$\alpha_v\beta_3$-integrin-dependent activation of focal adhesion kinase mediates NF-κB activation and motogenic activity by HIV-1 Tat in endothelial cells

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Summary

Once in the extracellular environment, the transactivator protein HIV-1 Tat exerts several pleiotropic effects by interacting with different cellular receptors, including integrin $\alpha_v\beta_3$. Real-time surface plasmon resonance analysis reveals that Tat/$\alpha_V\beta_3$ interaction occurs with rapid kinetics (association and dissociation rates equal to 1.16×10^7 M^{-1} s^{-1} and 3.78×10^{-1} s^{-1}, respectively) and high affinity (dissociation constant = 32 nM). Through this interaction, substratum-immobilized Tat promotes adhesion and motogenic activity in endothelial cells. Also, $\alpha_v\beta_3$/Tat interaction triggers the activation of focal adhesion kinase, RhoA and pp60^{src}. Overexpression of the dominant negative form of focal adhesion kinase, but not of an inactive $Leu_{1034}Ser$ substitution mutant isoform, impairs the activation of focal adhesion kinase and RhoA, but not that of pp60^{src}, without affecting endothelial cell adhesion and spreading. $\alpha_v\beta_3$/Tat interaction triggers the activation of NF-κB in endothelial cells in a focal adhesion kinase-, RhoA- and pp60^{src}-dependent manner, as shown in dominant negative focal adhesion kinase transfectants or using specific pharmacological inhibitors. Finally, the activation of focal adhesion kinase, RhoA, NF-κB and pp60^{src} are required to mediate the motogenic activity of Tat in endothelial cells.

Since Tat accumulates in an immobilized form in the extracellular matrix, these results provide new biochemical and biological insights about $\alpha_v\beta_3$/Tat interaction exploitable for the design of anti-Tat strategies.

Key words: HIV-1 Tat, $\alpha_v\beta_3$ integrin, endothelium, FAK, NF-κB

Introduction

The Tat protein is a cationic 86-101 amino acid polypeptide that acts as the main transactivating factor of HIV-1 (Gatignol and Jeang, 2000). Direct and indirect evidence indicate that Tat is actively released by HIV-1-infected cells (Noonan and Albini, 2000). Extracellular Tat targets different types of uninfected cells, including endothelial cells (ECs) (Rusnati and Presta, 2002), causing a variety of biological effects possibly related to distinct AIDS-associated pathologies, including neuropathies (Dewhurst et al., 1996), immune suppression (Caputo et al., 1999; Noonan and Albini, 2000) and increased tumorigenesis in AIDS patients (Caputo et al., 1999). The mechanisms by which Tat exerts its pathological effects are manifold and mediated by various signalling receptors in target cells, including the vascular endothelial growth factor (VEGF) receptor 2 (KDR) (Albini et al., 1996), chemochine receptors (Albini et al., 1998), and integrins (Barillari et al., 1999a; Barillari et al., 1999b; Fiorelli et al., 1999; Mitola et al., 2000).

Integrins are a family of heterodimeric receptors that, unlike growth factor receptors, lack intrinsic tyrosine kinase activity. Yet, an early event during integrin signalling is the tyrosine phosphorylation of the non-receptor tyrosine kinase focal adhesion kinase (FAK) (Kumar, 1998; Schlaepfer et al., 1999) that, in turn, leads to the activation of the GTPase RhoA and/or pp60^{src} in different cell types (Palazzo et al., 2004; Sharma-Walia et al., 2004; Zhai et al., 2003). In ECs, this signal transduction pathway can be activated upon $\alpha_v\beta_3$ integrin engagement and leads to nuclear translocation of NF-κB and cell survival (Scatena et al., 1998). Accordingly, integrins regulate EC proliferation in vitro (Eliceiri, 2001) and angiogenesis in vivo (Varner and Cheresh, 1996).

