The human immunodeficiency virus-1 Tat protein activates human umbilical vein endothelial cell E-selectin expression via an NF-κB-dependent mechanism

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Running Title: HIV Tat-mediated κB-dependent E-selectin induction
Summary

Human immunodeficiency virus infection is associated with inflammation and endothelial cell activation that cannot be ascribed to direct infection by the virus or to the presence of opportunistic infections. Factors related to the virus itself, to the host and/or to environmental exposures probably account for these observations. The HIV protein Tat, a viral regulator required for efficient transcription of the viral genome in host cells is secreted from infected cells and taken up by uninfected bystander cells. Tat can also act as a general transcriptional activator of key inflammatory molecules. We have examined whether Tat contributes to this endothelial cell activation by activating NF-κB. Human endothelial cells exposed to Tat in the culture medium activated E-selectin expression with delayed kinetics compared to TNF. Tat-mediated E-selectin up-regulation required the basic domain of Tat and was inhibited by a Tat antibody. Transfection of human E-selectin promoter-luciferase reporter constructs into Tat-bearing cells or into endothelial cells co-transfected with a Tat-expression vector resulted in induction of luciferase expression. Either Tat or TNF activated p65 translocation and binding to an oligonucleotide containing the E-selectin κB site 3 sequence. Tat-mediated p65 translocation was also delayed compared with TNF. Neither agent induced new synthesis of p65. A super-repressor adenovirus (AdIκBαSR) that constitutively sequesters IκB in the cytoplasm as well as cycloheximide or actinomycin D inhibited Tat- or TNF-mediated κB translocation and E-selectin up-regulation.

Introduction

Inflammation-mediated tissue destruction results from recruitment and extravasation of inflammatory cells at some tissue site, usually because of injury or infection. Host-pathogen interactions trigger the inflammatory response, and the balance between the pro- and anti-inflammatory forces will determine the outcome of the infection. The vascular endothelium is an essential component of this balance and contributes to the separation between vascular and interstitial spaces. At areas of inflammation, neutrophils are recruited by a combination of
adhesion molecule up-regulation and chemokine secretion. The leukocytes in turn activate their own inflammatory programs of increased reactive oxygen species generation and cytokine secretion. Endothelial cells respond in kind by becoming further activated. Accordingly, diseases that affect the endothelium result in marked vasculopathies and failure of the endothelial barrier.

In HIV infection there is a diffuse endothelial cell involvement, not often recognized, that includes increased susceptibility to inflammatory cytokines, increased adhesion molecule expression, and increased neutrophil and mononuclear cell adhesion. Cardiovascular complications, such as myocarditis with infiltrating neutrophils and mononuclear cells are often observed, even in the absence of infectious pathogens (1,2). Even though HIV may enter endothelial cells via transcytosis (3), productive infection is very hard to achieve unless the cells are proliferating in the presence of cytokines (4). On the other hand, transgenic mice carrying a replication-defective provirus develop smooth muscle hypertrophy as well as adventitial infiltration by T-lymphocytes. This vascular remodeling leads to narrowing of the blood vessels of different sizes with the resultant ischemia in organs such as brain, heart, kidney, pancreas and spleen (5). In view of these observations, the question remains as to what is causing this inflammation and vasculopathy when there is no obvious infectious or opportunistic pathogen.

The HIV-1 Tat protein is an early viral protein of 101 amino-acids when isolated from primary HIV isolates, or of 86 amino-acids when isolated from the laboratory strain HBX2, and which still retains full activity (6). Expression of Tat is critical for productive HIV infection. The tat gene consists of 2 coding exons. The first one, which encodes 72 amino acids, is sufficient to activate HIV LTR-mediated gene expression in co-transfection promoter-reporter assays (7). However, in the context of viral infection, where the integrated provirus is subject to chromatin influences, the second exon is required for trans activation of the long terminal repeat (LTR) (8). Exon 1 contains the cysteine-rich trans-activation domain, the core (9-11) and an arginine-rich motif (ARM) that is responsible for Tat-TAR RNA interactions. The ARM acts as a nuclear localization signal (NLS) as well (12,13) and is responsible for NF-κB activation in
HeLa cells (14). The second exon codes for the remaining 32 amino acids. Tat acts by binding to a region located at the 5'-end of the viral transcript. This region, called TAR (trans-activation response element), forms a stable stem-loop structure having a high affinity for Tat (7,11). The N-terminal trans-activation domain, when tethered to TAR, strongly interacts with cyclin T1, which is a component of transcription elongation factor β (7,15). The cyclin dependent kinase 9 that is part of this complex then phosphorylates the carboxyl-terminal domain of RNA polymerase II. This phosphorylation facilitates the elongation step by preventing premature termination and ensures production of the full-length viral transcript (16-18). The end-result is a significant increase in the production of viral proteins, essential for productive infection.

Tat can be secreted from infected cells and circulates in the bloodstream of infected individuals. From the circulation, Tat enters uninfected cells and once internalized, it alters cellular physiology by positively or negatively affecting gene expression. For example, Tat activates TGF-β expression in human chondrocytes (19), TNF expression in mononuclear cells (20), IL-8 secretion in endothelial cells (21) and T cell lines following CD3- and CD28-mediated costimulation (22). Tat increases IL-1β production in monocyctic cells, and IL-6 protein and mRNA in astrocytes, both effects independent of TNF-α production (23). Tat also increases FAS and FAS-ligand transcription, presumably via NF-κB (24), induces IL-10 in peripheral blood monocytes (25). Microglia exposed to Tat induce nitric oxide synthase (iNOS) and NO production, and this induction is also dependent upon NF-κB (26). On the other hand, negative effects include repression of MHC class-I gene promoter activity (27) and of the important antioxidant enzyme Mn-superoxide dismutase (28). Interestingly, a deregulation of cytokine expression and/or secretion is a hallmark of HIV infection (29-32).

These studies indicate that Tat may be having some of its positive transcriptional effects through NF-κB activation. Indeed, Tat activates the transcription factor NF-κB in HeLa cells. This activation is mediated by PKR, the double-stranded RNA-dependent protein kinase, which phosphorylates IκB leading to its degradation (14). Subsequent NF-κB translocation into the nucleus increases expression of a cascade of inflammatory genes (33). Tat introduced via
liposomes results in nuclear translocation of NF-κB (34) and induction of CD69 gene transcription in an erythroleukemia cell line (35) and interleukin-8 secretion in a T-cell line (22). Extracellular Tat is associated with an increase in both NF-κB binding and protein kinase C activity in primary fetal human astrocytes (36). On the other hand, Tat does not activate the NF-κB responsive reporter construct, (PRDII)(4)-CAT, but can synergize with NF-κB in the activation of both HIV-derived and non-HIV-derived promoters (37). Tat induces matrix metalloproteinase-9 in monocytes through protein tyrosine phosphatase-mediated activation of nuclear transcription factor NF-κB (38). In Jurkat T cells, Tat-mediated activation of NF-κB is dependent upon activation of the T cell-specific tyrosine kinase p56lck (33). Thus, Tat-mediated NF-κB activation may be via multiple signal transduction pathways.

