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Identification of Specific Molecular Structures of Human Immunodeficiency Virus Type 1 Tat Relevant for Its Biological Effects on Vascular Endothelial Cells

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Human immunodeficiency virus type 1 (HIV-1) Tat transactivates viral genes and is released by infected cells, acting as a soluble mediator. In endothelial cells (EC), it activates a proangiogenic program by activating vascular endothelial growth factor receptor type 2 (VEGFR-2) and integrins. A structure-activity relationship study was performed by functional analysis of Tat substitution and deletion variants to define the Tat determinants necessary for EC activation. Variants were made (i) in the basic and (ii) in the cysteine-rich domains and (iii) in the C-terminal region containing the RGD sequence required for integrin recognition. Our results led to the following conclusions. (i) Besides a high-affinity binding site corresponding to VEGFR-2, EC express low-affinity binding sites. (ii) The basic and the cysteine-rich variants bind only to the low-affinity binding sites and do not promote tyrosine phosphorylation of VEGFR-2. Furthermore, they have a reduced ability to activate EC in vitro, and they lack angiogenic activity. (iii) Mutants with mutations in the C-terminal region are partially defective for in vitro biological activities and in vivo angiogenesis, but they activate VEGFR-2 as Tat wild type. In conclusion, regions encoded by the first exon of *tat* are necessary and sufficient for activation of VEGFR-2. However, the C-terminal region, most probably through RGD-mediated integrin engagement, is indispensable for full activation of an in vitro and in vivo angiogenic program.

Tat is one of the regulatory proteins of human immunodeficiency virus type 1 (HIV-1). The protein is composed of 86 to 104 amino acids (aa) (according to the viral isolate) encoded by two exons. In the portion encoded by the first exon (72 amino acids) four distinct regions can be recognized (N-terminal, cysteine rich, core, and basic). The second exon encodes the C-terminal region, containing a RGD sequence (31). Tat plays an essential role in viral replication by up-regulating viral gene expression in infected cells by increasing the rates of transcriptional initiation and elongation by DNA polymerase II (31). Tat also modulates the expression of cellular genes involved in cell survival and proliferation or in coding for cytokines (11, 12, 23, 29, 37, 41, 54, 63, 66, 69). This newly acquired cell phenotype will contribute to the pathogenesis of specific diseases associate with HIV infection. Besides its intracellular effects, Tat may alter cellular behavior when it is released by infected cells in the microenvironment (10, 15). Tat easily enters different cell types contributing to the transactivation of the HIV-1 long terminal repeat (LTR) promoter in latently infected cells (20, 39). Alternatively, it acts as soluble mediator affecting the physiologic functions of cells of the immune (30, 35, 43, 49, 66) and nervous (33, 42, 48) systems. Moreover, it influences the apoptotic program in T cells (26, 36, 41, 65, 70) and in neurons (47, 59), thus favoring the progression of AIDS and the associated brain damage.

However, one of the most relevant targets for Tat is the vascular system, where it activates a proinflammatory and angiogenic program. Tat can up-regulate the expression of endothelial cell (EC) adhesion molecules (13, 28), resulting in leukocyte extravasion, which is essential for the homing of infected lymphomononuclear cells into lymphoid organs and for the tissue injury characteristic of some features of disease progression. Alone or combined with inflammatory cytokines, Tat induces EC to proliferate, release proteolytic enzymes, and migrate and is fully angiogenic in vivo (1, 3–5, 18). These features could be relevant to the chronic inflammatory damages characteristic of several AIDS-associated diseases (17). Furthermore, the ability of Tat to enter EC during the cell cycle could favor HIV-1 replication in some EC areas which are virus reservoirs (44, 45). Finally, Tat participates in the progression of Kaposi's sarcoma, both as a growth factor for spindle cells which represent the core of the tumor and as a means of sustaining its vascularization (14, 16). Furthermore, Tat transgenic mice generated by using either the HIV LTR (63) or the BK polyomavirus promoter (11) develop Kaposi's sarcoma-like lesions and tumors of different histotypes, supporting the pathogenetic role of Tat in Kaposi's sarcoma and in the vascularization of neoplasms associated with AIDS.

The molecular mechanisms leading to these broad and pleiotropic activities are largely unknown. By the use of peptides spanning specific domain of the Tat structure or of functional blocking antibodies, some investigators demonstrated that Tat binding to integrin through the RGD sequence near the C terminus (7, 62) is relevant for the activation of lymphocytes (70), EC (5, 13), monocytes (6, 35), and neuronal cells (48). Through its N-terminal structure, Tat interacts with

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dipeptidyl peptidase IV, located on the T cell surface, and suppresses antigen-induced cell activation (26, 68). Additionally, Tat binds to and activates the tyrosine kinase receptors encoded by the *KDR* and *Flt-1* genes in EC and Kaposi's sarcoma cells (4, 21) and monocytes (43), respectively.

We initiated a study of the structure-activity relationship of Tat to identify functionally important domains that are responsible for the activation of the angiogenic program in vascular EC. We report here that cysteine-rich and basic domains are relevant for functional activation of VEGFR-2 whereas the C-terminal region does not directly participate in the receptor activation but is required for full cell activation.

MATERIALS AND METHODS

Cells. Human EC from umbilical cord veins, prepared and characterized as previously described (9), were grown in M199 (Gibco, Grand Island, N.Y.) supplemented with 20% fetal calf serum (FCS) (Irvine, Santa Ana, Calif.), EC growth factor (100 μg/ml) (Sigma Chemical Co., St. Louis, Mo.), and porcine heparin (100 μg/ml) (Sigma). They were used at passage II and grown on a plastic surface coated with porcine gelatin (Sigma), unless specified.

Tat molecules. Recombinant wild-type HIV-1 Tat of 86 (Tat_{86}) and 101 (Tat_{101}) amino acids were expressed in *Escherichia coli* as maltose-binding protein (MBP) or glutathione *S*-transferase (GST) fusion proteins. They are referred to below as Tat (MBP-Tat) and *Tat (GST-Tat), respectively. Mutant constructs were obtained by a recombinant PCR procedure with overlapping oligonucleotides corresponding to the mutated sequences, and the specific mutations were verified by DNA sequencing. To obtain Tat mutants, a *Pst*I-*Bam*HI cDNA fragment of Tat_{86} containing the coding region of both exons was subcloned in the pALTER-Ex1 vector (Promega, Madison, Wis.). Site-directed mutagenesis was carried out with the Altered Sites mutagenesis kit (Promega) by using mutant oligonucleotides to introduce specific mutations (Tat D80E, 5′ CCC GAG GGG AAC CGA CAG GCC 3′; Tat R78K/D80E, 5′ CCC GAG GGG AAC CGA CAG CC 3′ and 5′ ACC TCC CAA TCC AAA GGG GAA CCG AC 3′; Tat R49G/K50I, 5′ CTC CTA TGG CGG GAT CAA GCG GAG AC 3′; Tat R49G/K50I/R52L/R53I, 5′ CTC CTA TGG CGG GAT CAA GCG GAG AC and 5′ CGG GAT CAA GCT AAT ACA GCG ACG AAG 3′). The mutated cDNAs (317-bp *Eco*RI-*Bam*HI fragments) were subcloned into the pMAL-c2 vector (New England Biolabs, Beverly, Mass.) and expressed as specified by the manufacturer. Tat_{86} and its mutants were purified to homogeneity from bacterial cell lysates by affinity chromatography on amylose resin, as specified by New England Biolabs, and used as fusion proteins.

*Tat_{86} and its mutated derivatives including the product of the first exon, *Tat_{72}, a nonconservative mutant with mutations in basic [*Tat R(49,52,53,55,56,57)A] and cysteine-rich [*Tat C(22,25,27)A] regions, were produced as previously described (40), as well as *Tat_{101} (61). Recombinant fusion proteins were purified by glutathione-Sepharose affinity chromatography (Sigma) (53). The purified MBP and GST fusion proteins gave a unique band after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) and silver staining. Tat_{86}, *Tat_{86}, and *Tat_{101} and their mutants were lipopolysaccharide free, as assessed by the *Limulus* assay (Sigma). Tat and *Tat were able to induce transcriptional activation of the HIV-1 LTR in HL3T1 cells that contain the bacterial gene of chloramphenicol acetyltransferase (CAT) directed by the HIV-1 LTR (67). Tat_{86} and *Tat_{101} dose dependently stimulated CAT expression. Briefly, Tat molecules were added to confluent HL3T1 cells in a 100-mm-diameter dish containing prewarmed phosphate-buffered saline (PBS). Cells were immediately scraped from the plastic surface, resuspended in fresh medium, and centrifuged. The cells were again plated in a CO_2 incubator, and a CAT assay was performed after 6 h as described previously (67). Under our experimental conditions, just scraping of the cells in absence of Tat molecules had no effect on LTR-directed CAT gene expression (125 ± 56 cpm of [^{3}H]acetylated chloramphenicol [$n = 3$]). However, CAT gene expression was markedly elevated in cells that received Tat_{86} at 0.5 μg/ml (423 ± 86 cpm of [^{3}H]acetylated chloramphenicol), 1 μg/ml (2,345 ± 167 cpm of [^{3}H]acetylated chloramphenicol), or 5 μg/ml (6,543 ± 567 cpm of [^{3}H]acetylated chloramphenicol) or *Tat_{86} at 0.5 μg/ml (512 ± 34 cpm of [^{3}H]acetylated chloramphenicol), 1 μg/ml (3,145 ± 343 cpm of [^{3}H]acetylated chloramphenicol), or 5 μg/ml (7,009 ± 671 cpm of [^{3}H]acetylated chloramphenicol).

