interaction of ECs with lymphocytes (7). It contributes to HIV cardiomyopathy (55) and to the accelerated bone resorption that leads to osteopenia and osteoporosis in HIV-seropositive patients (17). Unlike Tat, few data are available about the receptors that mediate the pathological effects of the extracellular free form of gp120.

At a molecular level, a "basic domain" is present in both Tat and gp120. In the C terminus of Tat, there is a stretch of Arg and Lys residues that is highly immunoreactive, conserved among the different HIV strains (21), and implicated in the interaction of Tat with different receptors, including HSPGs (reference 44 and references therein). The basic domain is necessary for Tat nuclear delivery (15), for interaction with nucleic acids (39), and for its neurotoxic (36), mitogenic, chemotactic, and angiogenic activities (2). Within the C terminus of gp120 is located the so-called "variable loop 3," which, despite its variability among the virus strains, maintains a strong basic nature (37). Variable loop 3 mediates HIV-embedded gp120 binding to cell surface HSPGs, CD4, and coreceptors and is thus implicated in HIV infection (33).

The basic natures of Tat and gp120 raised the possibility of using polyanionic heparin-like compounds as extracellular antagonists of the two viral proteins. In effect, unmodified, chemically modified, and biotechnological heparins (41, 59), suramin and distamycine derivatives (47), pentosan polysulfate (48), and dextrin-2-sulfate (62) have been demonstrated to bind Tat and to inhibit some of its biological activities. Similarly, unmodified, chemically modified, and biotechnological heparins (28, 33, 60), dextran sulfate (52), suramin (50), distamycine derivatives (10), and pentosan polysulfate (30) have been demonstrated to bind gp120, inhibiting HIV infection. No attempt has yet been made to evaluate polyanions as antagonists of the extracellular free form of gp120.

Synthetic sulfonic acid polymers (SSAPs) are a class of polyanionic heparin-mimicking compounds that have already been demonstrated to inhibit HIV-1 cytopathicity (32). Although the mechanism(s) of this inhibition has not been fully elucidated, a direct interaction of the SSAPs with HIV-embedded gp120 may be involved (32). On the other hand, SSAPs bind fibroblast growth factor 2 (FGF2) in the extracellular environment, hampering its interaction with ECs and inhibiting some of its biological effects in vitro and in vivo (26, 27). Relevant to this point, FGF2 shares with Tat several biological and biochemical features (43). On these bases, we investigated the capacity of SSAPs to bind the extracellular free forms of Tat and gp120 and to inhibit their cell interaction and biological activities.

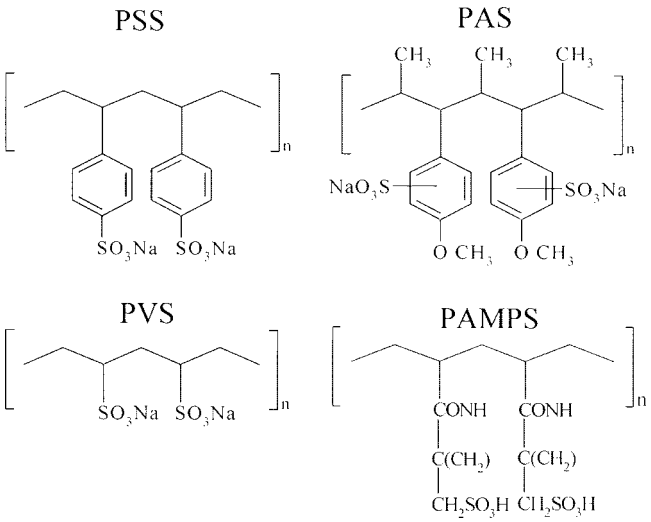

FIG. 1. Chemical structures of the SSAPs used in this study.

MATERIALS AND METHODS

Reagents. The 86-amino-acid Tat protein was expressed and purified from *Escherichia coli* as a glutathione *S*-transferase fusion protein (GST-Tat) or as GST-Tat fused to the green fluorescent protein (GST-Tat-GFP), as previously described (46). GST and GFP moieties do not interfere with the heparin-binding and long-terminal-repeat (LTR)-transactivating activities of Tat (48). gp120 was from the MRC AIDS Reagent Project (Potters Bar, United Kingdom). Biotinylated gp120 was provided by J. Arthos (NIH, Bethesda, MD). Poly(2-acrylamido-2-methyl-propanesulfonic acid) (PAMPS) (mass, 7,000 to 10,000 Da) was from Monomer-Polymer and Dajac Laboratories (Feasterville, PA). Poly(4-styrenesulfonic acid) (PSS) sodium salt (mass, 70,000 Da) was from Acros (Geel, Belgium). Poly(anetholesulfonic acid) (PAS) sodium salt (mass, 9,000 to 11,000 Da) was from ICN (Costa Mesa, CA). Poly(vinylsulfonic acid) (PVS) sodium salt (mass, 2,000 Da) was from Sigma (St. Louis, MO). Figure 1 shows the chemical structures of these compounds.