The adhesion molecule E-selectin is induced at inflammatory sites where it exhibits restricted and tightly regulated expression in endothelial cells. E-selectin up-regulation is accomplished via increased cytokines such as TNF, and mediated in part by two closely apposed NF-κB sites (39). Early studies demonstrated that as part of the immune activation seen in AIDS patients, soluble E-selectin levels were elevated (40,41). Furthermore, Tat increases E-selectin expression in human umbilical vein endothelial cells (42).

Because endothelial cell adhesiveness for neutrophils consists of an interplay between adhesion molecules and circulating neutrophils, and since Tat has been demonstrated to increase E-selectin expression in normal endothelial cells and activate NF-κB in some cells, we examined whether the Tat-mediated increases in E-selectin expression require NF-κB. Here we demonstrate for the first time that this Tat-mediated up-regulation of E-selectin requires NF-κB and the synthesis of new macromolecules. Furthermore, consistent with previous reports of Tat-mediated NF-κB activation in HeLa cells, we demonstrate that the basic domain of Tat is necessary for this induction.

**Experimental Procedures**

*Materials and Reagents:* Isopropyl β-D-thiogalactopyranoside (IPTG), antibiotics,
cycloheximide, actinomycin D, cell dissociation buffer, IGEPAL (NP-40), salts, bovine heart cAMP-dependent protein kinase and buffers were purchased from Sigma Chemical Co. (St. Louis, MO). GSH-sepharose and ECL chemiluminescence kit were from Amersham-Pharmacia; tumor necrosis factor-α (TNF) used for all the present studies was obtained from Pepro Tech, Inc. (New Jersey) FITC-conjugated Cd62E (clone 1.2B6) was obtained from Research Diagnostics Inc.; antibodies against p65, p52, RelB, Sp3 and actin were obtained from Santa Cruz Biotechnology; antibodies against cRel were obtained from Rockland, and p50 from Geneka Biotechnology Inc. Endothelial cell culture medium was EGM-2 (EGM-2 Bullet kit, BioWhittaker, San Diego), supplemented with 2% FCS, serum-free OPTI-MEM and neomycin was purchased from Life Technologies (Gaithersburg, MD). Bradford reagent for protein determination was purchased from BioRad (Hercules, CA). The Tat antibody was kindly provided by the AIDS Reference and Research Reagent Program and was originally contributed by Dr. Bryan Cullen (43). Superfect reagent for transfection was purchased from Qiagen and used according to manufacturer’s specifications.

**Plasmids:** Plasmid GST-Tat (GST-Tat 1 86R TK) contains the 2-exon 86-amino acid wild-type tat gene cloned into pGEX2TK (Pharmacia); pGST-Tat 1 48Δ TK is truncated after amino acid 48 and contains a functional activation domain; pGST-Tat 1 48Δ C22G contains a truncation after amino acid 48, and a non-functional activation domain because there is a point mutation at cysteine 22. All of these GST fusion vectors were obtained from the NIH AIDS Research and Reference Reagent Program and contributed by Dr. Andrew P. Rice. Parental vector pGEX-2TK was purchased from Pharmacia Biotechnology. This vector is designed for inducible expression of genes as fusions with the *Schistosoma japonicum* glutathione-S-transferase (GST). Because the fusion constructs are under the control of the tac promoter, induction with IPTG leads to high levels of expression. Affinity chromatography with GSH-Sepharose followed by thrombin cleavage is used to purify the protein of interest. Plasmid pMT/V5-His C (Invitrogen) is a *Drosophila* expression vector that carries the fly metallothionein promoter, which allows for CuSO₄ induction of cloned genes. The HIV-1 86-amino acid Tat was cloned into the multiple
cloning site of this vector to generate the plasmid pMT-tat (manuscript in preparation). Plasmid pELAM-Luc (pE-Luc), kindly provided by Dr. Tom McIntyre, contains sequences 840 base pairs upstream of the transcriptional start site of the human E-selectin gene driving firefly luciferase transcription. Control plasmid pGL3B containing the promoterless luciferase gene was obtained from Promega Biotechnology. Plasmid pCoHygro, obtained from Invitrogen, containing a hygromycin resistance gene under the control of the Drosophila copia promoter, was used for the generation of stable S2-tat cells. Plasmid pHOOK-3 (Invitrogen), an expression vector carrying the CMV promoter, was used to create a pHOOK-tat expression vector for transient transfections of mammalian cells.

**Cell culture and stimulation**: HUVEC were obtained from BioWhittaker and maintained in EGM-2. Cultures were maintained at 37°C in a 6.5% CO₂ humidified atmosphere. Adhesion molecule induction via NF-κB declines as passage number increases and is sensitive to growth state, therefore, expanded cells were used at passages 2-3 and cells at 3 days post-confluence were used for all the experiments. HeLa and HeLa-tat cells were obtained through the NIH AIDS Research and Reference Reagent Program and contributed by Drs. W. Haseltine and E. Terwilliger. These cells were cultured in OPTI-MEM with 3.75% fetal calf serum in the absence of antibiotics. The HeLa-tat cells were grown in the presence of 800 µg/ml neomycin. Schneider 2 (S2) Drosophila cells were purchased from Invitrogen and cultured in complete Drosophila Expression System (DES) medium according to manufacturer’s instructions. For transfection and selection of stable S2-tat cells, S2 cells were grown in 6-well culture dishes to a density of 1x10⁶ cells, collected, washed and co-transfected with pCoHygro/pMT-tat via Ca₃PO₄. Cells were plated and allowed to recover for 24 hours in complete DES medium. Stable clones were selected by growth in 300 µg/ml hygromycin for at least 2 months. Transgene expression was induced by 24 hour incubation in the presence of 500 µM CuSO₄.

**Transient Transfection and luciferase assays**: HeLa or HeLa-tat cells were seeded at a density of 5 x 10⁵ cells/well on 6-well tissue culture dishes and grown until 60% confluence. Cells were washed with sterile PBS and incubated in the presence of 2 µg of pE-Luc or pGL3B in Superfect
reagent for 18 hours. Cells were lysed in passive lysis buffer (Promega luciferase transfection kit) according to the manufacturer, lysate collected and total protein determined. Aliquots of 20 µl were assayed for light emission with a plate-reader luminometer. In a separate series of experiments, HUVEC were seeded in 6-well tissue culture dishes and grown to 60% confluence. Transfection and luciferase assays were performed as described above, except that the cells were co-transfected with equimolar amounts of either pHOOK-3/pE-Luc or pHOOK-tat/pE-Luc.