Tat molecules were stored at −80°C in aliquots of 5 μg/10 μl of PBS containing 0.1% human serum albumin (HSA; Farma Biagini, Lucca, Italy) and 1 mM dithiothreitol (Sigma).

Iodination of Tat molecules and binding studies. MBP, GST, Tat_{86}, *Tat_{86}, Tat R78K/D80E, Tat R49G/K50I/R52L/R53I, or *Tat C(22,25,27)A (2-μg samples) were dissolved in 200 μl of 20 mM sodium phosphate buffer (pH 7.4) without dithiothreitol and transferred in iodogen-coated tubes (50 μg/ml) (Pierce Europe B.V., Oud Beijerland, The Netherlands), where proteins were iodinated (5 min at 4°C) with 0.2 mCi of ^{125}I (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). A 20-μl volume of 20 mM phosphate buffer (pH 7.2) containing 1% HSA, 0.4 M NaCl, and 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS; Pierce) was added, and the reaction products were separated on Sephadex-G10. The specific activities of the tracers were as follows: MBP, 1 μCi/118 fmol; GST, 1 μCi/134 fmol; Tat_{86}, 1 μCi/108 fmol; *Tat_{86}, 1 μCi/100 fmol; Tat R78K/D80E, 1 μCi/128 fmol; Tat R49G/K50I/R52L/R53I, 1 μCi/112 fmol; and *Tat C(22,25,27)A, 1 μCi/103 fmol. [^{125}I]Tat_{86} and [^{125}I]*Tat_{86} retained their biological activity on Kaposi's sarcoma cells (8).

For binding studies, adherent EC on 24-well plates were incubated for 90 min at 22°C in 200 μl of M199 containing 20 mM HEPES (pH 7.4), 0.1% HSA, 0.2 U of heparin, 100 μg of soybean trypsin inhibitor per ml, bacitracin (binding buffer), and increasing concentrations of iodinated Tat molecules or control proteins in the presence of 100-fold excess of unlabeled proteins. After washes in buffer binding, the cells were extracted with 2% SDS in PBS. Specific binding (calculated by subtracting from the total the cpm bound after incubation with a 100-excess of unlabelled ligand) was approximately 80%. Curve displacement binding was obtained by incubating cells with 0.05 nM [^{125}I]Tat and processed as described above. Kinetic parameters were estimated with the Ligand program (Elsevier-Biosoft, Cambridge, United Kingdom).

Immunoprecipitation and immunoblotting. Confluent EC (10^7 cells/150-cm^2 dish) were made quiescent by 20 h of starvation in M199 containing 0.5% FCS and 0.1% HSA, preincubated for 15 min at 37°C with 1 mM Na_3VO_4, and then stimulated as detailed in Results in the presence of heparin (1 U/ml). The cells were lysed in a 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 1% Triton X-100, and protease and phosphatase inhibitors (pepstatin, 50 μg/ml; leupeptin, 50 μg/ml; aprotinin, 10 μg/ml; phenylmethylsulfonyl fluoride, 1 mM; soybean trypsin inhibitor, 500 μg/ml; $ZnCl_2$, 100 μM, Na_3VO_4, 1 mM [Sigma]). After centrifugation (20 min at 10,000 × *g*), supernatants were precleared by incubation for 1 h with protein A-Sepharose or with anti-mouse immunoglobulin-agarose (Sigma). Samples (1 mg of protein) were incubated with rabbit anti-VEGFR-2 polyclonal antibody (no. C-1158; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) or antiphosphotyrosine monoclonal antibody (MAb) (clone G410; Upstate Biotechnology Inc., Lake Placid, N.Y.) (5 to 10 μg/ml) for 1 h at 4°C, and immune complexes were recovered on protein A-Sepharose or anti-mouse Ig-agarose. Immunoprecipitates were washed four times with lysis buffer, twice with the same buffer without Triton X-100, and once with Tris-buffered saline. Proteins were solubilized under reducing conditions, separated by SDS-PAGE (8 or 10% polyacrylamide), transferred to Immobilon-P sheets (Millipore, Bedford, Mass.), and probed with antiphosphotyrosine MAb or with anti-VEGFR-2 antibody. The enhanced chemiluminescence technique (Amersham Pharmacia Biotech) was used for detection.

PI 3-kinase assay. A phosphoinositide 3-kinase (PI 3-kinase) assay was performed directly on antiphosphotyrosine immunoprecipitates exactly as described previously (58). Briefly, immunoprecipitates were incubated with 40 μM ATP, 50 to 100 μCi of [γ-^{32}P]ATP (Amersham), and a presonicated mixture of phosphatidylinositol-4,5-bisphosphate and phosphatidylserine (final concentration of both lipids, 50 μg/ml [Sigma]) in 25 mM HEPES (pH 7.4)–and 1 mM EGTA. The reaction was stopped after a 10-min incubation at room temperature by the addition of 1 volume of 1 M HCl and 2 volumes of chloroform-methanol (1:1). The lipids in the organic phase were separated by thin-layer chromatography (Silica Gel 60; Merck, Darmstadt, Germany) in 1-propanol–2 M acetic acid (65:35, vol/vol) and visualized by autoradiography.

Migration, proliferation, and adhesion assays. EC motility was studied by using a modified Boyden chamber technique exactly as previously described (9).

To evaluate EC proliferation, 2 × 10^3 cells were plated in M199 containing 20% FCS in 96-well plates (Falcon, Becton Dickinson Labware, Bedford, Mass.) coated with gelatin. After 24 h, the medium was removed and replaced with M199 containing 2.5% FCS. Tat molecules were added on days 0, 2, and 4, and the cell number was estimated on day 6 as previously described (9).

To study EC adhesion to immobilized Tat molecules, 96-well polysterene plates (Falcon) were coated overnight at 4°C with Tat molecules or control proteins (10 μg/well), washed, and then incubated for an additional 2 h at room temperature with 1% HSA in PBS. Cells were detached in cold PBS containing 2 mM EGTA, washed twice in M199 containing 1% FCS, and plated (5 × 10^4/0.1 ml) in the adhesion assay. After a 1-h incubation at 37°C, the plates were extensively washed in M199 containing 1% FCS, fixed, and stained with crystal violet (9). The absorbance was read at 540 nm in a microtiter plate spectrophotometer (EL340; Bio-Tek Instruments, Highland Park, Vt.).

In vivo angiogenesis. Matrigel (Becton Dickinson) supplemented with 10 U of heparin per ml was mixed with Tat molecules or control proteins and injected (0.7 ml) into the subcutaneous tissue of BALB/c male mice (Charles River, Conago, Italy) along the peritoneal midline. After 5 days, the mice were killed and gels were processed for histology, morphometric analysis, and hemoglobin content determination measured with a Drabkin reagent kit (Sigma) as previously described (8).

Statistical methods. One-way analysis of variance (ANOVA) and the Student-Neuman-Keuls test were used to test the difference within the experimental blocks of each biological assay. Statistical analyses were performed with STATISTICA for Windows, version 4.5 (StatSoft, Tulsa, Okla.).