Surface plasmon resonance assay. A BIAcore X apparatus (BIAcore Inc., Piscataway, NJ) was used to set up three different experimental models. (i) SSAPs were evaluated for their capacities to inhibit the binding of free GST-Tat or free gp120 to sensor chip-immobilized heparin. To this purpose, heparin was biotinylated and immobilized on an F1 sensor chip as described previously (48). GST-Tat (125 nM) or gp120 (45 nM) in the presence of SSAPs diluted in 10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20, pH 7.4 (HBS), was injected over the heparin surface for 5 min (to allow the association of the proteins with heparin), and the surface was then washed until dissociation was observed. (ii) GST-Tat or gp120 (the same doses described above) was injected for 4 min over the heparin surface and allowed to reach an equilibrium binding. Then, PSS was injected, and the detachment of the viral proteins was evaluated after 2 min. (iii) PSS was evaluated for its capacity to bind to GST-Tat or gp120 immobilized to the sensor chip. Fifty micrograms of Tat or gp120 per milliliter was allowed to react with a CM5 sensor chip activated as previously described (59), allowing the immobilization of 10,281 or 8,350 resonance units (RU), corresponding to approximately 300 or 70 fmol of GST-Tat or gp120, respectively. Similar results were obtained for the immobilization of bovine serum albumin, used as a negative control and for blank subtraction. The GST moiety immobilized over the sensor chip does not interact with polyanionic compounds (59). Increasing concentrations of PSS in HBS were then injected over the GST-Tat or gp120 surface for 4 min (to allow their association with immobilized proteins), and the surface was then washed until dissociation was observed. After every run, the sensor chip was regenerated by injection of HBS containing 2.0 M NaCl. Binding parameters were calculated with the nonlinear curve-fitting software package BIAevaluation 3.2, using a single site model with correction for mass transfer. Association and dissociation rates were calculated separately using

low (10 to 100 and 1 to 10 nM) and high (0.3 to 10 μM and 30 to 300 nM) concentrations of PSS for Tat and gp120, respectively (13).

Cell cultures. HL3T1 cells, derived from HeLa cells, contain integrated copies of the pL3CAT plasmid in which the chloramphenicol acetyltransferase bacterial gene is driven by the HIV-1 LTR (64). They were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco) with 10% fetal calf serum (FCS) (Gibco). Transformed fetal bovine aortic endothelial GM7373 cells (22) were grown in Eagle's minimal essential medium (Gibco) with 10% FCS, vitamins, and essential and nonessential amino acids.

MTT assay. The cytotoxicities of SSAPs on HL3T1 and GM7373 cells were assessed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assays according to the method of Alley et al. (5) with minor modifications.

Cell-binding assay of biotinylated gp120. Subconfluent cultures of GM7373 ECs in 96-well plates were incubated for 2 h at 4°C in phosphate-buffered saline containing 0.1 mg/ml $CaCl_2$, 0.1 mg/ml $MgCl_2$, 0.1% gelatin, and biotinylated gp120 (40 nM) in the presence of SSAPs. After the plates were washed with phosphate-buffered saline, the amount of cell-associated biotinylated gp120 was determined with horseradish peroxidase-labeled avidin (1/1,500) and the chromogen substrate ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] (Kierkegaard & Perry Laboratories, Gaithersburg, MA). For determination of the parameters of gp120 binding to ECs, the results were converted to a picomolar concentration using a standard curve of biotinylated gp120.

HIV-LTR transactivation assay. The HIV-LTR transactivation assay was performed by using HL3T1 cells and an enzyme-linked immunosorbent assay-based assay as previously described (46).

Tat internalization assays. Cell internalization of Tat was studied by using fluorescent GST-Tat-GFP and HL3T1 cells as previously described (46).

Caspase 3 activation assay. GM7373 ECs were seeded at 75,000/cm^2 in DMEM containing 10% FCS and incubated for 2 h at 37°C. Then, the cells were starved by a 24-h incubation in DMEM containing 0.5% FCS. Finally, the cells were incubated for 24 h at 37°C with DMEM containing gp120 (0.5 nM) in the presence of SSAPs. DMEM containing 10% FCS or colcemide (0.3 μg/ml) was used as a control. At the end of incubation, the cells were acetone fixed and incubated overnight at 4°C with anti-cleaved caspase 3 antibody (Cell Signaling Technology, Beverly, MA) and for another hour at room temperature with goat anti-rabbit immunoglobulin G-fluorescein isothiocyanate antibody. The nuclei were stained with DAPI (4-6-diamidino-2-phenylindole). The cells were observed under a fluorescence microscope. The numbers of DAPI-detected nuclei (corresponding to the numbers of cells) and the amounts of cleaved caspase 3 under the different experimental conditions were quantified by image analysis using the Image Pro-Plus analysis system (Media Cybernetics, Silver Spring, MD).