In ECs, Tat interacts with $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins (Barillari et al., 1999a; Barillari et al., 1999b; Fiorelli et al., 1999; Mitola et al., 2000). This interaction is required by Tat to induce neovascularization in vivo and chemotactic and mitogenic activity in cultured ECs (Barillari et al., 1999a; Barillari et al., 1999b; Mitola et al., 2000). Also, integrins mediate the adhesion and spreading of ECs to substratum-immobilized Tat (Barillari et al., 1999a; Barillari et al., 1999b; Fiorelli et al., 1999; Mitola et al., 2000). However, the molecular bases and biological relevance of this process remain poorly elucidated. Relevant to this point, Tat accumulates in the extracellular matrix (ECM) as an immobilized protein (Chang et al., 1997) by interacting with heparan sulphate proteoglycans (HSPGs) (Chang et al., 1997; Tyagi et al., 2001). These findings suggest that the concentration of Tat can increase in the microenvironment,

possibly representing a localized, persistent stimulus for EC adhesion and activation.

In this paper we demonstrate that HIV-Tat/$\alpha_v\beta_3$ interaction leads to activation of FAK, RhoA, NF-κB and pp60^{src}, which are required for the stimulation of motogenic activity in ECs.

Materials and Methods

Reagents

Cyclo(-Arg-Gly-Asp-D-Phe-Val) (cRGDfV) and cyclo(-Arg-Ala-Asp-D-Phe-Val) (cRADfV) peptides were from Bachem (Beidendorf, Switzerland). Integrin $\alpha_v\beta_3$ was purified to homogeneity as described previously (Rusnati et al., 1997). Fibronectin (FN) was from Sigma (St Louis, MO, USA). Anti-$\alpha_v\beta_3$ monoclonal LM 609 antibody was from Chemicon International (Temecula, CA, USA). Anti-β3 antiserum was from G. Tarone (University of Turin, Italy). Anti-FAK antibodies A17 and C20, anti-phosphotyrosine antibody PY99, anti-Src polyclonal antibody SRC-2 and anti-RhoA antibody 26C4 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-FAK phosphospecific antibody sampler kit was from BioSource International (Camarillo, CA, USA). Anti-haemagglutinin (HA) antibody was from Upstate Biotechnology (Lake Placid, NY, USA). The cell-permeable NF-κB inhibitory peptide SN50 and its inactive analogue SN50M were from Biomol (Plymouth Meeting, PA, USA). The pp60^{src} inhibitor PP_2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4-d] pyrimidine), its inactive analogue PP_3, the anti-Src phospho-specific Tyr_{416} antibody and the RhoA inhibitor exoenzyme C3 from *Clostridium botulinum* were from Calbiochem (La Jolla, CA, USA).

The 86 amino acid wild-type form of HIV-1 Tat and its mutants were expressed and purified from *Escherichia coli* as glutathione S-transferase (GST) fusion proteins as described previously (Rusnati et al., 1998).

Cell-free $\alpha_v\beta_3$ integrin/GST-Tat interaction

Human $\alpha_v\beta_3$ integrin purification, cell-free $\alpha_v\beta_3$ integrin/GST-Tat interaction and cell adhesion assay with GM7373 ECs were performed as previously described (Rusnati et al., 1997).

Real-time biomolecular interaction assay

A BIAcore X apparatus (BIAcore Inc, Piscataway, NJ, USA) was used. Surface plasmon resonance (SPR) was exploited to measure changes in refractive index caused by the ability of purified $\alpha_v\beta_3$ integrin to bind GST-Tat immobilized to a BIAcore sensorchip. For this, 50 μg/ml of GST-Tat were allowed to react with a flow cell of a CM5 sensorchip that was previously activated with 50 μl of a mixture of 0.2 M *N*-ethayl-*N*′-(3-diethylaminopropyl)carbodiimide hydrochloride and 0.05 M *N*-hydroxysuccinimide. These experimental conditions allowed the immobilization of 10281 resonance units (RU), corresponding to approximately 0.3 pmoles of GST-Tat. Similar results were obtained for the immobilization of BSA, here used as a negative control and for blank subtraction. Increasing concentrations of integrin $\alpha_v\beta_3$ in 10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20, pH 7.4 were then injected over the BSA or GST-Tat surfaces for 4 minutes (to allow their association with immobilized proteins) and then washed until dissociation was observed. The SPR signal was expressed in terms of RU.