**Cytoplasmic and nuclear protein extractions:** For nuclear and cytoplasmic protein extraction, confluent HUVEC cultures grown on 10 cm tissue culture dishes were washed with sterile PBS and fed 5 ml of serum-free OPTI-MEM containing 20 ng/ml recombinant human TNF, 500 ng/ml recombinant Tat, or the indicated control medium for 1 hour. After addition of 5 ml of EGM-2 containing 2% FCS, cells were incubated for the indicated times. Therefore, no serum was present during the first hour of incubation, and only 1% during the remainder. Cells were harvested by rinsing twice with PBS (calcium and magnesium-free), followed by incubation in a non-enzymatic cell dissociation buffer for 5 min at 37°C. Cells were detached by scraping, transferred to microcentrifuge tubes and pelleted at 5,000 x g for 1 min. The supernatant was removed, cells resuspended in 1 ml of cold hypotonic buffer (10 mM HEPES, 1.5 mM MgCl₂ and 10 mM KCl adjusted to pH 7.9 with KOH) containing 0.2 mM PMSF, 1 µg/ml aprotinin, 0.3 µg/ml leupeptin and 0.5 mM DTT and washed by centrifugation at 2,000 x g for 5 minutes at 4°C. The pellet was resuspended in 5 times the packed cell volume of cold hypotonic buffer with protease inhibitors as described above and allowed to swell on ice for 20 minutes. IGEPAL was then added to 0.1% and the swollen cells incubated an additional 5 minutes on ice followed by homogenization with 20 strokes of a microtube pestle homogenizer (Fisher Scientific). Cell lysis and nuclear integrity were verified by trypan blue exclusion analysis. Nuclei were pelleted at 13,000 x g for 5 minutes at 4°C. The supernatant containing the cytoplasmic fraction was transferred to another tube and stored at -80°C until further use. The nuclei were resuspended in 3-4 times the packed nuclear volumes of cold hypotonic buffer with protease inhibitors as above, washed once by centrifugation at 13,000 x g at 4°C, and resuspended gently in one-half packed
nuclear volume of cold low salt buffer (20 mM HEPES, 0.2 mM EDTA, 25% glycerol, 1.5 mM MgCl₂, 20 mM KCl pH 7.9). One-quarter packed nuclear volume of cold hypertonic buffer (10 mM HEPES, 0.1 mM EDTA, 50 mM KCl, 300 mM NaCl, 10% glycerol, 1.5 mM MgCl₂ at pH 7.9) was added in a dropwise manner to prevent lysis of the nuclei. Nuclear proteins were extracted at 4°C for 30 min, with a gently rotating motion. The nuclear protein extract was clarified by centrifugation at 13,000 x g for 20 min at 4°C. Nuclear and cytoplasmic protein concentrations were determined by the Bradford reagent microassay protocol using bovine serum albumin as a standard. To assess the purity of the fractions and to test our cell fractionation technique, 15 µg of nuclear or cytoplasmic proteins extracted from HUVEC, HeLa or HeLa-tat cells were immunoblotted with actin or Sp3 antibodies.

Electrophoretic mobility shift assays (EMSA): Nuclear proteins were diluted in a 2:1 mix of low salt:hypertonic buffer to a concentration of 0.75 µg/µl and 2.5 µg of this protein were used in the binding reaction. This procedure ensured that the salt as well as the protein concentration was the same in all the reactions. The oligonucleotide used contained the human E-selectin κB site 3 (39) flanked by additional E-selectin-specific sequences: 5'- GCCATTGGGGATTTCCTTTTT-3' (κB site underlined). Complementary oligos (GIBCO-BRL) were annealed and end-labeled with [γ-³²P]ATP and T4 polynucleotide kinase (Promega). Labeled oligos were separated from unincorporated nucleotides using a Sephadex G-25 spin column (5'→3 prime). The labeled probe was diluted to 8-12 fmol/µl (approximately 10,000 cpm) and 1 µl per binding reaction was used. The binding reaction also contained 4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM, Tris-HCl pH 7.5, 0.05 mg/ml poly (dI-dC)-poly (dI-dC) and 2.5 µg of nuclear protein (3.3 µl of 0.75 µg/ml dilution) in a final volume of 9 µl. For competition studies, 50-fold molar excess of cold competitor κB or an irrelevant oligonucleotide was included. The reaction was mixed by gentle rocking and incubated at room temperature for 20 min. After binding, 1 µl of 10X EMSA buffer (250 mM Tris-HCl pH 7.5, 0.2% bromphenol blue, and 40% glycerol) was added to each reaction and loaded onto a 4% Polyacrylamide gel (40:1 acrylamide:bis-acrylamide) containing 1 X TGE buffer (25 mM Tris-HCl pH 7.5, 190 mM
glycine, 1 mM EDTA, pH 8.3) and 10% glycerol. The protein complexes were separated at 12 mA/gel for approximately 2 hours. Gels were dried in vacuo and exposed to Bio-Max X-Ray (Kodak) film. Multiple exposures of the film were obtained to ensure that the signal was within the linear range of the film.

**Antibody supershift assays:** After 20 min incubation of the oligonucleotide and the nuclear protein extract, 1 µg of antibodies against p50, p65, RelB, cRel or p52 was added and the mixture incubated for an additional 15 minutes before electrophoresis.

**Purification of wild type and truncated Tat proteins:** The HIV-1 Tat protein was purified in our laboratory as described (44), using affinity chromatography. The truncated Tat mutants and the parental GST were purified using the same method. Expression from these vectors result in GST fusion proteins, linked by a thrombin-sensitive peptide. Host *E. coli* cells were grown to OD<sub>590</sub> = 0.5-0.7 in 500 ml culture volume. Induction of fusion proteins was achieved by growth in the presence of 0.1 mM IPTG for 2.5 hr. Cells were collected by centrifugation at 14,000 x g, the pellet was resuspended in 4 ml of EBC-DTT buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.1% IGEPAL and 5 mM DTT) and sonicated for 1 minute twice, with a 1-minute incubation on ice in-between sonications. The homogenate was clarified by centrifugation in microtubes at 13,000 x g for 15 minutes at 4°C. The supernatant was collected and frozen at -80°C. For GST-Tat purification, all of the buffers were degassed to minimize the exposure of Tat to oxygen. This treatment consistently gave biologically active Tat preparations. Glutathione-Sepharose beads (GSH-Sepharose, Pharmacia Biotechnology) were equilibrated in EBC-DTT buffer by washing twice with 1 ml of EBC-DTT buffer followed by resuspension in the same buffer to create 50% slurry. Two hundred fifty microliters of the 50% slurry were added to the bacterial supernatant containing the induced GST-fusion protein and the mixture incubated for 30 minutes at 4°C with constant rocking. After washing the beads extensively with 12 volumes of EBC-DTT twice and 5 volumes of thrombin cleavage buffer (50 mM Tris pH 7.6, 20 mM KCl and 1 mM DTT) once, the Tat peptide was cleaved by incubation with 12 U of thrombin A in 200 µl of thrombin cleavage buffer for 1.5 to 3 hours at room temperature with constant rocking. To
collect the cleaved Tat, the sample was incubated at 37°C for 3 minutes and then centrifuged at 13,000 x g for 30 seconds at room temperature and the supernatant containing the pure Tat transferred to a clean vial. To maximize the yield, the Sepharose beads were resuspended in 50 µl of thrombin cleavage buffer, heated to 37°C for 3 minutes and centrifuged at 13,000 x g again. This elution was performed a total of three times and the final supernatants pooled. The pure Tat preparation was divided into 100 µl aliquots, flash frozen and stored in liquid nitrogen. To determine Tat biological activity, the pure protein was electroporated into HeLa cells in the presence of the indicator plasmid pLUCA41, kindly provided by Dr. Gail Harrison, which contained the HIV Long Terminal Repeat (LTR) controlling firefly luciferase expression. Active Tat preparations consistently activated LTR-driven luciferase expression at least 5,000-fold. Bacterially expressed GST extracts purified as described for the GST-Tat fusion proteins were used as control. Presence of endotoxin was assessed by an E-TOXATE assay from Sigma.