RESULTS

Effects of SSAPs on the interaction of Tat and gp120 with immobilized heparin. In a first set of experiments, we evaluated the abilities of SSAPs to affect the interaction of Tat or gp120 with heparin immobilized on a BIAcore sensor chip, an experimental condition that resembles the binding of Tat (48) (and possibly of gp120) to cell-associated HSPGs. PSS, PVS, PAS, and PAMPS were used, since they encompass a large range of molecular masses (7,000 to 70,000 Da) and are representative of different chemical structures, such as aromatic rings (present in PSS and PAS) and linear chains (in PVS and PAMPS) (Fig. 1).

As shown in Fig. 2A, Tat bound sensor chip-immobilized heparin (dissociation constant [K_D], about 50 nM [48]). All the SSAPs tested were able to prevent such interaction in a dose-dependent way, with 50% inhibitory concentrations (IC_{50}) ranging from ≈3 nM (for PSS, PVS, and PAMPS) to 20 nM (for PAS) (Fig. 2C). Also, free gp120 interacted with sensor chip-immobilized heparin (Fig. 2B) (K_D = 600 pM) (data not shown). All the SSAPs tested prevented in a dose-dependent way the binding of gp120 to the heparin surface, with PSS being the most efficient inhibitor (IC_{50} = 0.3 nM) compared to the other SSAPs (the IC_{50} ranged between 0.6 and 20 nM)

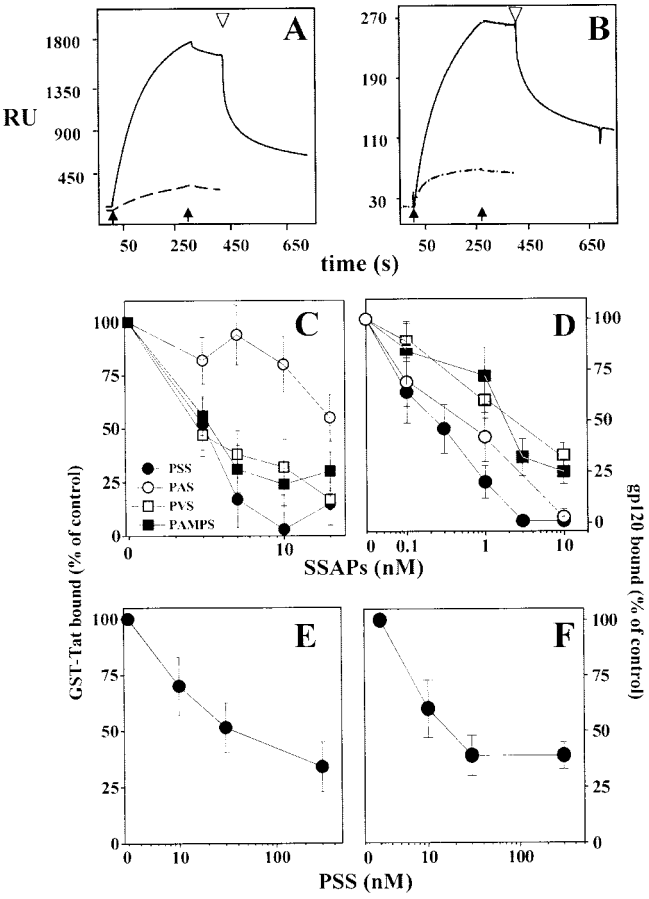

FIG. 2. Effects of SSAPs on the interaction of Tat and gp120 with immobilized heparin. Shown are representative sensorgrams of 125 nM GST-Tat (A) or 45 nM gp120 (B) injected alone (solid lines) or in the presence of 10 nM PSS (dashed lines) over a BIAcore sensor chip containing heparin. The black arrowheads indicate the beginning and the end of the injection phase. The white arrowheads indicate the injection of PSS (300 nM) on a sensor chip in which immobilized heparin was allowed to reach equilibrium binding with GST-Tat or gp120. GST-Tat (C) or gp120 (D) was injected over a BIAcore sensor chip containing immobilized heparin in the presence of the indicated SSAPs. Alternatively, GST-Tat (E) or gp120 (F) was injected over the BIAcore sensor chip containing immobilized heparin and allowed to reach equilibrium binding. Then, PSS was injected and evaluated for its capacity to destroy the already established Tat- or gp120-heparin complexes. In panels C to F, the responses (in RU) were recorded and plotted as percentages of the binding measured in the absence of SSAPs. Each point is the mean ± standard error of the mean of two or three determinations in duplicate.