Cell cultures

Transformed foetal bovine aortic endothelial GM7373 cells were obtained from the NIGMS Human Genetic Mutant Cell Repository (Institute for Medical Research, Camden, NJ, USA) (Grinspan et al., 1983). GM7373 cells were grown in Eagle's minimal essential medium containing 10% foetal calf serum (FCS) (Gibco, Grand Island, NY, USA), vitamins, essential and non essential amino acids.

BALB/c mouse aortic endothelial 22106 cells (MAECs) were from R. Auerbach (University of Wisconsin, Madison, WI, USA) and were grown in Dulbecco's minimal essential medium supplemented with 10% calf serum (Gibco).

Human umbilical vein ECs (HUVECs) were from Biowhittaker (Walkersville, MA, USA) and cultured in EGM-2 medium (Biowhittaker).

FRNK cDNA transfection

GM7373 cells were transfected with the expression vectors pCDNA 3.1 FRNK or pCDNA 3.1 FRNK-Ser1034 encoding for the FAK C-terminal domain (FRNK) or the inactive $FRNK_{L1034S}$ mutant, respectively (Schlaepfer and Hunter, 1996), both tagged with a triple haemagglutinin (HA) (Sieg et al., 1999). To obtain stable transfectants, GM7373 cells were plated at 7×10^5 cells/100 mm plates and were transfected with 8 μg of plasmid DNA using lipofectamin (Gibco) according to manufacturer's instructions. After 72 hours, 200 μg/ml of hygromycin-B (Gibco) were added to cell cultures. Hygromycin-B-resistant clones were isolated and tested for HA-tag expression by western blotting.

FAK phosphorylation analysis

Tissue culture dishes (10 cm in diameter) containing subconfluent cultures of GM7373 or MAE cells were incubated at 37°C in serum-free medium for 24 hours. Then, cells were added with GST-Tat in absence or in presence of the different inhibitors and further incubated at 37°C for different periods of time. Alternatively, GM7373 cells maintained in serum-free medium for 24 hours were detached from culture dishes by 15 minutes incubation in PBS containing 15 mM EDTA and resuspended in medium containing 1% FCS and 25 mM Hepes, pH 7.5. Aliquots of 500,000 cells were then maintained in suspension or allowed to adhere to non-tissue culture dishes (60 mm in diameter) coated with GST-Tat or poly-L-lysine (PL) for 30 minutes at 37°C.

At the end of incubation, cells were lysed in RIPA modified lysis buffer [50 mM Tris-HCl pH 7.4 containing 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycolate and a protease inhibitor mixture (1 mM phenylmethylsulphonylfluoride, 4 mM amino-n-caproic acid, 10 μg/ml leupeptin, 1 mM Na_3VO_4, 50 mM NaF)] and centrifuged (10 minutes at 15,000 ***g***). Protein concentration was evaluated in the supernatants and 600 μg protein/sample were incubated for 3 hours at room temperature with anti-FAK rabbit polyclonal antibody A17 (1.5 μg/sample) and Ultra-link Immobilon Protein A (20 μl/sample) (Pierce, Rockford, IL, USA). At the end of incubation, samples were centrifuged (1 minute at 100 ***g***) and pellets were washed extensively with RIPA modified lysis buffer, resuspended in reducing SDS-PAGE sample buffer and incubated for 5 minutes at 90°C. In some experiments, cells cultures were directly lysed in reducing SDS-PAGE sample buffer and incubated for 5 minutes at 90°C. Immunoprecipitates and total cell extracts were analysed on SDS-7% PAGE under reducing conditions followed by western blotting using anti-phosphotyrosine monoclonal antibody PY99 or the FAK phosphorylation site-specific antibodies, respectively. Parallel experiments with antibodies directed against the unphosphorylated form of FAK were performed for each experiment.

The extent of FAK phosphorylation was quantified by using the Image Pro-Plus analysis system (Media Cybernetics, Silver Spring, MD, USA). Briefly, the autoradiographs for total or phosphorylated FAK were digitized on a high resolution monitor and stored within the Pro-Plus analysis system's memory. The integrated densities of the bands were then evaluated and the values of those corresponding to phosphorylated FAK were normalized to total FAK protein levels.

pp60^{src} phosphorylation analysis

GM7373 cells were allowed to adhere to the different substrata or were maintained in suspension exactly as described above. At the end of incubations, cells were lysed and immunoprecipitated with anti-Src antibody SRC-2 (1.5 μg/sample) as described above, resuspended in reducing SDS-PAGE sample buffer, incubated for 5 minutes at 90°C and analysed on SDS-12% PAGE under reducing conditions followed by western blotting using antibodies directed against the phosphorylated form of pp60^{src}.