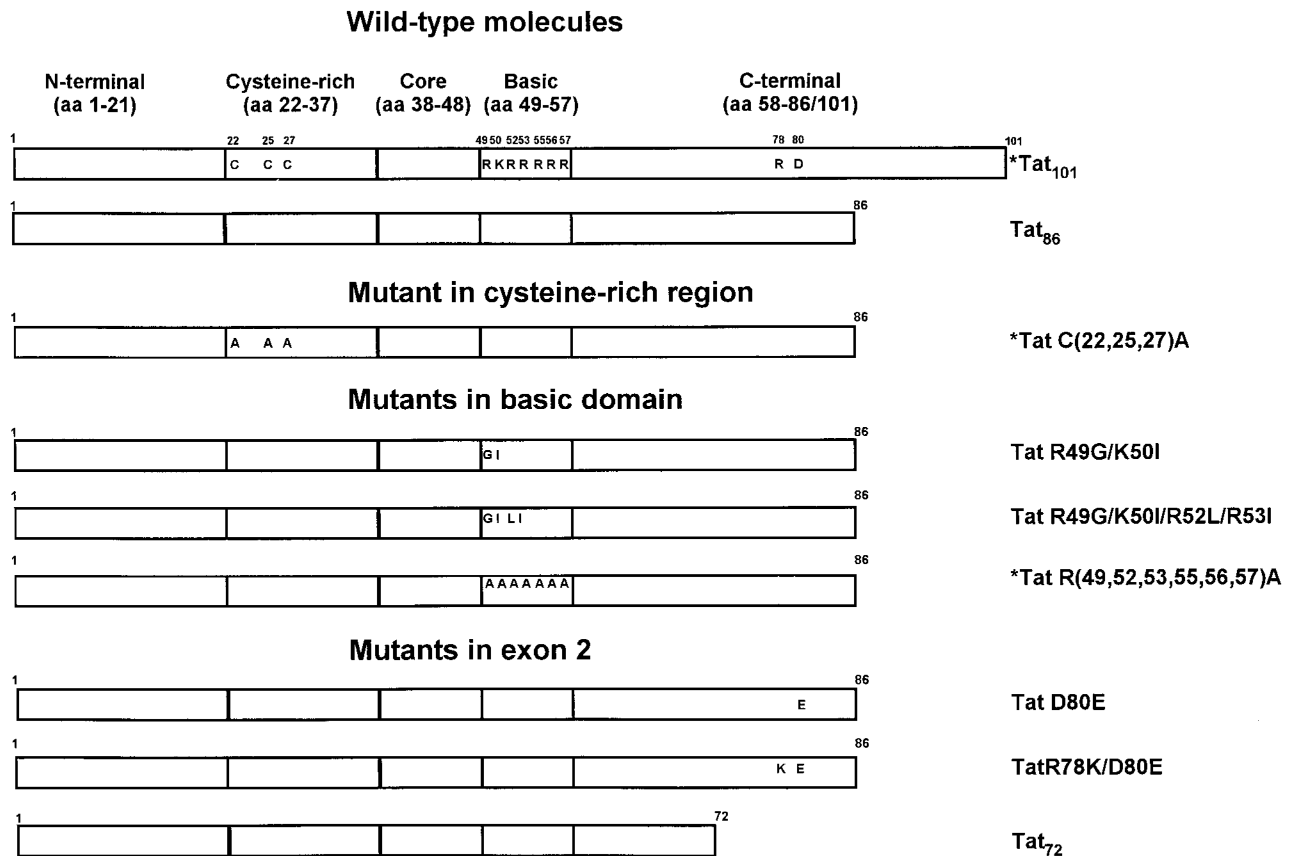

FIG. 1. Domain structure of the HIV-1 Tat protein and mutations introduced. The arbitrary domain structure is that of Kuppuswamy et al. (34). The number of mutated amino acids is indicated.

RESULTS

Effect of Tat and Tat mutants on biological functions of EC. It has been reported that soluble Tat induces the activation of vascular endothelium and of Kaposi's sarcoma cells through the engagement of VEGFR-2 (4, 21) and the integrin system (5, 13, 16). Furthermore, Tat mimics the extracellular matrix protein and favors cell adhesion through the amino acid sequence RGD (7) or the basic domain (62, 64). To examine the Tat regions involved in EC activation, a set of Tat mutants was constructed (Fig. 1), expressed as fusion proteins in *E. coli*, and studied for their ability to induce cell migration, proliferation, and adhesion.

First we studied the effect of mutations in the basic region. Tat R49G/K50I, Tat R49G/K50I/R52L/R53I, and *Tat R(49,52,53,55,56,57)A had a reduced effect in terms of migration in the Boyden chamber (Fig. 2) and proliferation (Fig. 3) of human EC compared to wild-type molecules (Tat_{86} or $^*Tat_{101}$) ($P < 0.005$), with Tat R49G/K50I being more active than Tat R49G/K50I/R52L/R53I and *Tat R(49,52,53,55,56,57)A ($P < 0.005$). Finally, the adhesion of EC to Tat R49G/K50I/R52L/R53I and *Tat R(49,52,53,55,56,57)A was markedly reduced with respect to the adhesion to Tat_{86} or Tat R49G/K50I ($P < 0.005$) (Fig. 4). These data suggest that the basic domain is relevant for all the EC biological activities considered.

Analysis of the mutants with mutations in the RGD sequence indicated that this sequence is also necessary for full activation of the migration (Fig. 2) and proliferation (Fig. 3) of EC and is required for the adhesion process (Fig. 4). Tat R78K/D80E showed reduced proliferation and migration activities compared to Tat_{86} ($P < 0.005$). Tat D80E had an effect similar to Tat_{86}, indicating that a unique mutation is insufficient to impair the activities of Tat_{86} on EC. Deletion of the sequence encoded by exon 2 ($^*Tat_{72}$) resulted in a molecule with biological activities (migration and proliferation) similar to those of Tat R78K/D80E (Fig. 2 and 3). Similarly, $^*Tat_{72}$ and Tat R78K/D80E were not permissive for EC adhesion (Fig. 4).

*Tat C(22,25,27)A, the mutant with the mutation in the cysteine-rich region, was a weak activator of EC migration and proliferation and showed little impairment of the adhesive property (Fig. 2 to 4). This suggests that the cysteine-rich region of Tat is required for migration and proliferation of EC rather than for their adhesion to the extracellular matrix.

In an effort to better define the activation of EC by the different Tat domains, the migration of EC was triggered by different concentrations of Tat_{86}, Tat R49G/K50I/R52L/R53I, Tat R78K/D80E, and *Tat C(22,25,27)A. All concentrations of the mutants tested (5 to 100 ng/ml) showed a reduced activity compared to Tat_{86}. However, the highest concentrations of Tat R78K/D80E (50 to 100 ng/ml) tended to reach the activity of Tat_{86} (Fig. 5).

MBP and GST, the two proteins fused to the different Tat molecules used, did not promote migration, proliferation, or adhesion at any concentration tested (0.1 to 200 ng/ml) (Fig. 2 to 4).

In vivo effect of Tat and Tat mutants in an angiogenesis model. The capacity of Tat to induce migration and prolifera-

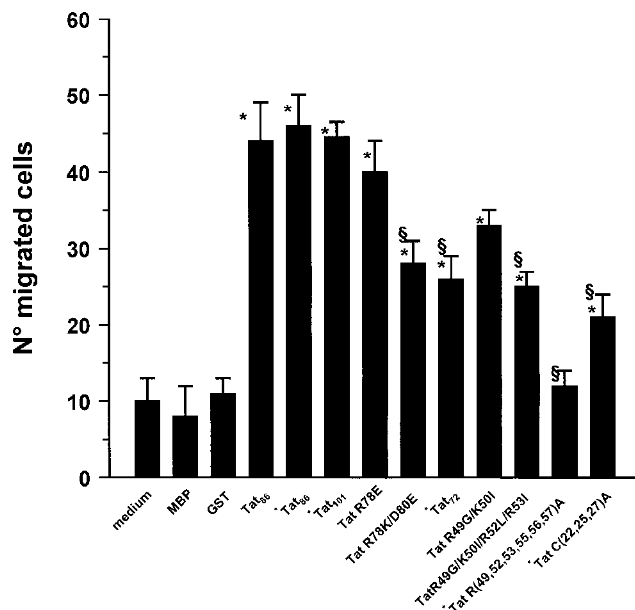

FIG. 2. Effect of Tat molecules on the migration of EC. The migration of EC across a 5-μm-pore-size polycarbonate filter in response to Tat molecules (20 ng/ml) or vehicle was evaluated by using a Boyden chamber. At the end of the incubation (37°C for 6 h), the filters were removed and stained and five high-power oil immersion fields were counted (400× magnification). Results (mean and standard deviation) of one experiment (performed in triplicate) representative of at least three independent experiments are shown. Data were analyzed by ANOVA (F = 41.11) and the Student-Newman-Keuls test. * indicates $P < 0.05$ within Tat-stimulated EC and unstimulated or control protein-stimulated cells; § indicates $P < 0.005$ within wild-type Tat molecule-stimulated EC and Tat mutant-stimulated cells.

tion of EC has as in vivo counterpart, i.e., the formation of new blood vessels in rabbit and mouse models (3, 4). Analysis of the effects of Tat mutants in a murine angiogenesis assay is reported in Table 1. Progressive mutations in the basic region or in the RGD sequence were associated with an increasing loss of angiogenic activity compared to the effect of wild-type molecules (Tat_{86} or $*Tat_{101}$). The deletion of the amino acids encoded by exon 2 of *tat* led to negligible angiogenic activity. Also, the mutation in the cysteine-rich region did not promote angiogenesis. Overall, the in vivo data clearly indicate that the angiogenic program elicited by Tat is dependent on the molecular integrity of its basic and cysteine-rich domains as well as that of the exon 2 product.

Binding of Tat and Tat mutants to EC. We have previously demonstrated that EC express high-affinity binding sites for Tat, identified as VEGFR-2 (4). In this study we used a large amount of $[^{125}I]Tat_{86}$ with high specific activity, which permitted us to study also the presence of low-affinity binding sites on EC. The binding of Tat was evaluated by using saturation binding curves and by competitive displacement of $[^{125}I]Tat_{86}$. The direct ligand binding curves suggested that Tat binding approached saturability without reaching full saturation. Scatchard analysis suggested the presence of two classes of binding sites. Besides a high-affinity binding site (K_d = 15.7 ± 5.3 pM; B_{max} = 31.9 ± 7.2 fmol [n = 3]), a low-affinity (K_d = 8.5 ± 3.2 nM; B_{max} = 2.6 ± 1.4 pmol [n = 3]) and high-capacity binding site was also observed (Fig. 6; Table 2). The competitive displacement experiments of $[^{125}I]Tat_{86}$ with increasing amounts of cold ligand confirmed the presence of two binding sites (Table 2).

By using Tat mutants, we examined the regions of Tat required for binding to EC. Saturation binding curves of $[^{125}I]$Tat R49G/K50I/R52L/R53I and of $[^{125}I]$*Tat C(22,25,27)A (Fig. 6) as well competitive displacement of $[^{125}I]Tat_{86}$ with the two unlabeled peptides (Table 2) indicated that these mutants bound EC only with low affinity. The number of low-affinity sites bound by $[^{125}I]$Tat R49G/K50I/R52L/R53I and $[^{125}I]$*Tat C(22,25,27)A was increased compared to that bound by $[^{125}I]Tat_{86}$ (Table 2). The same experimental approaches showed that Tat R78K/D80E bound endothelium in almost the same manner as did Tat_{86} (Fig. 6; Table 2). $[^{125}I]$MBP and $[^{125}I]$GST did not show any specific binding to EC, and the unlabeled proteins were unable to displace $[^{125}I]$Tat (data not shown).