(Fig. 2D). In parallel experiments, the two viral proteins were allowed to reach an equilibrium binding with the heparin surface, and only then was PSS injected (Fig. 2A and B). Under these experimental conditions, PSS retained its capacity to inhibit in a dose-dependent way the binding of Tat and of gp120 to heparin (Fig. 2E and F), suggesting that it is able not only to prevent the binding of the two viral proteins to heparin/HSPGs, but also to disrupt already established complexes.

To investigate the mechanism of inhibition, increasing concentrations of PSS were tested for the capacity to inhibit heparin interaction of increasing concentrations of Tat or gp120. The binding data were then analyzed by the double-reciprocal

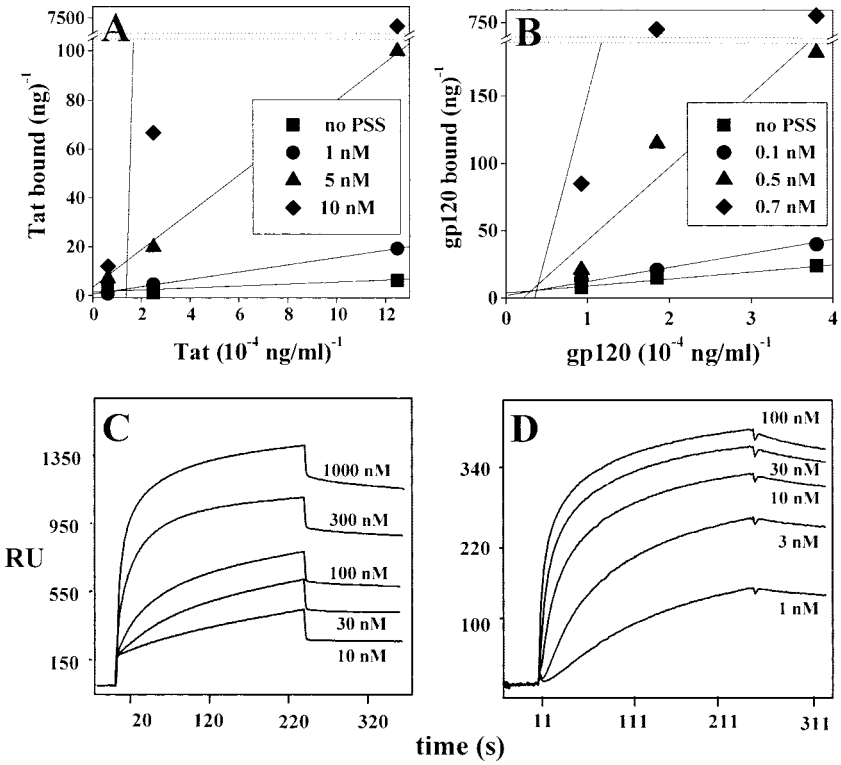

FIG. 3. Interaction of PSS with Tat and gp120. A BIAcore sensor chip containing immobilized heparin was incubated with GST-Tat (25, 125, or 500 nM) (A) or with gp120 (25, 45, or 90 nM) (B) with the indicated concentration of PSS. Then, the amounts of the two viral proteins associated with the sensor chip were measured and expressed as the reciprocals of concentrations versus the reciprocals of the amounts of bound proteins. Increasing concentrations of PSS were injected over a BIAcore sensor chip containing immobilized GST-Tat (C) or gp120 (D). The responses (in RU) were recorded at the ends of injections and plotted as a function of time.

method. For both viral proteins, the data fit straight lines whose slope increased with increasing PSS concentrations (Fig. 3A and B). All the lines intersected at a point, thus supporting the competitive nature of the inhibitions, which in turn suggest that PSS directly binds the two viral proteins, sequestering them in the extracellular environment. To evaluate this possibility, Tat and gp120 were immobilized to a BIAcore sensor chip and evaluated for the ability to bind free PSS. As shown in Fig. 3C and D, PSS effectively interacted with the Tat and gp120 surfaces in a dose-dependent way. Evaluation of the binding parameters (Table 1) showed that PSS/gp120 binding occurs with an association rate that is 11-fold higher and with a dissociation rate that is 2-fold lower than those of PSS/Tat binding, indicating that the former interaction is stronger than the latter. Accordingly, the affinity of PSS/gp120 interaction is 40-fold higher than that of PSS/Tat interaction.