**Adenovirus transduction:** An adenovirus vector containing an IκB mutant (S32A/S36A) super-repressor insert (AdIκBαSR) or a Green Fluorescent Protein (AdGFP) insert was used. The recombinant protein contained an HA epitope tag to facilitate detection of the transduced construct. Cells were grown to 100% confluence, medium removed and replaced with serum-free medium containing the virus at a multiplicity of transduction of 30 plaque forming units/cell. After an overnight incubation, monolayers were washed and exposed to Tat or TNF for 6 hours. The cells were collected and processed for nuclear protein extraction or for E-selectin cell surface expression.

**Western blot analysis:** Cytoplasmic or nuclear proteins were resolved by SDS polyacrylamide gel electrophoresis in 5-15% gradient pre-cast gels, electroblotted in a buffer containing 25mM Tris, 192mM glycine and 10% methanol onto a polyvinyl difluoride membrane (PVDF, BioRad), stained with antibodies against, p65, actin or Sp3 and a horseradish peroxidase-coupled secondary antibody (ECL, Amersham) and detected on film. Molecular weight standards on an adjacent lane were the Benchmark standards from Bio Rad. Autoradiographs were quantitated by densitometry. Membranes were stripped and equal loading of proteins assessed by colloidal
gold staining (BioRad). For all p65 immunoblots, 0.5 µg of protein (nuclear, cytoplasmic or whole cell) was loaded per well; for actin and Sp3 immunoblots, 15 µg of protein (nuclear or cytoplasmic) were loaded per well.

**Cell surface E-selectin expression:** Cells were grown on 6-well tissue culture dishes until confluent, and incubated for 3 additional days. Then, cells were incubated with serum-free OPTI-MEM supplemented with 500 ng/ml Tat +/- Tat antibody, 500 ng/ml of truncated Tat mutants, 20 ng/ml TNF, 500 ng/ml GST control extracts, 500 ng/ml of boiled Tat or with serum-free media alone. After 1 hour, 1 ml of EGM-2 with 2% serum was added and the cells incubated for an additional 5 hours. Cells were incubated for 30 min with 5 µg/ml cycloheximide or 2 µg/ml actinomycin D, prior to addition of Tat or TNF for total 6 h incubation. For endotoxin exposure, cells were exposed to 500 ng/ml *E. coli* LPS (055:B5, Sigma) for 6 hours in the presence or absence of serum. Cells were exposed for 5 min to cell dissociation buffer (Sigma) at 37°C after washing with calcium and magnesium-free PBS. Cells were harvested by scraping followed by centrifugation at 600 x g for 5 min. The pellet was resuspended in 400 µl of FACS binding buffer (1% FCS, 0.2% NaN₃ in PBS), and 200 µl aliquots transferred to round-bottom 96-well plates. Cells were centrifuged at 200 x g for 5 min at 4°C, supernatant removed by gentle tapping against the sink, and pellets resuspended in 100 µl of binding buffer containing 5 ng/µl of FITC-conjugated anti human E-selectin (mouse monoclonal, clone 1.2B6, IgG1). In control experiments, an IgG isotype antibody was used. After incubation on ice for 15 min, cells were washed twice by centrifugation with binding buffer. After the last wash, the cells were resuspended in 700 µl of binding buffer and analyzed on a FACSscalibur fluorescence cytometer (Becton Dickinson). In experiments with the AdGFP, secondary antibody used was conjugated to biotin, and detection was via cychrome-streptavidin-mediated fluorescence. For quantitation of E-selectin expression, 10,000 cells were acquired per experimental set. Voltage and gain parameters were established and gates assigned during the acquisition of unstained cells. In the analysis, regions M1 and M2 were gated and set according to the fluorescence of cells exposed to medium alone. Region M1 was determined to
be of low fluorescence intensity and consistently contained cells with mean fluorescence intensities (MFI) less than 10. Region M2 was determined to be of high fluorescence intensity and consistently contained cells with mean fluorescence intensities higher than 50. Region M1 was therefore set between 0 and 10 and region M2 between 10 and $10^4$. Cells having an MFI less than 10 were considered negative for E-selectin. Cells with an MFI higher than 10 were considered positive for E-selectin. The results are therefore expressed as the percentage of cells in region M2. For all experiments, TNF treatment of HUVEC was used as the positive control. This exposure allowed us to control for differences in source, passage number and growth state of the HUVEC used in each particular experiment.

**Statistical analyses:** With the exception of autoradiographs and Western blot analyses, all results shown represent means +/- SEM. Results were analyzed by one-way ANOVA.

**RESULTS:**

**Purity of the Tat preparation:**

Recombinant Tat protein was purified from bacteria (*E. coli* SURE cells) harboring a pGST-tat fusion construct under the control of the *tac* promoter. Induction with IPTG increased expression of the fusion protein (Fig. 1A, lane 2). After binding to GSH-Sepharose beads, the fused Tat was cleaved with thrombin and subsequently isolated. Western blot analyses with a Tat polyclonal antibody confirmed the presence of Tat in the final preparation (Fig. 1A, lane 4). There was some GST-tat in the uninduced bacterial extracts (Fig. 1A, lane 1) possibly due to leakiness of the *tac* promoter, even in the absence of IPTG. A significant amount of GST-tat was detected in the flow-through (Fig. 1A, lane 3); this effect is more evident when the GSH-Sepharose beads have been in storage for prolonged periods of time (not shown). When newly purchased beads or beads whose reduced glutathione is regenerated in the presence of DTT are used, the amount of GST-tat in the flow-through is considerably reduced (data not shown). A representative colloidal gold-stained membrane of a Tat preparation is shown in Fig. 1B, left panel. The right panel shows a different Tat preparation. The band denoted by the open arrow
represents a 70 kDa bacterial chaperonin, the product of the *E. coli* gene dnaK, which co-purifies with recombinant proteins (45). This association can be disrupted by incubation of the lysate in the presence of ATP and MgSO₄, prior to affinity purification (45). Presence of this protein is not responsible for the Tat effects observed since control GST extracts do not activate NF-κB translocation or E-selectin expression (discussed later). Furthermore, extracts where this protein was absent retained Tat activity (Fig. 1B, Tat 2).

The pGST-Tat fusion construct includes a cardiac muscle cAMP-dependent protein kinase recognition sequence downstream of the thrombin cleavage site, which allows the detection of cleaved fusion products by phosphorylation *in vitro*. To further establish the purity of the Tat preparation, the purified product as well as extracts from cells harboring the control parental plasmid (pGST) were phosphorylated *in vitro* with bovine heart cAMP-dependent protein kinase and [γ-³²P]ATP for 30 min on ice. The results shown in Fig. 1C demonstrate that only the extract purified from the GST-Tat-harboring bacteria contains a phosphorylated protein of the correct molecular weight.

Finally, we compared bacterially purified Tat (rTat, Fig. 1D) to Tat expressed in a *Drosophila* cell line engineered to stably produce Tat (S2-tat) upon CuSO₄ induction. The Tat-specific band was only detected in the induced S2 cell cultures and not in uninduced cells or in cells transfected with the parental pMT vector. The upper band seen in all the S2 extracts is a non-specific product. In our hands, bacterially expressed Tat always migrates as 3 separate bands on denaturing gels. Presumably, the reducing environment of the bacterial host (SURE cells, Stratagene) is responsible for this observed behavior. These cells have been engineered with mutations in several recombination and DNA repair genes. Nevertheless, the Tat purified from bacteria co-migrated with the Tat present in the induced S2 cells. Taken *in toto*, these results demonstrate that the bacterially purified Tat is approximately 95% pure and comparable to Tat expressed in eukaryotic expression systems.