Rho pull-down assay

GM7373 cells were allowed to adhere to the different substrata or were maintained in suspension exactly as described above. At the end of incubations, cells were lysed in Rho binding lysis buffer (50 mM Tris-HCl pH 7.4 containing 100 mM NaCl, 1% Nonidet P-40, 2 mM $MgCl_2$, 10% glycerol and the protease inhibitors mixture described above) and centrifuged (20 minutes at 16,000 ***g***). Protein concentration was evaluated in the supernatants and 300 μg protein/sample were incubated for 1 hour at 4°C with GST-Rho-GTPase-binding domain (30 μg/sample) immobilized to glutathione-Sepharose 4B (Sigma). Beads were then centrifuged (1 minute at 1000 ***g***) and pellets were washed extensively with Rho binding lysis buffer, resuspended in reducing SDS-PAGE sample buffer, incubated for 5 minutes at 90°C, and analysed on SDS-15% PAGE under reducing conditions followed by western blotting using anti-RhoA antibodies.

NF-κB activation assay

GM7373 cell cultures (24 well plates) were incubated at 37°C in serum-free medium for 24 hours. Then, cells were added with GST-Tat in the absence or presence of the inhibitors under test and further incubated at 37°C for different periods of time. Alternatively, GM7373 cells maintained in serum-free medium for 24 hours were detached from culture dishes by 15 minutes incubation in PBS containing 15 mM EDTA and resuspended in medium containing 1% FCS and 25 mM Hepes, pH 7.5. Aliquots of 1×10^6 cells were then allowed to adhere to non-tissue culture dishes (35 mm in diameter) coated with 20 μg/ml GST-Tat, FN, or PL for 3 hours at 37°C. At the end of incubation, the amount of activated NF-κB p50 or p65 subunit present in the cell extracts was determined using the TransAM NF-κB p50 or p65 Assay Kits (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions (Renard et al., 2001).

EC monolayer wound healing assay

GM7373 cells were allowed to reach confluence onto 3.5 cm polystyrene tissue culture plates or non-tissue culture plates coated with 20 μg/ml of GST-Tat, FN or PL in medium containing 10% FCS. After extensive washing, EC monolayers were wounded with a rubber policeman and incubated at 37°C with medium containing 0.4% FCS in the absence or presence of free GST-Tat (100 ng/ml) or of the different antagonists. Photomicrographs were taken under an inverted microscope (Olympus 1×51 microscope with a Camedia C-4040 digital camera, ×10/0.25; Olympus Biosystem, Munich, Germany) and wound repair was evaluated by measuring the area of the wound by computerized image analysis using the Image Pro-Plus analysis system.

Results

Characterization of Tat/$\alpha_v\beta_3$ interaction

Human $\alpha_v\beta_3$ integrin was assessed for its capacity to interact with GST-Tat in two different cell-free models. Firstly, BSA, FN, GST-Tat and GST devoid of the Tat moiety were immobilized onto non-tissue culture plastic and assessed for their capacity to bind $\alpha_v\beta_3$. As shown in Fig. 1A, $\alpha_v\beta_3$ binds to immobilized GST-Tat but not to FN, BSA or GST. Next, increasing concentrations of $\alpha_v\beta_3$ were injected over GST-Tat immobilized onto a BIAcore CM5 sensorchip and a set of sensorgrams was obtained (Fig. 1B). After subtraction of the blank, sensorgrams were used to calculate kinetic parameters: an association rate constant (k_{on}) equal to 1.16×10^7 M^{-1} s^{-1} and a dissociation rate constant (k_{off}) equal to 3.78×10^{-1} s^{-1} characterized the interaction of $\alpha_v\beta_3$ with immobilized GST-Tat. Thus, GST-Tat/$\alpha_v\beta_3$ interaction occurs with a dissociation constant (K_d)=k_{off}/k_{on} equal to 32 nM. In some experiments, the association phase of GST-Tat/$\alpha_v\beta_3$ interaction was allowed to proceed to equilibrium and, after subtraction of the blank, the data were used to calculate an affinity value independent of the kinetics of binding. This analysis demonstrates that GST-Tat/$\alpha_v\beta_3$ interaction occurs with a K_d equal to 36 nM, a value consistent with our kinetics analysis. The association of $\alpha_V\beta_3$ with GST-Tat is specific, since the integrin binds poorly to a BSA-coated sensorchip, with RU values similar to those obtained after the injection of buffer alone onto GST-Tat- or BSA-coated sensorchips (Fig. 1C).