Effect of Tat and Tat mutants on VEGFR-2 phosphorylation and signal transduction. Figure 7 shows the tyrosine phosphorylation of VEGFR-2 immunoprecipitated from EC stimulated with Tat_{86} and different Tat variants. Tat_{86}, $*Tat_{72}$, Tat R78K/D80E, and TatD80E induced the phosphorylation of VEGFR-2. Moreover, we consistently observed that Tat R78K/D80E phosphorylated the receptor more efficiently than Tat_{86} did. In contrast, mutants with mutations in the basic domain [(Tat R49G/K50I, Tat R49G/K50I/R52L/R53I, and *Tat R(49,52,53,55,56,57)A] showed a reduced ability to phosphorylate the receptor. This feature depends on the number of basic residues mutated, with Tat R49G/K50I being more active than Tat R49G/K50I/R52L/R53I and *Tat R(49,52,53,55,56,57)A. These data confirm that the binding and activation of VEGFR-2 are mediated by the basic domain, while the RGD sequence seems to be not involved in receptor phosphorylation. Mutations in the cysteine-rich region [*Tat C(22,25,

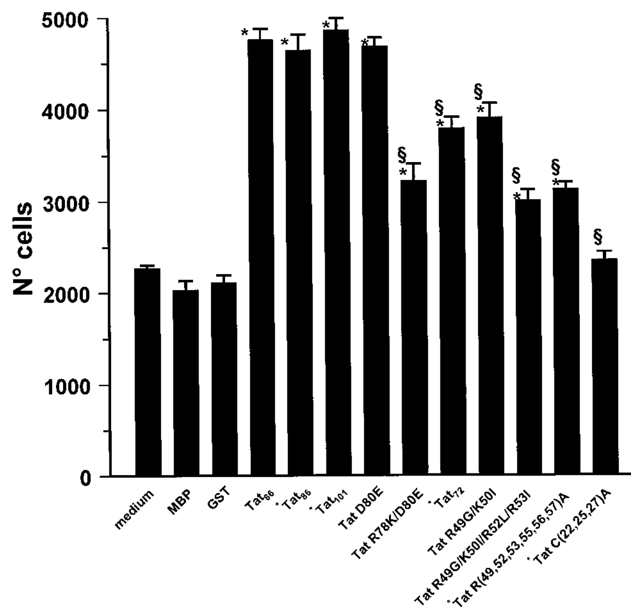

FIG. 3. Effect of Tat molecules on EC proliferation. A total of 2×10^3 EC were plated in 96-well plates and grown for 12 h in M199 containing 20% FCS. After 24 h, the medium was removed and replaced with M199 containing 2.5% FCS. Tat molecules (20 ng/ml) were added on days 0, 2, and 4, and the cell number was estimated on day 6. Cells were fixed and stained with crystal violet, and the absorbance was read at 540 nm. Results (mean and standard deviation) of one experiment (performed in quadruplicate) representative of at least four independent experiments are shown. Data were analyzed by ANOVA (F = 86.31) and the Student-Newman-Keuls test. * indicates $P < 0.05$ within Tat molecule-stimulated EC and unstimulated or control protein-stimulated cells; § indicates $P < 0.005$ within wild-type Tat molecule-stimulated EC and Tat mutant-stimulated cells.

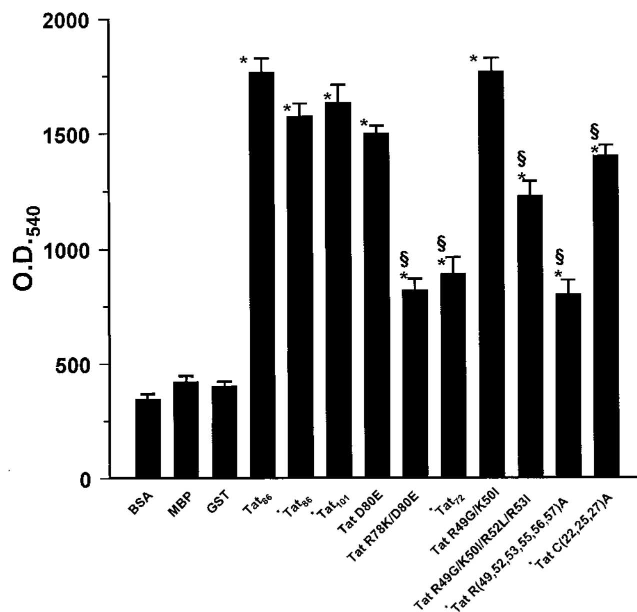

FIG. 4. Effect of immobilized Tat molecules on adhesion of endothelial cells. A plastic surface was coated overnight at 4°C with Tat molecules or control proteins (10 μg/well) and then saturated with 1% HSA. Suspended cells (5×10^4/0.1 ml) were seeded, and after a 1-h incubation at 37°C, plates were extensively washed in M199 containing 1% FCS, fixed, and stained with crystal violet. The optical density (O.D.) was read at 540 nm in a microtiter plate spectrophotometer. Results (mean and standard deviation) of one experiment (performed in quadruplicate) representative of at least four independent experiments are shown. Data were analyzed by ANOVA ($F = 71.16$) and the Student-Newman-Keuls test. * indicates $P < 0.05$ within Tat molecule-stimulated EC and unstimulated or control protein-stimulated cells; § indicates $P < 0.005$ within wild-type Tat molecule-stimulated EC and Tat mutant-stimulated cells.

27)A] also abrogated the ability of Tat to promote VEGFR-2 phosphorylation. MBP and GST did not phosphorylate VEGFR-2 (Fig. 7).

It has been recently demonstrated that PI 3-kinase activation is associated with the signals elicited by VEGFR-2 activation elicited by VEGF-A and Tat_{86} (25, 58). To support the notion that the basic domain of Tat_{86} is involved in the signalling pathways downstream of VEGFR-2, we tested the activity of PI 3-kinase in EC stimulated with Tat R49G/K50I/R52L/R53I and Tat R78K/D80E. The results reported in Fig. 8 show that Tat R49G/K50I/R52L/R53I activated PI 3-kinase activity to a lesser extent than Tat_{86} or Tat R78K/D80E did.

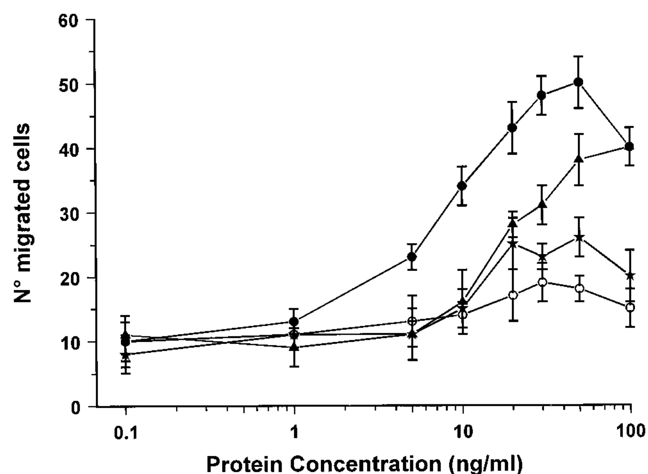

FIG. 5. Dose-dependent effect of Tat_{86} (●), Tat R49G/K50I/R52L/R53I (★), TatR78K/D80E (▲), and *Tat C(22,25,27)A (○) on EC migration. The cell migration was studied by the Boyden chamber technique as described in the legend to Fig. 1. The data obtained with $^{*}Tat_{86}$ have not been reported as superimposable on those obtained with Tat_{86}. Results (mean and standard deviation) of one experiment (performed in triplicate) of two are shown.

TABLE 1. Quantitative analysis of the angiogenic effect of Tat molecules[a]

Conditions	No. of positive implants/total no. of implanted Matrigel plugs[b]	Morphometric result (% of the total Matrigel area that is vascularized)[c]
1. Control	3/13	6 ± 3
2. Tat_{86}	10/11	56 ± 7
3. Tat_{72}	2/7	13 ± 3
4. $^{*}Tat_{101}$	3/3	56 ± 13
5. Tat D80E	4/4	49 ± 8
6. Tat R78K/D80E	3/9	5 ± 3
7. Tat R49G/K50I	9/13	36 ± 9
8. Tat R49G/K50I/R52L/R53I	1/8	7 ± 8
9. *Tat C(22,25,27)A	1/5	11 ± 7
10. MBP	1/5	9 ± 7
11. GST	1/5	8 ± 3

[a] Matrigel (0.5 ml) containing 10 U of heparin per ml was mixed with factors, and the angiogenic response was evaluated after 4 days by measurement of the hemoglobin content and morphometric analysis done on hematoxylin-eosin-stained histological sections (4).

[b] Implants were considered positive with a hemoglobin content ≥0.2 g/dl. $R \times C$ tables of contingency gave a χ^2 of 58.66 with $P < 0.05$.