**Tat up-regulates E-selectin expression in human endothelial cells:**

Tat is secreted into the extracellular compartment and once taken up by target cells can
affect the expression of cellular genes. To establish whether Tat may affect adhesion molecule expression in endothelial cells, we exposed HUVEC to recombinant Tat purified from bacteria. Confluent monolayers were exposed to culture medium alone, Tat, TNF or GST extracts for 6 hours and E-selectin expression measured by FACS. Histograms of cell counts versus fluorescence intensity are shown in Fig. 2A. Neither culture medium alone nor control extracts from bacteria harboring the parental GST vector induced E-selectin expression. Cells grown in the presence of 500 ng/ml Tat show an increase in the percentage of cells in region M2, (E-selectin positive) from 4 to 93% when compared to cells grown in medium alone. This concentration of Tat was deemed optimal because higher concentrations (between 500 ng/ml to 1 µg/ml) did not induce further, and lower concentrations (between 50–400 ng/ml) were not as efficient. A similar fluorescence shift was observed in TNF-treated cells. The kinetics of E-selectin up-regulation showed a similar pattern for both Tat and TNF, with TNF inducing an increase in expression in 20% of the population by 2 hours, while Tat reached a level of 4% at this time point. Both agents stimulated expression maximally by 5 hours (Fig. 2B). Treatments for a total of 6 hours were therefore chosen for all of the subsequent experiments.

To address which functional domains of Tat may be responsible for the E-selectin up-regulation, truncation mutants with or without a functional activation domain were tested. Figure 2C shows that neither the truncated Tat product with a functional activation domain (48∆TK), nor the truncated product with a non-functional domain (48∆C22G) activated E-selectin expression. Tat boiled for 10 minutes, purified GST extracts or culture medium alone also failed to significantly induce E-selectin expression. These treatments increased E-selectin expression to less than 20%, compared to an average of 87% +/- 1.5 for active Tat. Thus, the difference between the Tat-mediated changes and the control treatments is due to Tat activity. Tat that had been incubated in the presence of a Tat antibody (anti-Tat) for 30 minutes prior to treatment resulted in only 23% E-selectin expression, demonstrating the specificity of the effect. Endotoxin treatment for 6 hours did not increase E-selectin, regardless of whether serum was present or not (data not shown). TNF, used as a positive control, induced E-selectin to levels
comparable to Tat.

To test whether the E-selectin promoter is responsive to Tat, HeLa or HeLa-tat cells were transfected with a plasmid containing upstream regulatory sequences of the human E-selectin promoter driving expression of luciferase (pE-Luc, Fig. 3A). The promoterless luciferase vector pGL3B was used as a control. Luciferase expression was increased approximately 6-fold compared to the pGL3B alone in the HeLa-tat, but not in the HeLa cells. When endothelial cells were co-transfected with a Tat-expressing plasmid and pE-Luc, there was a 15-fold increase in luciferase expression when compared to co-transfection with empty vector (pHOOK) and the E-Luc. These results suggest that the presence of Tat is necessary for the induction, regardless of whether the cells are stably expressing the Tat protein or whether the \textit{tat} gene is introduced with the reporter construct.

\textbf{Tat stimulates NF-κB translocation in endothelial cells:}

We compared the ability of Tat or TNF to up-regulate κB family members by using an oligonucleotide containing the \textit{k}b site 3 of the human E-selectin promoter in gel shift assays with nuclear proteins extracted from HUVEC. After 20 min incubation with Tat there was very little NF-κB activation, while incubation with TNF strongly resulted in considerable translocation at this time point (Fig. 4A). Neither medium alone nor boiled Tat had any effect on κB translocation. The composition of the nucleoprotein complexes was investigated by antibody supershift analyses. The slower migrating complexes contained p65/p50 heterodimers (top arrow), while the faster migrating ones contained p50 homodimers (bottom arrow). When the extracts were incubated with the RelB antibody, nucleoprotein complexes disappeared in the TNF-treated, but not in the Tat-treated cells, suggesting the presence of RelB in some of the complexes. At 6 hours, Tat or TNF strongly induced κB binding to the oligonucleotide (Fig. 4B). Furthermore, cells incubated with medium alone showed an increased binding, suggesting that the addition of serum after the first hour of incubation may be stimulatory to NF-κB. The c-Rel antibody resulted in a weakly supershifted product in the TNF, but not in the Tat-treated samples, suggesting that there are qualitative differences between Tat and TNF-stimulated
complexes. In a separate experiment, gel shift assays were performed with nuclear proteins from cells exposed to control GST extracts for 6 hours (Fig. 4B). NF-κB translocation was not detected in these extracts.

Western blot analyses with 0.5 µg of either cytoplasmic or nuclear proteins corroborated that TNF-mediated translocation of p65 is observed by 20 minutes, while Tat resulted in a delayed response that was first detected at 2 hours (Fig. 5A). Again, increased p65 expression in the cytoplasm of cells exposed to medium after 2 hours probably is a reflection of the re-introduction of serum into the system at 1 hr. Interestingly, at later time points, steady-state levels of nuclear p65 in TNF-treated extracts were higher than in Tat-treated extracts. Boiled Tat had no effect at any of the time points examined. To exclude the possibility that the nuclear proteins may have been contaminated with cytoplasmic proteins during the extraction, 15 µg of nuclear or cytoplasmic proteins from cells exposed to the indicated treatments were immunoblotted with an actin antibody (Fig. 5B). Levels of immunoreactive actin were readily evident in the cytoplasm, but very little in the nucleus, regardless of treatment. As an additional control for cross-contamination, HeLa or HeLa-tat cells were fractionated using the same procedure as for the endothelial cells, and 15 µg of nuclear or cytoplasmic proteins subjected to Sp3 immunoblotting. Fig. 5C shows that Sp3 is only found in the nucleus, but not the cytoplasm of the cells. Furthermore, immunoblots for p65 were performed with only 0.5 µg of protein per well, while in order to detect Sp3 or actin, 15 µg of proteins were required. Thus, it was not possible to detect both p65 and actin on the same membrane. The fact that there is very little detectable actin in the nucleus, or Sp3 in the cytoplasm at these protein amounts makes the possibility of cross-contamination unlikely. Independently prepared extracts have yielded exactly the same results, validating our laboratory’s cell fractionation technique (data not shown). These results indicate that the presence of p65 in the nucleus of Tat or TNF-treated cells, but not cells exposed to media alone, is due to translocation and not contamination. In contrast, when whole cell extracts were subjected to p65 immunoblot analyses, the intensity of all the p65 bands was the same for Tat or TNF at all time points tested (Fig. 6). These results
demonstrate that neither Tat nor TNF resulted in increased expression of p65 during the exposure suggesting that the NF-κB-mediated effects are due to its translocation and not to increased expression.