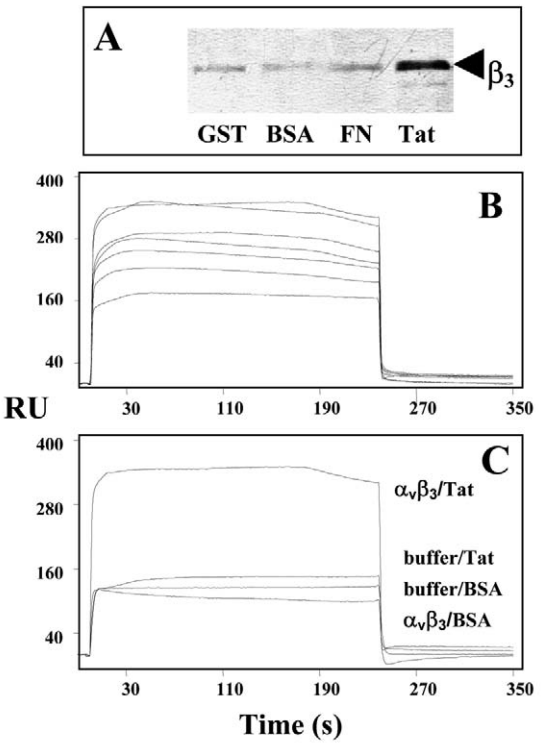


Fig. 1. Cell-free interaction of Tat with $\alpha_v\beta_3$ integrin. (A) Aliquots of purified human $\alpha_v\beta_3$ were incubated on plastic coated with BSA, FN, GST or GST-Tat. At the end of incubation, plastic-bound proteins were extracted and analysed by western blotting with anti-β_3 antibodies. (B) Purified $\alpha_v\beta_3$ was injected at (from top to bottom) 100, 50, 25, 18, 15, 10, and 5 nM for 4 minutes over a BIAcore sensorchip coated with GST-Tat. (C) $\alpha_v\beta_3$ (100 nM) or the buffer alone were injected over BIAcore sensorchips coated with GST-Tat or BSA. In all the experiments the responses (in RU) were recorded as a function of time.

Tat/$\alpha_v\beta_3$ interaction promotes adhesion and motogenic activity in ECs

Plastic coated with GST-Tat promotes adhesion of endothelial GM7373 cells with a maximum effect at 20 μg/ml, corresponding to about 96,000 adherent cells/cm^2 (Fig. 2A). Under the same experimental conditions, FN allows the adhesion of about 120,000 cells/cm^2, whereas no significant cell adhesion was observed on plastic coated with BSA or GST protein. Endothelial GM7373 cells express $\alpha_v\beta_3$ (Rusnati et al., 1997). Accordingly, the highly specific anti-$\alpha_v\beta_3$ monoclonal LM 609 antibody (Cheresh, 1987), but not irrelevant IgGs, inhibits cell adhesion to GST-Tat but not to FN (Fig. 2B). Integrin $\alpha_v\beta_3$ binds to both the RGD motif and the basic domain present in several adhesive proteins (Gehlsen et al., 1992; Mitola et al., 2000; Vogel et al., 1993). Accordingly, the Tat mutants GST-Tat 1e (characterized by the deletion of the amino acid sequence that contains the RGD sequence) and GST-Tat$_{R49/52/53/55/56/57A}$ (in which the arginine residues 49, 52, 53, 55, 56, 57 within the basic domain were mutated to alanine residues) are characterized by a significantly lower cell-adhesive capacity with respect to the wild-type protein. In contrast, the mutant GST-Tat$_{\Delta 1\text{-}21}$ (containing a deletion of the amino acid sequence 1-21 that represents an absolute requirement for Tat transactivating activity) (Demarchi et al., 1996) retains a full cell-adhesive capacity (Fig. 2C). Also, GST-Tat loses its cell adhesive capacity after heat denaturation (Fig. 2C). These data indicate that the RGD motif, the basic domain, and a proper tridimensional conformation are required for Tat to interact with $\alpha_v\beta_3$ integrin present on the surface of ECs.