TABLE 1. Binding parameters of the interactions of PSS with GST-Tat or gp120 immobilized to a BIAcore sensorchip[a]

Protein	k_{ass}[b] (M^{-1} s^{-1})	k_{diss}[c] (s^{-1})	K_D[d]
GST-Tat	$3.85 \pm 2.85 \times 10^5$	$4.00 \pm 1.55 \times 10^{-4}$	1.0 nM
gp120	$4.20 \pm 1.56 \times 10^6$	$1.98 \pm 1.34 \times 10^{-4}$	23 pM

[a] Each amount is the mean ± standard deviation of three or four independent determinations.
[b] k_{ass}, association rate.
[c] k_{diss}, dissociation rate.
[d] The dissociation constant (K_D) was derived from the k_{diss}/k_{ass} ratio (kinetics).

Effects of SSAPs on Tat-induced biological activities. HSPGs expressed on the surfaces of HL3T1 cells specifically interact with Tat, mediating its internalization and gene transactivation (56). We next evaluated the effects of SSAPs on these biological activities. As shown in Fig. 4A and B, SSAPs inhibited the internalization of GST-Tat-GFP in HL3T1 cells and the LTR transactivation induced by GST-Tat in the same cells. In the latter assay, PSS was again demonstrated to be the most efficient inhibitor (IC_{50} = 1 nM) compared to the other SSAPs (the IC_{50} ranged between 4 and 9 nM). PSS did not affect LTR transactivation exerted by native Tat endogenously produced by HL3T1 cells following their transient transfection with an expression vector harboring the HIV-1 Tat cDNA (data not shown), indicating that PSS does not exert cytotoxic effects or interfere nonspecifically with the intracellular machinery required for the transactivation process. Also, in HL3T1 cells cultured in the presence of the different SSAPs (from 1 nM to 1 μM), cell viability always remained higher than 91% (data not shown).

As already mentioned, PSS is able to disrupt already established Tat-heparin interaction (Fig. 1E). We thus investigated the time dependency of the inhibition exerted by SSAPs on Tat-transactivating activity. SSAPs were administered to HL3T1 cells together with GST-Tat or at different times after GST-Tat administration. As shown in Fig. 4C, PSS lost only 30% of its inhibitory capacity when added 5 h after GST-Tat administration. On the other hand, the decrease in the inhib-

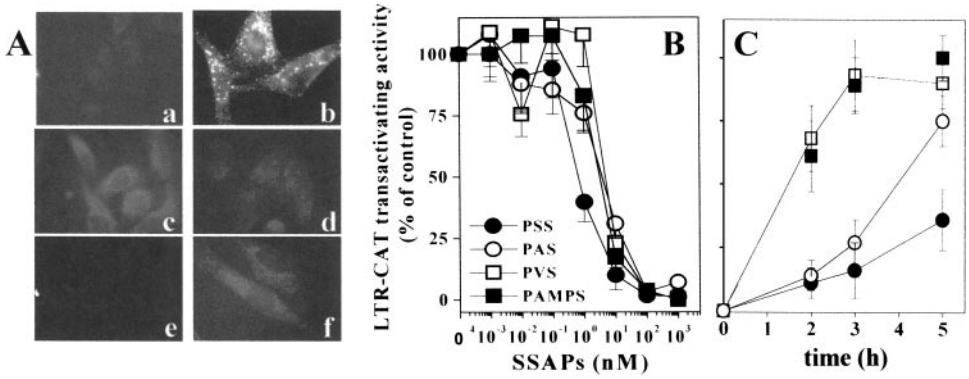

FIG. 4. Effects of SSAPs on Tat internalization and LTR transactivating activity. (A) Photomicrographs (original magnification, ×600) of HL3T1 cells incubated for 6 h at 37°C without (a) or with GST-Tat-GFP (6 nM) in the absence (b) or in the presence of PSS (c), PAS (d), PVS (e), or PAMPS (f) (all at 100 nM). (B) HL3T1 cells were incubated with GST-Tat (6 nM) in the presence of the indicated SSAPs. (C) Alternatively, cells were incubated with GST-Tat, and SSAPs (all at 100 nM) were added at the indicated times after Tat administration (symbols as in panel B). At the end of the treatments, cell extracts were assayed for the levels of chloramphenicol acetyltransferase antigen. In panels B and C, the data are expressed as percentages of the activity measured in the absence of SSAPs, and each point is the mean ± standard error of the mean of three to five determinations in duplicate.

itory capacity of PAS over time was more pronounced (25% and 74% reductions when administered 3 and 5 h after GST-Tat, respectively). Under the same experimental conditions, PAMPS and PVS lost their inhibitory capacities very rapidly (60% and 100% reductions when administered 2 to 3 h after GST-Tat).