**Tat-mediated E-selectin up-regulation requires new macromolecule synthesis.**

To determine if Tat-mediated E-selectin up-regulation requires transcription or translation, cells were pre-treated with either actinomycin D (ActD) or cycloheximide (CHX) for 30 minutes prior to exposure to Tat followed by measurements of E-selectin expression. 60% of cells were positive for E-selectin after exposure to Tat (Fig. 7). ActD or CHX treatment inhibited this up-regulation almost completely, demonstrating that the Tat-mediated effects require the synthesis of new macromolecules. Neither ActD nor CHX resulted in endothelial cell apoptosis, as measured by Annexin V staining (data not shown).

**Tat-mediated E-selectin up-regulation requires NF-κB activation:**

Translocation of NF-κB requires phosphorylation and subsequent degradation of the inhibitory cytoplasmic IκB. Phosphorylation is therefore an essential component of this activation. HUVEC were transduced with an adenovirus vector coding for a recombinant IκB super-repressor protein. This construct contains two site-directed mutations at serines 32 and 36 preventing their phosphorylation. The result is a constitutively inactive NF-κB. To control for any effects due to adenovirus transduction itself, an adenovirus containing a GFP insert was used. EMSA analyses demonstrated that the expression of this super-repressor completely abolished Tat or TNF-mediated NF-κB binding to the E-selectin κB oligo (Fig. 8). The IκB super repressor adenovirus completely inhibited p65 translocation to the nucleus in response to either Tat or TNF (Fig. 9), and abolished Tat-mediated E-selectin up-regulation (Fig. 10 A & B). A recombinant adenovirus-GFP control had no effect on E-selectin expression. Examination of GFP fluorescence indicated that close to 100% of the cells had been transduced with the virus (not shown).

**DISCUSSION**
Abnormalities in leukocyte-endothelial cell interactions have been reported in HIV infection. For example, infection of monocytes results in elevation of adhesion molecule LFA-1 (46) and a marked increase in adherence to human capillary endothelial cell monolayers derived from brain, lung and skin.

E-selectin, one of the adhesion molecules activated in HIV infection, is tightly regulated by multiple NF-κB sites in its promoter (47), and is transcriptionally silent until its rapid induction by inflammatory cytokines or reactive oxygen species (48). Previous reports have demonstrated that Tat increases E-selectin expression, a phenomenon that is augmented in the presence of TNF (49). Furthermore, studies have demonstrated increased monocyte adhesion to endothelium exposed to Tat (50). Because E-selectin up-regulation in response to cytokines requires an intact κB site in the promoter (48,51), the possibility that Tat-mediated E-selectin up-regulation also requires NF-κB activation was investigated in these studies.

The closely apposed κB sites in the promoter of the human E-selectin gene are essential for maximal expression in response to TNF (39). Instead of using a κB consensus oligonucleotide in the gel-shift studies described here, we chose to test an oligonucleotide containing the E-selectin κB site 3, located within -99 to -80 bases from the transcriptional start site. NF-κB binds preferentially to sites 2 and 3 and mutations within these sites are more specific at inhibiting cytokine-mediated E-selectin expression (39). TNF activates translocation and binding of p65/p50 heterodimers to this site 3 (51). Likewise, Tat activated this heterodimer in endothelial cells, albeit with delayed kinetics. Translocation of p65 and binding to the E-selectin oligonucleotide were observed as early as 20 minutes after initiation of TNF exposure, when only 20% of the cells were positive for E-selectin after 2 hours. In contrast, Tat-dependent translocation and binding of p65 after 20 minutes and E-selectin expression after 2 hours were undetectable, suggesting that these agents activate NF-κB via different mechanisms. Indeed, Tat-mediated activation of NF-κB in lymphocytes is dependent upon a functional Lck57 tyrosine kinase, while TNF-mediated activation is not (33). However, E-selectin levels were comparable after treatment with Tat or TNF for 5 hours. The delayed response after Tat may be a reflection
of the fact that TNF recruits other factors to the E-selectin promoter, forming higher order structures that include the high mobility group protein Y(I), and ATF2/c-Jun heterodimers and CRE binding proteins (52,53). Even though Tat activates NF-κB translocation and binding to the site 3 of the E-selectin promoter, there may be a delay in the recruitment of other unknown accessory proteins to the E-selectin promoter, also delaying transcription of the E-selectin gene. In fact, other investigators have demonstrated that the rate-limiting step in E-selectin expression is transcription initiation (54). Therefore, anything that delays recruitment of factors to the transcriptional initiation complex will delay expression of the gene. Tat induces the expression of the adhesion molecules vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) and the chemokine monocyte chemoattractant protein 1 (MCP-1) and the cytokine interleukin 6 (IL-6). The expression of these genes is partly regulated by NF-κB. The pattern of induction in response to Tat differed from that to TNF, with higher peak levels that occurred earlier in response to Tat (55). In these studies, Tat and TNF activated comparable levels of NF-κB, consistent with our present findings.

In order to map the region of Tat responsible for the E-selectin up-regulation, truncated mutants with/without a functional activation domain were tested along with the wild type 86 amino acid Tat. All mutants were tested as recombinant proteins. Even though the activation domain was present in the mutants, amino acids beyond residue 48 have been deleted and therefore the basic domain (amino acids 49 to 58) as well as the region encoded by the second exon are absent. None of the mutants retained the ability to induce E-selectin, suggesting that carboxyl-terminal amino acids are important. Interestingly, Demarchi et al. (14) have demonstrated that Tat-mediated NF-κB activation in HL3T1 cells requires the integrity of the basic domain of Tat. Thus, deleting this basic domain would abolish Tat-mediated NF-κB activation and E-selectin up-regulation.

The family of NF-κB transcription factors includes c-Rel, RelB, p52, p50 and p65. NF-κB is constitutively bound to the inhibitory IκB in the cytoplasm of non-B cells. Upon stimulation, IκB is phosphorylated, ubiquitinated, and degraded by the proteasome (56). This
releases NF-κB, which translocates to the nucleus and activates transcription of specific genes. The diverse stimuli that result in IκB phosphorylation are still being investigated, but the downstream effects are fairly well known. In the present study, either TNF or Tat resulted in NF-κB activation by IκB phosphorylation because the κB super-repressing adenovirus resulted in complete inhibition of NF-κB translocation, particularly p65. The inhibition was also reflected in decreased binding of p65 to the E-selectin oligonucleotide and in decreased E-selectin expression. This effect was specific to NF-κB and not an artifact of adenovirus transduction because the control adenovirus-GFP had no effect. This control adenovirus also had no effect on p65 translocation as measured via Western blot or gel shift analyses (not shown).

Our experiments have corroborated the ability of extracellular Tat to affect cellular physiology. Tat in the culture medium could be binding to a receptor whose activation and subsequent signal transduction may result in NF-κB translocation. In fact, the transfection studies with E-selectin luciferase reporter constructs (Fig. 3) suggest that the presence of a tat gene in the nucleus also activates the E-selectin promoter. The reporter construct used for these studies contains 840 base pairs upstream of the transcriptional start site of the human E-selectin gene, which includes all 3 κB sites. At this time, we cannot exclude the contribution of κB sites other than site 3 to the Tat-responsiveness of this promoter. Interestingly, E-selectin promoter-driven luciferase expression was much higher in the endothelial cells, maybe reflecting the restricted expression of the E-selectin gene to these specific cells. When Tat is introduced via liposomes into HL3T1 cells, the interferon-inducible protein kinase is activated, which in turn phosphorylates Iκb and results in NF-κB translocation (14). This activation is independent of CDC42 and RAC, two pathways shown to be indispensable for TNF-mediated E-selectin transcription (51). Thus, different pathways are activated in response to Tat or TNF. Nevertheless, kinetics of E-selectin expression are consistent with previous reports in HUVEC, where peak levels are detected by 4 hours, returning to baseline by 24 hours after TNF induction (57,58). On the other hand, even without transfection, Tat is able to enter cells, since its positively charged protein transduction domain stimulates cellular uptake (59,60) and its RGD
domain is responsible for binding to cell surface integrins (10). The RGD and basic domains of Tat stimulate and modulate the VEGF receptor Flk-1/KDR and components of the focal adhesion kinase in Kaposi’s sarcoma cells (61,62).