The cell adhesive capacity of GST-Tat is not restricted to GM7373 cells. Indeed, under the same experimental conditions, ECs of different origin, including HUVECs and MAECs, adhere to immobilized GST-Tat or FN but not to BSA (Fig. 2D).

Integrin-dependent EC adhesion can be considered the first step of a process that, together with EC migration and proliferation, leads to neovascularization (Folkman and Klagsbrun, 1987). EC activation following a mechanic wound of an EC monolayer resembles, at least in part, this process (Lauder et al., 1998). On this basis, we investigated the ability of ECs to repair a mechanically wounded monolayer (motogenic activity) when cells were allowed to adhere to immobilized GST-Tat. As shown in Fig. 2E,F, ECs adherent to immobilized GST-Tat show an improved capacity to repair the wounded monolayer when compared to cells seeded on FN or PL. The effect is inhibited by the $\alpha_v\beta_3$ antagonist cRGDfV but not by the control cRADfV peptide. At variance with substratum-immobilized GST-Tat, free GST-Tat does not induce motogenic activity when added to wounded GM7373 cell monolayers adherent to tissue culture plastic or to non-tissue-culture plastic coated with PL, FN or GST-Tat (Fig. 2E).

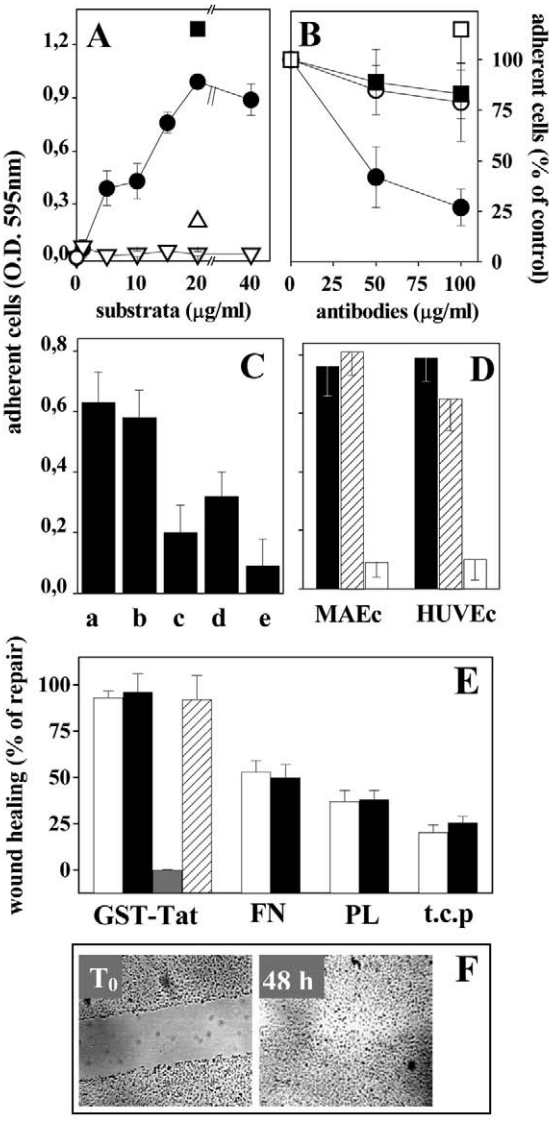


Fig. 2. Immobilized Tat promotes adhesion and motogenic activity in ECs. (A) GM7373 cells were allowed to adhere to plastic coated with the indicated concentrations of native GST-Tat (●), GST (▽), FN (■) or BSA (△). (B) GM7373 cells were incubated for 1 hour at 4°C with the indicated concentrations of anti-$\alpha_v\beta_3$ neutralizing monoclonal antibody LM 609 (closed symbols) or irrelevant IgG (open symbols). Then, cells were allowed to adhere to plastic coated with 20 μg/ml of GST-Tat (circles) or FN (squares). (C) GM7373 cells were allowed to adhere to plastic coated with 20 μg/ml of native (a) or heat denatured (e) wild-type GST-Tat, GST-Tat$_{\Delta 1\text{-}21}$ (b), GST-Tat$_{K49/52/53/55/56/57A}$ (c), GST-Tat-1e (d). (D) HUVE cells or MAE cells were allowed to adhere on plastic coated with 20 μg/ml of GST-Tat (black bars), FN (hatched bars), or BSA (white bars). At the end of incubation, cell adhesion was quantified. In B, data are expressed as the percentage of cells adherent to the different substrata in the absence of antibodies. Each point is the mean ± s.e.m. of three or four determinations in duplicate. (E) GM7373 cell monolayers adherent to the indicate plastic-immobilized proteins or to tissue culture plastic (t.c.p.) were wounded and incubated for 48 hours in culture medium containing 0.4% FCS in the absence (white bars) or in the presence of free GST-Tat (100 ng/ml; black bars), cRGDfV (grey bars) or cRADfV (hatched bars) (both at 3 μM). At the end of incubation the area of the wound was quantified by image analysis. Each point is the mean ± s.e.m. of four to five fields measured in one experiment out of two or three that gave similar results. The results are expressed as percentage of repair in respect to untreated monolayers adherent to immobilized Tat.