Effects of SSAPs on gp120-induced biological activities. The capacities of SSAPs to bind gp120 and to prevent its interaction with heparin prompted us to evaluate the capacities of SSAPs to sequester gp120 in the extracellular environment, inhibiting its interaction with and pathological effects on ECs, which are a preferential target of the extracellular free form of gp120 (57). Since little is known about gp120/EC surface interaction, we performed a preliminary set of experiments with the aim of characterizing it. As shown in Fig. 5A, the binding of biotinylated gp120 to GM7373 ECs was dose dependent and, under our experimental conditions, nonsaturable, suggesting the existence of high-capacity binding sites. Transformation of binding data into a Scatchard plot (Fig. 5B) demonstrated a classic biphasic plot that identified two classes of receptors with dissociation constants (K_D) equal to 1.94×10^{-10} M (high-affinity binding) and 6.4×10^{-8} M (low-affinity binding). It was estimated that there are 2.5×10^3 and 2.3×10^6 high- and low-affinity binding sites/cell, respectively. We then evaluated the capacities of SSAPs to inhibit the interaction of gp120 with ECs. As shown in Fig. 5C, PSS and PAS inhibited the binding of gp120 to endothelial GM7373 ECs in a dose-dependent way, with IC_{50} equal to 20 and 60 nM, respectively, while PVS and PAMPS turned out to be poor inhibitors ($IC_{50} > 300$ nM).

Caspases mediate apoptosis induced by extracellular gp120 in ECs (57). We then evaluated the capacities of SSAPs to inhibit gp120-dependent activation of caspase 3 in ECs. Colcemide and 10% serum were used as positive and negative controls, respectively. As shown in Fig. 6, SSAPs inhibited in a dose-dependent way the apoptosis induced by extracellular free gp120 in ECs with the following potencies: PSS > PAMPS > PVS > PAS (IC_{50} = 0.2, 4.0, 30.0, and 70.0 nM, respectively). An MTT assay demonstrated that in GM7373 cells cultured in the presence of the different SSAPs (from 1 nM to 1 μM), cell viability always remained higher that 93% (data not shown).

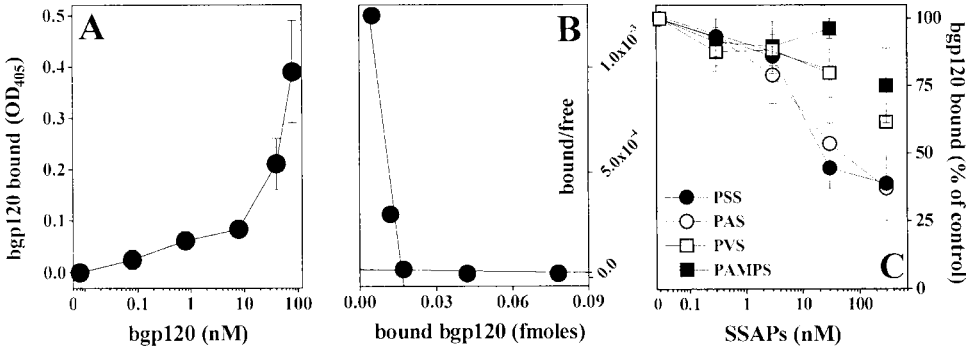

FIG. 5. Binding of gp120 to ECs. (A) GM7373 ECs were incubated with increasing concentrations of biotinylated gp120 (bgp120). Then, the amount of biotinylated gp120 bound to cells was measured. Each point is the mean ± standard error of the mean of two determinations in duplicate. (B) Scatchard plot regression of the biotinylated-gp120 binding data to GM7373 ECs. (C) GM7373 ECs were incubated with biotinylated gp120 (40 nM) in the presence of the indicated SSAPs. Then, the amounts of cell-associated biotinylated gp120 were measured and expressed as percentages of biotinylated gp120 bound to cells in the absence of SSAPs. Each point is the mean ± standard error of the mean of three determinations in duplicate. OD_{405}, optical density at 405 nm.

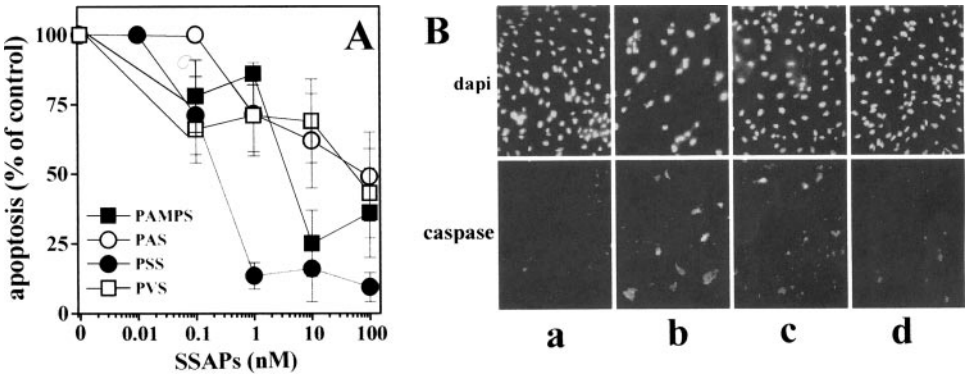

FIG. 6. Effects of SSAPs on gp120-dependent caspase 3 activation in ECs. (A) GM7373 ECs were treated with gp120 in the presence of the indicated SSAPs. Then, caspases 3 activation was evaluated. Each point is the mean ± standard error of the mean of three to six random fields. The data are expressed as percentages of caspase 3 activation measured in the absence of SSAPs. (B) Representative images of nuclear immunostaining with DAPI (top row) or of cytoplasmic immunostaining with anti-cleaved caspase 3 antibody (bottom rows) of GM7373 ECs treated with 10% FCS (a), colcemide (b), or gp120 (0.5 nM) alone (c) or in the presence of PSS (10 nM) (d).