[c] Morphometric analysis was performed on four representative implants for each group. Results are mean ± standard deviation. One-way ANOVA gave $F = 39.62$ with $P < 0.0001$. The Student-Newman-Keuls test gave a $P < 0.05$ in 1 versus 2, 4, 5, and 7; 2 versus 3, 6, 7, 9, 10, and 11; 3 versus 4, 5, and 7; 4 versus 6, 7, 8, 9, 10, and 11; 5 versus 6, 7, 8, 9, 10, and 11; 6 versus 7; and 7 versus 8, 9, 10, and 11.

DISCUSSION

HIV-1 Tat is essential for viral replication in infected cells and acts as a soluble mediator in the microenvironment. Several studies on the structure-activity relationship of Tat have determined the domains involved in gene transcription. The basic domain (aa 49 to 57) mediates the binding of Tat to *trans*-activation element RNA (22, 27), directs it to the nucleus (27, 52), and is essential for the recruitment of the p300/CBP transcriptional coactivator (40). A cluster of acidic residues (Glu2, Asp5, and Glu9) in the proline-rich domain, the cysteine-rich domain (aa 22 to 37), and the core domains (aa 32 to 47) all contribute to LTR-directed transcriptional activation (22, 38, 51, 52, 56). More recently, it has been reported that the C-terminal domain encoded by exon 2 also makes a small contribution to viral replication (60).

In this study we have analyzed Tat mutants for binding to and activation of EC, which are among the more relevant extracellular targets for this viral protein (1, 3–5, 13, 18, 28).

In a previous work (4), we detected only a high-affinity binding site corresponding to VEGFR-2, as demonstrated by the ability of VEGF-A to displace [^{125}I]Tat. However, it has been recently demonstrated that Tat binds to heparan sulfate (3, 53) and to integrins (7, 62) that both have features of low-affinity, high-capacity binding sites. Therefore, we have revised these binding studies by using larger amounts of [^{125}I]Tat than in our previous study, labeled at high efficiency. Under these experimental conditions, we detected two binding

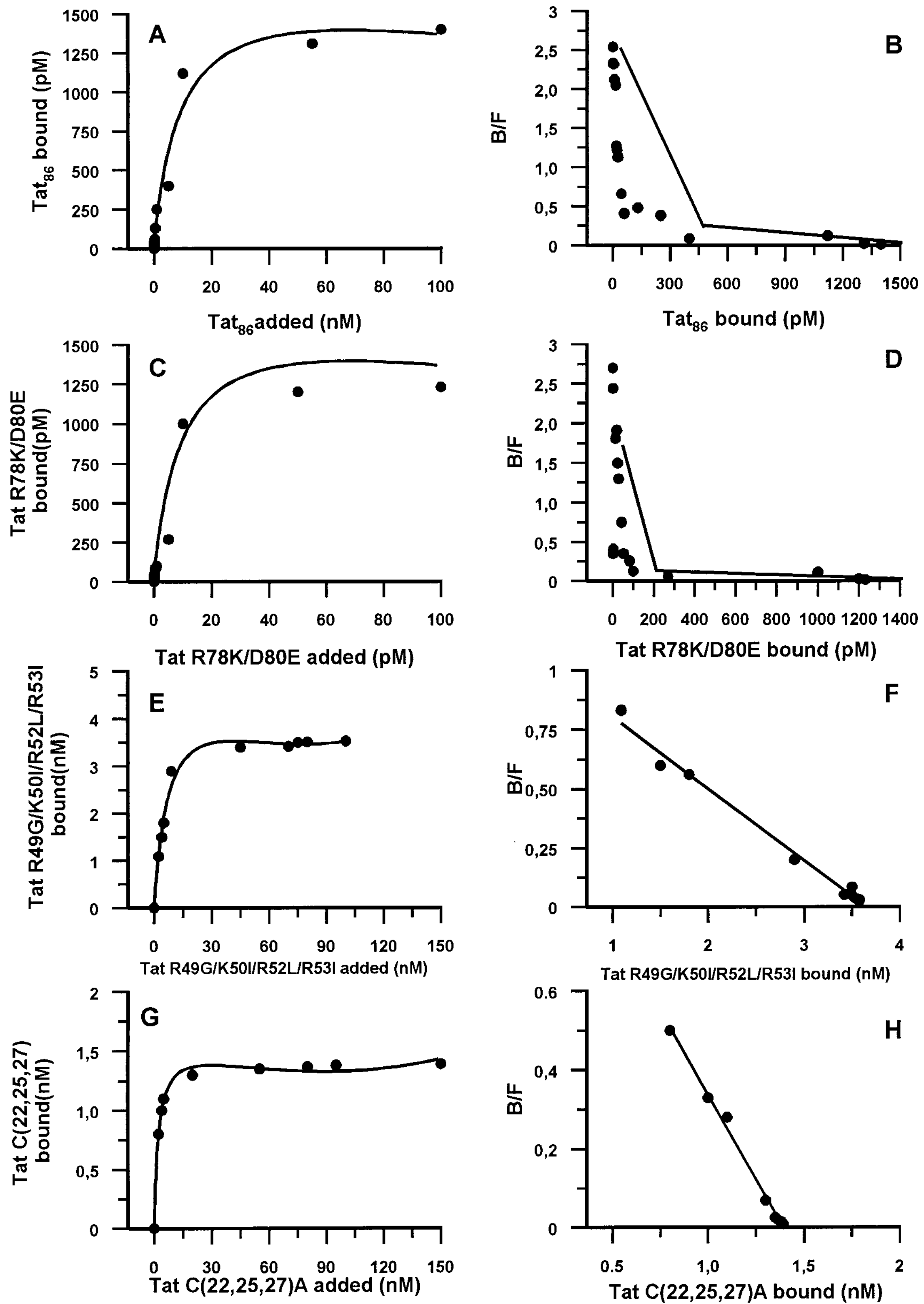

FIG. 6. Specific binding at equilibrium and Scatchard plot of [^{125}I]Tat$_{86}$ (A and B), [^{125}I]Tat R78K/D80E (C and D), [^{125}I] Tat R49G/K50I/R52L/R53I (E and F), and [^{125}I] *Tat C(22,25,27)A (G and H) to EC. Monolayers (10^5 cells) were incubated for 90 min at 22°C with the indicated concentrations of iodinated molecules in the presence of a 100-fold excess of cold ligands. Results of one experiment of three are shown. Tat$_{86}$: K_d (site 1) = 12.9 pM; B_{max} (site 1) = 28.9 fmol; K_d (site 2) = 4.21 nM; B_{max} (site 2) = 1.32 pmol. Tat R78K/D80E: K_d (site 1) = 23.8 pM; B_{max} (site 1) = 38.4 fmol; K_d (site 2) = 13.2 nM; B_{max} (site 2) = 1.50 pmol. Tat R49G/K50I/R52L/R53I: K_d (site 1) = 4.58 nM; B_{max} (site 1) = 3.51 pmol. *Tat C(22,25,27)A: K_d (site 1) = 1.15 nM; B_{max} (site 1) = 1.39 pmol.

TABLE 2. Binding affinity of Tat molecules to EC

Protein	High-affinity sites[a]			Low-affinity sites[a]		
	K_d[b] (pM)	B_{max}[b] (fmol)	IC_{50}[c] (pM)	K_d[b] (nM)	B_{max}[b] (pmol)	IC_{50}[c] (nM)
Tat_{86}	15.7 ± 7.3	31.9 ± 7.2	12.3 ± 7.1	8.5 ± 3.2	2.6 ± 1.4	17.0 ± 6.2
Tat R78K/D80E	25.6 ± 1.6	4.6 ± 1.2	10.2 ± 4.1	11.2 ± 4.3	4.3 ± 2.1	8.5 ± 3.2
Tat R49G/K50I/R52L/R53I	ND[d]	ND	ND	5.6 ± 2.1	5.5 ± 2.4	12.3 ± 6.3
*Tat C(22,25,27)A	ND	ND	ND	4.8 ± 2.8	3.1 ± 0.2	10.7 ± 3.5

[a] Mean ± standard deviation of three experiments.
[b] Calculated from binding-affinity curves.
[c] Calculated from displacement binding curves.
[d] ND, not done.

sites for Tat on the surface of EC. The presence of MBP did not interfere with the binding, because it did not show any specific binding to the cells and did not displace [^{125}I]Tat. The K_d of the first was in the picomolar range, and that of the second was in the nanomolar range. We can hypothesize that this low-affinity site reflects the binding of Tat to membrane glycosaminoglycans and/or to integrins.

To elucidate the functional importance of the basic domain of Tat, two, four, or six nonconservative mutations were made. Variants with a double mutations exhibited only a slightly reduced activity compared to Tat_{86} or Tat_{101} in term of migration, proliferation, and adhesion of EC and of angiogenesis induction. However, the variants with four or six substitutions showed a marked decrease in the activities studied. Because Tat signals inside the cells through the tyrosine kinase VEGFR-2 (4, 21, 58), we investigated the tyrosine phosphorylation of VEGFR-2. Tat R49G/K50I/R52L/R53I and *Tat R(49,52,53,55,56,57)A failed to activate the receptor. Tat R49G/K50I induced VEGFR-2 phosphorylation but to a lesser extent. The variants with two or four mutations did not activate PI 3-kinase, which is downstream of Tat_{86} and VEGF-A-activated VEGFR-2 (58). These results are in agreement with the similar observations obtained with a peptide encompassing the basic domain, which mimics the effect of Tat_{86} on VEGFR-2 (4, 21). The variant with four mutations did not retain the ability to bind to the high-affinity sites on EC, which correspond to VEGFR-2 (4). Taken together with the previous data, this suggests that the Tat basic domain is crucial for the binding and activation of VEGFR-2. The relevance of the positively charged amino acids in performing these functions is consistent with the observation that the charged residues R82, K84, and H86 of VEGF-A are important for VEGFR-2 recognition (32).