The Tat concentration required to up-regulate E-selectin was optimal at 500 ng/ml. A small percentage of HIV-infected patients have detectable serum levels of Tat that are in the ng/ml range (63,64). However, this may be an underestimation of the actual levels because Tat may be sequestered in lymphoid tissues for example (64). When pure Tat preparations were analyzed via Western blots, additional Tat-specific bands were detected. Because Tat has 7 cysteine residues in tandem (65,66), these bands probably represent complexes formed by intra and intermolecular disulfide bridges, which are difficult to dissociate, even in the presence of the denaturing and reducing conditions of the gel. These Tat complexes may be inactive and therefore, the actual concentration of active Tat may be considerably lower than the protein concentration would suggest. These additional Tat bands were not present in the S2-tat cell extracts possibly because the reducing environments in the S2 and the E. coli cells are different. Nevertheless, the Tat expressed in the S2 cells co-migrated with one of the Tat-specific bands seen in bacteria.

The up-regulation of E-selectin also requires new macromolecular synthesis, as demonstrated by the inhibitory effect of cycloheximide or actinomycin D. Several reports have suggested that cycloheximide in combination with TNF treatment induces apoptosis in endothelial cells (67). However, we did not detect any evidence of apoptosis in the time frame of exposure to TNF (not shown). Because growth state and passage number markedly affect cytokine-mediated adhesion molecule expression in endothelial cells, the experiments were performed in cells 3 days post-confluence. Studies that report increased apoptosis in response to protein synthesis inhibitors and TNF have been performed on exponentially growing endothelial cells. Interestingly, agents such as endostatin have a pro-apoptotic effect selective for proliferating endothelial cells (68,69). Furthermore, in the present studies, the inhibitors were added prior to the exposure to Tat or TNF, and therefore, it is unlikely that increased apoptosis
would have been detected (67). Whether Tat is having a growth-inducing effect in these cells is unlikely, since cells were quiescent and non-proliferating. In other systems, and depending on the cell type, Tat may induce apoptosis (63,70) or act as a growth factor (71).

Recombinant Tat purified from a bacterial expression system may have some level of LPS contamination, potentially stimulating endothelial cells to produce TNF, and in an autocrine fashion, induce E-selectin expression. However, IL-1β production in monocytic cells, and IL-6 protein and mRNA in astrocytes, are Tat-mediated NF-κB-mediated effects that are independent of TNF-α production (23). Furthermore, endotoxin contamination is unlikely because boiled Tat or GST control extracts had only a minor effect (<20%) on activation of these pathways and a Tat-specific antibody successfully inhibited Tat-mediated E-selectin up-regulation (Fig. 2C). GST extracts alone had no effect when NF-κB translocation was assessed in endothelial cells (Fig 4B). Therefore, any co-purified bacterial proteins or endotoxin responsible for the observed effects would have been present in these extracts. Endothelial cells are able to secrete TNF in response to LPS, but these effects are observed after prolonged incubation. In addition, endotoxin-mediated effects on HUVEC require the presence of LPS-binding protein and soluble CD14, both present in serum (72,73). In the present studies, there was no serum during the first hour of incubation with Tat, and the incubations were for no longer than 6 hours, again arguing against endotoxin contamination.

Endothelial cells play a central role in the inflammatory process by secreting chemoattractants that recruit circulating leukocytes to the site of injury. Thus, mechanisms that recruit neutrophils or mononuclear cells to the interstitium, will contribute to endothelial cell injury and failure. A major route of HIV entry into the brain is proposed to be through a virus-induced up regulation of E-selectin and V-CAM-1 expression. The infected monocytes bring HIV and Tat in contact with the endothelium, activate surrounding microglia, and prime the surrounding endothelial cells (74). Because selectins mediate rolling, which is the initial step in leukocyte adhesion to endothelial cells (75), increased expression of this molecule would result in enhanced interactions between the endothelium and the circulating leukocytes. By taking
advantage of the host inflammatory response, Tat allows the virus to escape the vascular space and invade interstitial spaces. Macrophages and monocytes, as viral reservoirs, secrete Tat, in conjunction with cytokines and oxidants, which brings these molecules near the by-stander endothelial cells. Given the long list of immune system mediators whose expression is increased by Tat-mediated NF-κB activation, it is not surprising that HIV-infected patients exhibit exuberant inflammatory responses. In conclusion, these studies have linked the ability of Tat to activate NF-κB and E-selectin with the potential to modulate the interactions between leukocytes and the endothelium and reset the balance between pro- and anti-inflammatory forces.

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**Footnotes:**
PMSF = phenyl methyl sulfonyl fluoride  
LTR = long terminal repeat  
FCS = fetal calf serum  
FITC = fluoresceine isothiocyanate  
PBS = phosphate buffered saline  
DTT = dithiothreitol  
SDS = sodium dodecyl sulfate  
FACS = fluorescence activated cell sorter  
VEGF = vascular endothelial growth factor  
LPS = lipopolysaccharide  
ARM = arginine rich motif  
TAR = *trans*-activation response element

**Acknowledgements:**
The NIH/NHLBI grant numbers HL59785 (SCF and ACG) and HL58344 (JS) supported this work. The authors are grateful to Dr. Tom McIntyre for kindly providing the plasmid pELAM-Luc and to John Marecki for critical review of the manuscript. The NIH AIDS Reference and Reagent Program generously provided some of the materials used in the present studies.
Figure Legends

Figure 1. **Purity of the Tat preparation.** Recombinant Tat protein was purified from *E. coli* SURE cells harboring a pGST-tat fusion construct under the control of the tac promoter. **A.** Western blot analyses of 50 ng of the total bacterial extract before (uninduced) and after induction (induced) with IPTG, the flow through (FT) after binding the fusion protein to GSH-Sepharose and the final Tat eluate. The proteins were resolved by SDS-PAGE, transferred to PVDF and pure Tat detected using a polyclonal antibody. The arrows point to Tat, the arrowhead points to GST-Tat. **B.** Colloidal gold stained membrane with 650 ng of the total bacterial extract (lysate), FT and two different Tat preparations. The arrows point to Tat, the arrowhead points to GST and the open arrow points to a 70 kDa bacterial chaperonin that frequently co-purifies with recombinant proteins. **C.** *In vitro* phosphorylation of the purified bacterial products from the pGST or pGST-tat plasmids with protein kinase from bovine heart. **D.** Western blot analyses of purified recombinant Tat (rTat) compared to Tat expressed in S2-tat cells after CuSO4 induction. pMT refers to S2 cells stably transfected with the empty expression vector.