DISCUSSION

Tat and gp120 are released by HIV-infected cells, and once in the extracellular environment, they can alter the functions of HIV-nonpermissive cell types, contributing to the rise of AIDS-associated diseases. Tat is detected in sera of HIV^+ individuals at 0.01 to 0.1 nM (63), but due to its capacity to accumulate in the HSPG-rich ECM (8) and subendothelial matrix (C. Urbinati, unpublished observation), it is highly possible that its concentration significantly increases in the microenvironment, in close contact with the surfaces of target cells. Relevant to this point, Tat can induce its effects in target cells without being mobilized from its storage compartments (58). Free gp120 has been reported in the sera of HIV^+ individuals at concentrations ranging between 2 and 20 pM and 0.1 and 0.8 nM (reference 25 and references therein). However, as already demonstrated for Tat, it has been proposed that gp120 can accumulate in the HSPG-rich ECM, increasing its concentration in the microenvironment (25).

The aim of this paper was to evaluate the possibility of blocking extracellular Tat and gp120 simultaneously by interfering with their interaction with putative common receptors expressed on target cells, among which are HSPGs. It has been effectively demonstrated that HSPGs act as Tat receptors, mediating several of its effects in different cell types (44, 56). Here, we demonstrated that SSAPs, and in particular PSS, efficiently prevent HSPG-dependent cell internalization and the transactivating activity of Tat. This occurs by a direct PSS/Tat interaction that sequesters the transactivating factor in the extracellular environment, hampering its binding to the cell surface. Relevant to this point, the affinity of PSS-Tat interaction (K_D = 1 nM) is 10 to 50 times higher than that of Tat/heparin interaction (46). Accordingly, PSS inhibits HSPG-dependent Tat transactivating activity with a potency (IC_{50} = 0.8 nM) that is 25 times higher than that of heparin (20 nM) (41) and comparable to those of other polyanions previously assayed for their Tat antagonist potentials (47, 48, 59).

As mentioned above, Tat accumulates in an immobilized form in the ECM. Relevant to this point, PSS is able not only to prevent the binding of Tat to heparin/HSPGs, but also to disrupt already established complexes. Accordingly, PSS and PAS retain their capacities to inhibit Tat transactivating activity in a delayed administration (see Fig. 4C), an experimental condition under which PAMPS and PVS, as well as heparin (47), exert their inhibitory effects only if added before or within the first 2 h after Tat administration (Fig. 4C). Also, PAS shows a limited potency in inhibiting Tat/heparin interaction in a very short-term assay (Fig. 2C), while it proved to be much more efficient when tested in long-term cell culture assays, approaching the potency of PSS (Fig. 4C). Taken together, these data suggest that an antagonist able to efficiently "prevent" the action of Tat is not necessarily also able to inhibit the transactivating factor already bound to the cell surface, with important implications for the choice of the methods of in vitro screening of Tat (and possibly gp120) antagonists. At a structural level, it is interesting that, compared to PVS and PAMPs, PSS and PAS are characterized by longer chains and by the presence of aromatic rings, implicating these structural features in the "long-term" inhibitory potential of SSAPs.

Little is known about the receptors that mediate the pathological effects exerted by gp120 in target cells. In particular, although it is well established that HSPGs act as receptors for HIV-embedded gp120 (33), the possibility that this class of receptors plays the same role for the extracellular free form of gp120 has never been explored. Here, we found that, on the EC surface, free gp120 interacts with two classes of receptors, one of which is characterized by high capacity (2.3×10^6 binding sites/cell) and low affinity (K_D = 64 nM). Interestingly, these values are similar to those calculated for Tat (31) and FGF2 (49) binding to HSPGs, suggesting that HSPGs represent the low-affinity, high-capacity receptors of gp120 on the EC surface. This possibility is further supported by the observation that free heparin (used as an HSPG antagonist to determine the extent of HSPG-dependent binding) (49) inhibits gp120 binding to ECs (65% inhibition when tested at 3.7 mM) (data not shown). However, the residual, heparin-resistant binding of gp120 to ECs suggests that receptors other than HSPGs exist on the endothelial surface, likely corresponding to the low-capacity (2.5×10^3 binding sites/cell), high-affinity (K_D = 194 pM) receptors identified here. Relevant to this point, CCR5 and CXCR4 have been proposed to act as recep-

tors for extracellular free gp120 and to mediate gp120-dependent apoptosis in ECs (23).