To ascertain whether the RGD sequence is required for Tat binding or activity, mutants with single (D80E) and double (R78K/D80E) mutations and a variant truncated after the residue 72 (Tat_{72}) were obtained. Tat R78K/D80E and Tat_{72} had reduced biological activity in vitro and in vivo, which is consistent with previous reports showing that $\alpha_v\beta_3$-, $\alpha_v\beta_5$-, and $\alpha_5\beta_1$-integrin participate in Tat-induced activation of EC (5, 13) as well as of other cell types (6, 7, 35, 48, 62, 70). Moreover, the RGD sequence and the C-terminal domain are not required for VEGFR-2 activation, as shown by the ability of Tat R78K/D80E and Tat_{72} to induce VEGFR-2 phosphorylation. Furthermore, the variant with the double mutations activated PI 3-kinase activity and retained the ability to bind both low- and high-affinity Tat binding sites on the EC membrane. In our experiments, we have observed that the activity of Tat R78K/D80E on VEGFR-2 phosphorylation was greater than that elicited by Tat_{86}. The explanation of this result is lacking, but it will be interesting to determine the role of VEGFR-2 regulatory pathways associated with integrins. For instance, we have recently shown that β_3-integrin is associated with VEGF-

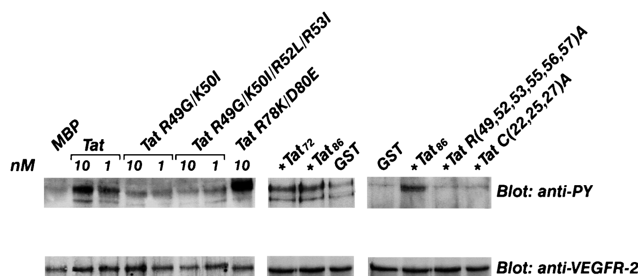

FIG. 7. Effect of Tat molecules on tyrosine phosphorylation of VEGFR-2. Quiescent, confluent EC were preincubated for 15 min at 37°C with 1 mM Na_3VO_4 and then stimulated with Tat molecules (20 ng/ml) for 10 min. The cells were lysed and immunoprecipitated with anti-VEGFR-2 antibody. The immunoprecipitate was analyzed by SDS-PAGE followed by immunoblotting with antiphosphotyrosine MAb. Subsequently, the blots were reprobed with anti-VEGFR-2 antibody. Immunoreactive bands were detected by enhanced chemiluminescence. The results are representative of three similar experiments.

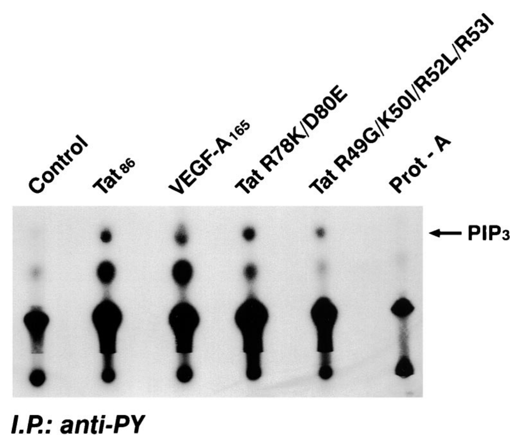

FIG. 8. Effect of Tat molecules on PI 3-kinase in endothelial cells. Quiescent, confluent ECs were stimulated with Tat molecules (20 ng/ml) for 15 min at 37°C. The PI 3-kinase assay was performed on immune complexes done with MAb antiphosphotyrosine (anti-PY) antibodies from lysates of EC in the presence of 40 μM ATP, 50 μCi of [γ-^{32}P]ATP, and 50 μg of a presonicated mixture of phosphatidylinositol-4,5-bisphosphate and phosphatidylserine per ml in 25 mM HEPES (pH 7.4)–1 mM EGTA. The extracted lipids were separated by thin-layer chromatography and visualized by autoradiography. PIP_3, phosphatidylinositol-3,4-bisphosphate; Prot-A, a PI 3-kinase assay done on protein A alone. Spots corresponding to PIP_3 were recovered and counted (n = 3): control, 420 ± 132 cpm; Tat_{86}, 1,342 ± 231; Tat R78K/D80E, 1,280 ± 280; Tat R49G/K50I/R52L/R53I, 750 ± 201. I.P., immunoprecipitate.

A-stimulated VEGFR-2 and that a MAb against this integrin inhibits the activation of the receptor (58). Alternatively, this variant can assume a spatial conformation more favorable for receptor activation, independent of the interaction with integrins. Therefore, the activation of the biological activities of EC related to angiogenesis most probably requires the engagement of VEGFR-2 as well as integrins by two specific molecular determinants of Tat: the basic domain and the product of exon 2 containing the RGD motif. Many of the integrin-induced signalling pathways are also normally activated by binding of soluble growth factors to their receptors, which suggests the existence of coordinate mechanisms between integrins and growth factors in the control of cell functions, and there is increasing evidence that growth factors can induce an appropriate cellular response only when the target cells express defined sets of integrins (24, 55). Normally, growth factors do not directly bind to and activate integrin. The data reported here and those showing that anti-$\alpha_v\beta_3$ or anti-VEGFR-2 neutralizing antibodies (4, 5, 58) inhibit the EC response to Tat suggest that this viral protein is a unique example of a molecule which turns on intracellular signals by direct activation of both receptor and integrin systems. Furthermore, besides playing a direct role in integrin activation, the C terminus region of Tat could regulate VEGFR-2 through the presence of other determinants relevant for the engagement of neuropilin-1, a coreceptor of this tyrosine kinase receptor (57).

*Tat C(22,25,27)A was used to study the role of the cysteine-rich region of Tat in EC activation. This variant was less potent than Tat_{86} or Tat_{101} in terms of migration, proliferation, adhesion, and in vivo angiogenesis, bound only the low-affinity binding site, and showed low efficiency in VEGFR-2 phosphorylation. Because it has been suggested that Tat forms a dimer bridging cysteine-rich regions from each monomer (19) and that two cysteine residues are pivotal for the VEGF-A dimerization and the subsequent binding to and activation of endothelium (46, 50), it could be hypothesized that the active form of Tat on the endothelium has a dimeric structure. The relevance of this domain has also been emphasized by Albini et al., who demonstrated its role in the migration of monocytes (2).

Except for Tat D80E, all mutants studied showed a reduced ability to activate EC functions, but their effects are only partially overlapping. For example, mutants with mutations in the RGD sequence have reduced biological activities but fully activated VEGFR-2 phosphorylation. Tat R49G/K50I promoted cell adhesion as Tat_{86}, but its migratory and proliferative capacities were consistently lower than those of Tat_{86}. Mutants with mutations in basic and in cysteine-rich domains had similar kinetic binding parameters, which differed from those of TatR78K/E80D. Furthermore, Tat E80D was similar to Tat_{86} or $*Tat_{101}$ in all assays performed. Taken together, these observations exclude the possibility that the effects of the Tat mutants are caused merely by an unfolded protein, as also reported for other studies performed with Tat mutants (22, 27, 38, 40, 51, 52, 56, 60).

In conclusion, this study demonstrates that the product of the first exon of *tat* is crucial for the activation of VEGFR-2 and for induction of the activation of an angiogenic program in EC. However, to obtain full activation of this program, Tat also requires the C-terminal region containing the RGD sequence. The C-terminal region could influence the VEGFR-2 response by interacting with integrin subunits or with neuropilin-1 (57), which modulate the functions of this tyrosine kinase receptor, or with other unidentified coreceptors. Further studies of the hypothesized role of Tat dimerization by intermolecular cysteine bridge formation should make it possible to define the receptor-ligand interaction more precisely.

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ADDENDUM IN PROOF

Boykins et al. (R. A. Boykins, R. Mahieux, U. T. Shankavaram, Y. S. Gho, S. F. Lee, I. K. Hewlett, L. M. Wahl, H. K. Kleiman, J. N. Brady, K. M. Yamada, and S. Dhawan, J. Immunol. **163**:15–20, 1999) recently reported that a peptide containing six cysteine residues (from amino acids 21 to 40 of the Tat molecule) is angiogenic in chicken chorioallantoic membranes.