Figure 2. **Effects of Tat on HUVEC E-selectin expression.** Confluent, HUVEC monolayers were exposed to Tat, TNF, GST or cell culture medium alone. After exposure, cells were collected and processed for detection of surface E-selectin by FACS as described in Experimental Procedures. 10,000 cells per experimental set were acquired. Regions M1 and M2 were set according to the fluorescence of the cells exposed to medium alone. Region M1 is of low fluorescence intensity (background fluorescence) while region M2 is of high fluorescence intensity and considered positive for E-selectin induction. **A.** Cells were incubated in the presence of the indicated agents for a total of 6 hours. Percentage of cells in region M2 with medium alone was 4%, with GST, 11%, with Tat, 93%, with TNF, 95%. **B.** Kinetics of E-selectin surface expression, ▲ medium alone, ■ 500 ng/ml Tat, ○ 20 ng/ml TNF. At 2 hr, 20% of the cells with TNF, while 4% of the cells with Tat or media alone were positive for E-selectin. Results expressed as means +/- SEM, n=6. **C.** Summary of E-selectin expression in response to the indicated agents for 6 hours. 48ΔTK and 48ΔC22G are truncated Tat products with or without a functional activation domain, respectively while anti-Tat refers to polyclonal Tat antiserum.

Figure 3. **Effects of Tat on an E-selectin promoter-reporter construct.** A. HeLa or HeLa-tat cells were transfected with plasmid pE-Luc, containing upstream regulatory sequences of the human E-selectin promoter driving expression of luciferase or with the promoterless luciferase vector, pGL3B. Light emission was detected with a luminometer and expressed as luciferase light units. **B.** HUVEC were co-transfected with a mammalian Tat-expression vector (pHOOK-tat) and pE-Luc and luciferase activity was measured. pHOOK is the empty vector.

Figure 4. **Tat-dependent activation of NF-κB.** Confluent HUVEC monolayers were incubated with Tat, TNF, cell culture medium alone or boiled Tat (bTat). Nuclear extracts of cells exposed to the control GST preparation for 6 hours are shown in panel B. After exposure to these agents for 20 minutes (Panel A) or 6 hours (Panel B), the cells were collected and nuclear proteins extracted as described in Experimental Procedures. The nuclear extracts were assayed for the presence of active NF-κB complexes by EMSA using an oligonucleotide containing the proximal
κB site of the human E-selectin promoter. Arrows point to two visible NF-κB complexes. The identity of the protein in the complexes was analyzed by supershifting with 1 µg of p65, p50, p52, cRel or RelB specific antibodies. Results shown are representative of 5 independent experiments.

Figure 5. **Kinetics of nuclear translocation of NF-κB p65 in response to Tat.** Confluent HUVEC were exposed to Tat, TNF, cell culture medium or boiled Tat (bTat) for the indicated times. Cells were collected and nuclear proteins extracted as described in Experimental Procedures. A. 0.5 µg aliquots of cytoplasmic (CF) and nuclear fractions (NF) were analyzed for nuclear translocation of p65 by western blotting. Results shown are representative of 3 separate experiments. B. 15 µg of HUVEC CF and NF were analyzed for α-actin. C. 15 µg of CF and NF from HeLa or HeLa-tat cells were probed for the nuclear transcription factor, Sp3 by Western blot.

Figure 6. **Expression levels of p65 after Tat exposure.** Confluent HUVEC exposed to Tat, TNF, or cell culture medium for the indicated times were collected and whole cell proteins extracted as described in Experimental Procedures. Whole cell lysates (0.5 µg) were analyzed for p65 expression levels via Western blots. Results are representative of 3 separate experiments.

Figure 7. **Effects of cycloheximide or actinomycin D on Tat-stimulated E-selectin expression.** Confluent HUVEC were exposed to Tat for 6 hours after a 30 minute pre-incubation in the presence or absence of either 5 µg/ml cycloheximide (CHX) or 2 µg/ml actinomycin D (ActD). After exposure, the cells were collected and processed for E-selectin measurements via FACS as described in Experimental Procedures. Regions M1 and M2 were defined as described in Fig. 2. Results expressed as means +/- SEM, n=4.

Figure 8. **Inhibition of NF-κB activation by AdIκBαSR.** Confluent HUVEC were transduced with an adenovirus construct encoding a super-repressor (AdIκBαSR) form of IκBα at a multiplicity of transduction = 30 plaque forming units/cell. 24-hours after transduction, cells were exposed to Tat, TNF, or cell culture medium for 6 hours. After exposure, the cells were collected and nuclear proteins extracted as described in Experimental Procedures. The nuclear extracts were assayed for the presence of active NF-κB complexes by EMSA. The arrow points to the shifted complexes.

Figure 9. **Inhibition of NF-κB p65 translocation by AdIκBαSR.** After transduction and exposure as described in Fig. 8, cells were collected, fractionated, and nuclear proteins assayed for evidence of p65 nuclear translocation by Western blot. The top panel shows p65 in the CF while the bottom panel shows p65 in the NF.

Figure 10. **Inhibition of Tat-mediated E-selectin expression by AdIκBαSR.** After transduction and exposure as described in Fig. 8, cells were collected and processed for E-selectin surface expression measurements via FACS. Since GFP fluorescence would interfere with FITC fluorescence, E-selectin expression in the cells transduced with the control adenovirus (AdGFP) was measured via streptavidin-biotin/cyochrome fluorescence. Panel A shows representative histograms. Panel B shows the average +/- SEM, n=4.
**Fig. 2A**

Medium

GST

Tat

TNF

**Fig. 2B**

% Cells in M2

Time (hrs)
Fig. 2C

% Cells in M2

Medium  GST  TNF  Tat  anti-Tat  Boiled Tat  48ΔC22G  48ΔTK
Fig. 3A

Open bars: pGL3B
Closed bars: pE-Luc

Fig. 3B
Fig. 4A

Fig. 4B

20 minutes

6 hours
Fig. 7

% Cells in M2

Medium  Tat  Tat+CHx  Tat+ActD

0  10  20  30  40  50  60  70
Fig. 8

Medium  Tat  TNF  Medium  Tat  TNF

+AdIkBαSR
Fig. 9

Medium

AdIkBαSR

Tat

TNF

AdIkBαSR

+ Tat

AdIkBαSR

+ TNF
**Fig. 10A**

Fluorescence Counts

Fluorescence

Tat + AdIkBαSR

Counts

M1

M2

Fluorescence

Tat

Counts

M1

M2

Fluorescence

Tat + AdIkBαSR

Counts

M1

M2

Fluorescence

Tat + AdGFP

Counts

M1

M2

Fluorescence

**Fig. 10B**

% Cells in M2

Medium + AdIkBαSR

Tat + AdIkBαSR

Tat

Tat + AdGFP

% Cells in M2

Medium + AdIkBαSR

Tat + AdIkBαSR

Tat

Tat + AdGFP
The human immunodeficiency virus-1 Tat protein activates endothelial cell E-selectin expression via an NF-κ-dependent mechanism

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*J. Biol. Chem.* published online February 4, 2002

Access the most updated version of this article at doi: [10.1074/jbc.M108591200](http://dx.doi.org/10.1074/jbc.M108591200)

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