Polyanionic compounds bind HIV-embedded gp120, exerting an anti-HIV effect. In particular, SSAPs were found to be efficient anti-HIV agents, inhibiting viral infection with an IC_{50} of 0.08 to 0.1 μM (32) compared to 0.9 to 3.0 μM for dextran sulfate (24), 0.1 to 4.0 μM for K5 derivatives (60), and 0.1 to 35 μM for sulfonated/phosphonated analogs of distamycin (10). However, polyanions have never been taken into consideration as inhibitors of the extracellular free form of gp120. Here, we found that PSS binds gp120 with an affinity (K_D = 23 pM) that is 26 times higher than that of heparin/gp120 interaction (K_D = 600 pM) (data not shown). Also, it inhibits the interaction of free gp120 with substrate-immobilized heparin with an efficiency (IC_{50} = 200 pM) that is 40 times higher than that of free heparin (IC_{50} = 8.3 nM) (61). Accordingly, PSS inhibits the binding of gp120 to ECs with an efficiency that is higher than that of heparin (IC_{50} = 40 nM for PSS and 1,000 nM for heparin) (data not shown). Finally, SSAPs inhibit the activation of proapoptotic caspase 3 induced by free gp120 in ECs, suggesting that, at least in part, HSPGs contribute to the induction of apoptosis in ECs. Relevant to this point, gp120 has been demonstrated to induce EC apoptosis in a biphasic way, with maximal effects obtained at 1 pM and 2 nM, respectively (23).

However, it must be pointed out that PAMPS, which scarcely affects HSPG-dependent binding of gp120 to the EC surface, inhibits gp120-induced apoptosis with an efficiency (IC_{50} = 3 nM) that is lower only than that of PSS (IC_{50} = 0.3 nM). Also, PSS inhibits apoptosis with an efficiency that is 60 times higher than that measured for its inhibition of HSPG-dependent gp120/EC interaction (IC_{50} = 0.3 and 20 nM, respectively). Taken together, these data imply the possibility that the antiapoptotic effects of SSAPs cannot be simply attributed to an inhibition of gp120/HSPG interaction. Rather, it is likely that SSAPs inhibit gp120 binding to cell receptors other than HSPGs. Interestingly, SSAPs have been demonstrated to inhibit the binding of FGF2 to both HSPGs and tyrosine-kinase receptors on ECs.

HIV infection and AIDS-associated pathologies are extremely redundant processes mediated by different viral products that, in turn, act by complex mechanisms (i.e., by activating different cell surface receptors). Here, we demonstrated that SSAPs simultaneously inhibit cell interaction and biological activities of the extracellular form of HIV gp120 and Tat. On the other hand, SSAPs had already been demonstrated to inhibit HIV infection (32). Taken together, these data point to SSAPs as candidates for the development of drugs with the capacity to simultaneously inhibit HIV infection and the pathological effects of HIV proteins.

Despite their anti-HIV efficacy in vitro, polyanions proved to be of limited benefit for AIDS patients after oral or intravenous administration. This may be due to their poor bioavailability and/or to their anticoagulant/toxic activities, which limit their dosages. To overcome these limitations, recent papers have focused on the possibility of chemically modifying and/or synthesizing new polyanions with tailored structural modifications in order to remove deleterious side effects and to improve their bioavailabilities and therapeutical efficacies (reviewed in reference 42).

The importance of the development of efficacious microbicides has been raised (54). Relevant to this point, sulfate and sulfonate polymers differ in their pharmacological behavior. Whereas the sulfate groups can be subject to hydrolysis (release) by sulfatases present in the vaginal ecosystem, resulting in inactivation of the compounds, the sulfonate groups are tightly linked to the polymers and highly metabolically stable. Accordingly, a high-molecular-weight PSS was demonstrated to be an efficacious and safe vaginal contraceptive antimicrobial against HIV infection in phase I clinical trials (19, 66). Alternatively, SSAPs may be employed for intralesional administration, as in Kaposi's sarcoma, where HIV-Tat and FGF2 synergize in the induction of the lesions (16). Relevant to this point, SSAPs have also been demonstrated to inhibit the angiogenic activity of FGF2 (26).

In conclusion, the exploitation of polyanions as HIV antagonists, despite their definite potential (29), deserves a more articulated approach that must start with a large number of compounds with distinct structural features being subjected to a wide array of biological assays, possibly representative of the various aspects of HIV pathology, with the aim of identifying/producing selected compounds with multitarget activities against HIV.

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