REFERENCES

1. **Albini, A., G. Barillari, R. Benelli, R. C. Gallo, and B. Ensoli.** 1995. Angiogenic properties of human immunodeficiency virus type 1 Tat protein. Proc. Natl. Acad. Sci. USA **92**:4838–4842.
2. **Albini, A., R. Benelli, D. Giunciuglio, T. Cai, G. Mariani, S. Ferrini, and D. M. Noonan.** 1998. Identification of a novel domain of HIV tat involved in monocyte chemotaxis. J. Biol. Chem. **273**:15895–15900.
3. **Albini, A., R. Benelli, M. Presta, M. Rusnati, M. Ziche, A. Rubartelli, G. Paglialunga, F. Bussolino, and D. Noonan.** 1996. HIV-tat protein is a heparin-binding angiogenic growth factor. Oncogene **12**:289–297.
4. **Albini, A., R. Soldi, D. Giunciuglio, E. Giraudo, R. Benelli, L. Primo, D. Noonan, M. Salio, G. Camussi, W. Rockl, and F. Bussolino.** 1996. The angiogenesis induced by HIV-1 tat protein is mediated by the Flk-1/KDR receptor on vascular endothelial cells. Nat. Med. **2**:1371–1375.
5. **Barillari, G., C. Sgadari, V. Fiorelli, F. Samaniego, S. Colombini, V. Manzari, A. Modesti, B. C. Nair, A. Cafaro, M. Sturzl, and B. Ensoli.** 1999. The Tat protein of human immunodeficiency virus type-1 promotes vascular cell growth and locomotion by engaging the $\alpha5\beta1$ and $\alpha v\beta3$ integrins and by mobilizing sequestered basic fibroblast growth factor. Blood **94**:663–672.
6. **Benelli, R., R. Mortarini, A. Anichini, D. Giunciuglio, D. M. Noona, S. Montalti, C. Tacchetti, and A. Albini.** 1998. Monocyte-derived dendritic cells and monocytes migrate to HIV-Tat RGD and basic peptides. AIDS **12**:261–268.
7. **Brake, D. A., C. Debouk, and G. Biesecker.** 1990. Identification of an Arg-Gly-Asp (RGD) cell adhesion site in human immunodeficiency virus type i transactivation Tat protein. J. Cell Biol. **111**:1275–1281.
8. **Bussolino, F., M. Arese, G. Montrucchio, L. Barra, L. Primo, R. Benelli, F. Sanavio, M. Aglietta, D. Ghigo, M. R. Rola-Pleszczynski, A. Albini, and G. Camussi.** 1995. Platelet activating factor produced in vitro by Kaposi's sarcoma cells induces and sustains in vivo angiogenesis. J. Clin. Investig. **96**: 940–952.
9. **Bussolino, F., M. F. Di Renzo, M. Ziche, E. Bocchietto, M. Olivero, L. Naldini, G. Gaudino, L. Tamagnone, A. Coffer, and P. M. Comoglio.** 1992. Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. J. Cell Biol. **119**:629–641.
10. **Chang, H. C., F. Samaniengo, B. C. Nair, L. Buonaguro, and B. Ensoli.** 1997. HIV-1 Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulphate proteoglycans through its basic region. AIDS **11**:1421–1431.
11. **Corallini, A., G. Altavilla, L. Pozzi, F. Bignozzi, M. Negrini, P. Rimessi, F. Gualandi, and G. Barbanti-Brodano.** 1993. Systemic expression of HIV-1 tat gene in transgenic mice induces endothelial proliferation and tumours of different histotypes. Cancer Res. **53**:5569–5575.
12. **Cupp, C., J. P. Taylor, K. Khalili, and S. Amini.** 1993. Evidence for stimulation of the transforming growth factor beta 1 promoter by HIV-1 Tat in cells derived from CNS. Oncogene **8**:2231–2236.
13. **Dhawan, S., R. K. Puri, A. Kumar, H. Duplan, J. Masson, and B. B. Aggarwal.** 1997. Human immunodeficiency virus-1-Tat protein induces the cell surface expression of endothelial leukocyte adhesion molecule-1, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 in human endothelial cells. J. Immunol. **90**:1535–1544.
14. **Ensoli, B., G. Barillari, S. Z. Salahuddin, R. C. Gallo, and F. Wong-Staal.** 1990. Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. Nature **344**:84–86.
15. **Ensoli, B., L. Buonaguro, G. Barillari, V. Fiorelli, R. Gendelman, R. A. Morgan, P. Wingfield, and R. C. Gallo.** 1993. Release, uptake, and effects of

extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation. J. Virol. **67:**277–287.
16. **Ensoli, B., R. Gendelman, P. Markham, V. Fiorelli, S. Colombini, M. Raffeld, A. Cafaro, H. K. Chang, J. N. Brady, and R. C. Gallo.** 1994. Synergy between basic fibroblast growth factor and HIV-1 Tat protein in induction of Kaposi's sarcoma. Nature **371:**674–680.
17. **Fauci, A. S.** 1987. AIDS: immunopathogenic mechanism and research strategies. Clin. Res. **35:**503–510.
18. **Fiorelli, V., R. Gendelman, F. Samaniego, P. D. Markham, and B. Ensoli.** 1995. Cytokines from activated T cells induce normal endothelial cells to acquire the phenotypic and functional features of AIDS-Kaposi's sarcoma spindle cells. J. Clin. Investig. **95:**1723–1734.
19. **Frankel, A. D., D. S. Bredt, and C. O. Pabo.** 1988. Tat protein from human immunodeficiency virus forms a metal-linked dimer. Science **240:**70–73.
20. **Frankel, A. D., and C. O. Pabo.** 1988. Cellular uptake of the Tat protein from human immunodeficiency virus. Cell **55:**1189–1193.
21. **Ganju, R. K., N. Munshi, B. C. Nair, L. Zy, P. Gill, and J. E. Groopman.** 1998. Human immunodeficiency virus Tat modulates the Flk-1/KDR receptor, mitogen-activated protein kinases, and components of focal adhesion in Kaposi's sarcoma cells. J. Virol. **72:**6131–6137.
22. **Garcia, J. A., D. Harrich, E. Soultanakis, F. Wu, R. Mitsuyasu, and R. B. Gaynon.** 1989. Human immunodeficiency virus type 1 LTR TATA and TAR region sequences required for transcriptional regulation. EMBO J. **8:**765–778.
23. **Garza, H. H., O. Prakash, and D. J. Carr.** 1996. Aberrant regulation of cytokines in HIV-1 TAT_{72}-transgenic mice. J. Immunol. **156:**3631–3637.
24. **Giancotti, F. G.** 1997. Integrin signaling: specificity and control of cell survival and cell cycle progression. Curr. Opin. Cell Biol. **9:**691–700.
25. **Guo, D., Q. Jia, H. Y. Song, R. S. Warren, and D. B. Donner.** 1995. Vascular endothelial cell growth factor promotes tyrosine phosphorylation of mediators of signal transduction that contain SH2 domains. Association with endothelial cell proliferation. J. Biol. Chem. **270:**6729–6733.
26. **Gutheil, W. G., M. Subramanyam, G. R. Flentke, D. G. Sanford, E. Munoz, B. T. Huber, and W. W. Bachovchin.** 1994. Human immunodeficiency virus 1 Tat binds to dipeptidyl aminopeptidase IV (CD26): a possible mechanism for Tat's immunosuppressive activity. Proc. Natl. Acad. Sci. USA **91:**6594–6598.
27. **Hauber, J., H. M. Malim, and B. R. Cullen.** 1989. Mutational analysis of the conserved basic domain of human immunodeficiency virus *tat* protein. J. Virol. **63:**1181–1187.
28. **Hofman, F. M., A. D. Wright, M. M. Dohadwala, F. Wong-Staal, and S. M. Walker.** 1993. Exogenous tat protein activates human endothelial cells. Blood **82:**2774–2780.
29. **Howcroft, T. K., K. Strebel, M. A. Martin, and D. S. Singer.** 1993. Repression of MHC class I gene promoter activity by two-exon Tat of HIV. Science **260:**1320–1322.
30. **Huang, L., C. J. Li, and A. B. Pardee.** 1997. Human immunodeficiency virus type 1 TAT protein activates B lymphocytes. Biochem. Biophys. Res. Commun. **237:**461–464.
31. **Jones, K. A., and M. B. Peterlin.** 1994. Control of RNA initiation and elongation at the HIV-1 promoter. Annu. Rev. Biochem. **63:**717–743.
32. **Keyt, B. A., H. V. Nguyen, L. T. Berleau, C. M. Duarte, J. Park, H. Chen, and N. Ferrara.** 1996. Identification of vascular endothelial growth factor determinants for binding KDR and FLT-1 receptors. Generation of receptor-selective VEGF variants by site-directed mutagenesis. J. Biol. Chem. **271:**5638–5646.
33. **Kolson, D. L., J. Buchhalter, R. Collman, B. Hellmig, C. F. Farrell, C. Debouck, and F. Gonzalez-Scarano.** 1993. HIV-1 Tat alters normal organization of neurons and astrocytes in primary rodent brain cell cultures: RGD sequence dependence. AIDS Res. Human Retroviruses **9:**677–685.
34. **Kuppuswamy, M., T. Subramanian, A. Srinivasan, and G. Chinnadurai.** 1989. Multiple functional domains of Tat, the trans-activator of HIV-1, defined by mutational analysis. Nucleic Acids Res. **17:**3551–3561.
35. **Lafrenie, R. M., L. M. Wahl, J. S. Epstein, I. K. Hewlett, K. M. Yamada, and S. Dhawan.** 1996. HIV-1-Tat protein promotes chemotaxis and invasive behavior by monocytes. J. Immunol. **157:**974–977.
36. **Li, C. J., D. J. Friedman, C. Wang, V. Metelev, and A. B. Pardee.** 1995. Induction of apoptosis in uninfected lymphocytes by HIV-1 Tat protein. Science **268:**429–431.
37. **Li, C. J., C. Wang, D. J. Friedman, and A. B. Pardee.** 1995. Reciprocal modulations between p53 and Tat of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **92:**5461–5464.
38. **Luo, Y., and B. M. Peterlin.** 1993. Juxtaposition between activation and basic domains of human immunodeficiency virus type 1 Tat is required for optimal interactions between Tat and TAR. J. Virol. **67:**3441–3445.
39. **Marcuzzi, A., J. Weinberger, and O. K. Weinberger.** 1992. Transcellular activation of the human immunodeficiency virus type 1 long terminal repeat in cocultured lymphocytes. J. Virol. **66:**4228–4232.
40. **Marzio, G., M. Tyagi, M. I. Gutierrez, and M. Giacca.** 1998. HIV-1 tat transactivator recruits p300 and CREB-binding protein histone acetyltransferases to the viral promoter. Proc. Natl. Acad. Sci. USA **95:**13519–13524.
41. **McCloskey, T. W., M. Ott, E. Tribble, S. A. Khan, S. Teichberg, M. O. Paul, S. Pahwa, E. Verdin, and N. Chirmule.** 1997. Dual role of HIV Tat in regulation of apoptosis in T cells. J. Immunol. **158:**1014–1019.
42. **Milani, D., M. Mazzoni, P. Borgatti, G. Zauli, L. Cantley, and S. Capitani.** 1996. Extracellular human immunodeficiency virus type-1 Tat protein activates phosphatidylinositol 3-kinase in PC12 neuronal cells. J. Biol. Chem. **271:**22961–22964.
43. **Mitola, S., S. Sozzani, W. Luini, M. Arese, A. Borstatti, H. A. Weich, and F. Bussolino.** 1997. Tat-HIV-1 induces human monocyte chemotaxis by activation of vascular endothelial growth factor receptor-1. Blood **90:**1365–1372.
44. **Moses, A., F. E. Bllom, C. D. Pauza, and J. A. Nelson.** 1993. Human immunodeficiency virus infection of human brain capillary endothelial cells occurs via a CD4/galactosylceramide-independent mechanism. Proc. Natl. Acad. Sci. USA **90:**10474–10478.
45. **Moses, A. S., S. G. Stenglein, J. G. Strussenberg, K. Wehrly, B. Chesebro, and J. A. Nelson.** 1996. Sequences regulating trophism of human immunodeficiency virus type 1 for brain capillary endothelial cells map to a unique region on the viral genome. J. Virol. **70:**3401–3406.
46. **Muller, Y. A., B. Li, H. W. Christinger, B. C. Cunningham, and A. M. de Vos.** 1997. Vascular endothelial growth factor: crystal structure and functional mapping of the kinase domain receptor binding site. Proc. Natl. Acad. Sci. USA **94:**7192–7197.
47. **New, D. R., S. B. Maggirwar, L. G. Epstein, S. Dewhurst, and H. A. Gelbard.** 1998. HIV-1 Tat induces neuronal death via tumor necrosis factor-alpha and activation of non-N-methyl-D-aspartate receptors by a NFkappaB-independent mechanism. J. Biol. Chem. **273:**17852–17858.
48. **Orsini, M. J., C. M. Debouck, C. L. Webb, and P. G. Lysko.** 1996. Extracellular human immunodeficiency virus type 1 Tat protein promotes aggregation and adhesion of cerebellar neurons. J. Neurosci. **16:**2546–2552.
49. **Ott, M., J. L. Lovett, L. Mueller, and E. Verdin.** 1998. Superinduction of IL-8 in T cells by HIV-1 Tat protein is mediated through NF-kappaB factors. J. Immunol. **160:**2872–2880.
50. **Potgens, A. J., N. H. Lubsen, M. C. van Altena, R. Vermeulen, A. Bakker, J. G. Schoenmakers, D. J. Ruiter, and R. M. de Waal.** 1994. Covalent dimerization of vascular permeability factor/vascular endothelial growth factor is essential for its biological activity. Evidence from Cys to Ser mutations. J. Biol. Chem. **269:**32879–32885.
51. **Rice, A. P., and F. Carlotti.** 1990. Structural analysis of wild-type and mutant human immunodeficiency virus type 1 Tat proteins. J. Virol. **64:**6018–6026.
52. **Ruben, S., A. Perkins, R. Purcell, K. Joung, R. Sia, R. Burghoff, W. A. Haseltine, and C. A. Rosen.** 1989. Structural and functional characterization of human immunodeficiency virus tat protein. J. Virol. **63:**1–8.
53. **Rusnati, M., G. Tulipano, C. Urbinati, E. Tanghetti, R. Giuliani, M. Giacca, M. Ciomei, A. Corallini, and M. Presta.** 1998. The basic domain in HIV-1 Tat protein as a target for polysulfonated huparin-mimicking extracellular Tat antagonists. J. Biol. Chem. **273:**16027–16037.
54. **Scala, G., M. R. Ruocco, C. Ambrosino, M. Mallardo, V. Giordano, F. Baldassarre, E. Dragonetti, I. Quinto, and S. Venuta.** 1994. The expression of the interleukin 6 gene is induced by the human immunodeficiency virus 1 TAT protein. J. Exp. Med. **179:**961–971.
55. **Schwartz, M. A.** 1997. Integrins, oncogenes, and anchorage independence. J. Cell Biol. **139:**575–578.
56. **Siderovski, O. P., T. Matsuyama, E. Frigerio, S. Chui, X. Min, H. Erfle, M. Sumner-Smith, R. W. Barnett, and T. W. Mak.** 1992. Random mutagenesis of the human immunodeficiency virus type-1 *trans*-activator of transcription (HIV-1 Tat). Nucleic Acids Res. **20:**5311–5320.
57. **Soker, S., S. Takashima, H. Q. Miao, G. Neufeld, and M. Klagsbrun.** 1998. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell **92:**735–745.
58. **Soldi, R., S. Mitola, S. Strasly, P. Defilippi, G. Tarone, and F. Bussolino.** 1999. Role of αvβ3 integrin in the activation of vascular endothelial growth factor receptor-2. EMBO J. **18:**734–740.
59. **Taylor, J. P., C. Cupp, A. Diaz, M. Chowdhury, K. Khalili, S. A. Jimenez, and S. Amini.** 1992. Activation of expression of genes coding for extracellular matrix proteins in Tat-producing glioblastoma cells. Proc. Natl. Acad. Sci. USA **89:**9617–9621.
60. **Verhoef, K., M. Bauer, A. Meyerhans, and B. Berkhout.** 1998. On the role of the second coding exon of the HIV-1 Tat protein in virus replication and MHC class I downregulation. AIDS Res. Hum. Retroviruses **14:**1553–1559.
61. **Verhoef, K., S. E. C. Koken, and B. Berkhout.** 1993. Electroporation of the HIV Tat trans-activator protein into cells. Anal. Biochem. **210:**210–214.
62. **Vogel, B. E., S. J. Lee, A. Hildebrand, W. Craig, M. D. Pierschbacher, F. Wong-Staal, and E. Ruoslahti.** 1993. A novel integrin specificity exemplified by binding of the alpha v beta 5 integrin to the basic domain of the HIV Tat protein and vitronectin. J. Cell Biol. **121:**461–468.
63. **Vogel, J., S. H. Hinrichs, K. R. Reynolds, P. A. Luciw, and G. Jay.** 1988. The HIV tat gene induces dermal lesions resembling Kaposi's sarcoma in transgenic mice. Nature **335:**606–611.
64. **Weeks, B. S., K. Desai, P. M. Loewenstein, M. E. Klotman, P. E. Klotman, M. Green, and H. K. Kleinman.** 1993. Identification of a novel cell attachment domain in the HIV-1 Tat protein and its 90-kDa cell surface binding protein. J. Biol. Chem. **268:**5279–5284.
65. **Westendorp, M. O., R. Frank, C. Ochsenbauer, K. Stricker, J. Dhein, H.**

Walczak, K. M. Debatin, and P. H. Krammer. 1995. Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. Nature **375:**497–500.

66. **Westendorp, M. O., V. A. Shatrov, K. Schulze-Osthoff, R. Frank, M. Kraft, M. Los, P. H. Krammer, W. Droge, and V. Lehmann.** 1995. HIV-1 Tat potentiates TNF-induced NF-kappa B activation and cytotoxicity by altering the cellular redox state. EMBO J. **14:**546–554.
67. **Wright, C. M., B. K. Felber, H. Paskalis, and G. N. Pavlakis.** 1986. Expression and characterization of the *trans* activator of HTLV-III/LAV virus. Science **234:**988–992.
68. **Wrenger, S., T. Hoffmann, J. Faust, C. Mrestani-Klauss, W. Brandt, K. Neubert, M. Kraft, S. Olek, R. Frank, S. Ansorge, and D. Reinhold.** 1997. The N-terminal structure of HIV-1 Tat is required for suppression of CD26-dependent T cell growth. J. Biol. Chem. **272:**30283–30288.
69. **Zauli, G., D. Gibellini, A. Caputo, A. Bassini, M. Negrini, M. Monne, M. Mazzoni, and S. Capitani.** 1995. The human immunodeficiency virus type-1 Tat protein upregulates Bcl-2 gene expression in Jurkat T-cell lines and primary peripheral blood mononuclear cells. Blood **86:**3823–3834.
70. **Zauli, G., D. Gibellini, C. Celeghini, C. Mischiati, A. Bassini, M. La Placa, and S. Capitani.** 1996. Pleiotropic effects of immobilized versus soluble recombinant HIV-1 Tat protein on CD3-mediated activation, induction of apoptosis, and HIV-1 long terminal repeat transactivation in purified $CD4^+$ T lymphocytes. J. Immunol. **157:**2216–2224.