(F) Representative microphotographs (magnification 50×) of Tat-adherent GM7373 cell monolayers at T_0 and 48 hours after the wounding.

In conclusion, immobilized Tat induces adhesion and motogenic activity in ECs by interacting with $\alpha_v\beta_3$ integrin.

Tat/$\alpha_v\beta_3$ interaction promotes FAK activation

The activation of FAK, as well as of the downstream second messengers RhoA and pp60^{src}, are early integrin signalling events following EC adhesion and spreading (Kumar, 1998; Schlaepfer et al., 1999). To investigate whether these second messengers are activated following EC adhesion to immobilized Tat, GM7373 cells were seeded on plastic coated with GST-Tat or PL and incubated for 30 minutes at 37°C. GM7373 cells maintained in suspension for the same period of time were used as controls. As shown in Fig. 3A, FAK, RhoA, and pp60^{src} are activated in GM7373 cells adherent to immobilized GST-Tat but not in cells adherent to PL or maintained in suspension, indicating the specificity of the effect.

$\alpha_v\beta_3$ is expressed both at the basal and luminal aspects of ECs (Conforti et al., 1992) and Tat is present in the serum of AIDS patients (Westendorp et al., 1995). To assess whether the engagement of luminal $\alpha_v\beta_3$ by free Tat could also activate FAK in ECs, GM7373 cells adherent to tissue culture plastic were exposed to free GST-Tat. As shown in Fig. 3B,C, free GST-Tat induces FAK phosphorylation in GM7373 cells in a dose- and time-dependent manner (maximum effect being observed at 100 ng/ml and 30 minutes, respectively). Also, by using phosphospecific anti-FAK antibodies, we found that GST-Tat promotes the phosphorylation of FAK at position Y397 and Y577 in GM7373 cells, without affecting the levels of phosphorylation at Y407, Y576, Y861, and Y925 (Fig. 3D).

To demonstrate that FAK phosphorylation by free Tat is triggered via $\alpha_v\beta_3$ engagement, free GST-Tat was administered to GM7373 cells in the absence or in the presence of the anti-$\alpha_v\beta_3$ antibody LM609 or of cRGDfV cyclic peptide. As shown in Fig. 3E, both LM609 and cRGDfV inhibit FAK phosphorylation by free GST-Tat. However, no effect is exerted by an irrelevant antibody or by the cRADfV control peptide. Also, cRGDfV does not affect the basal levels of FAK phosphorylation in the absence of GST-Tat. Finally, free GST-Tat also triggers FAK phosphorylation in MAECs without affecting the level of unphosphorylated FAK protein (Fig. 3F).

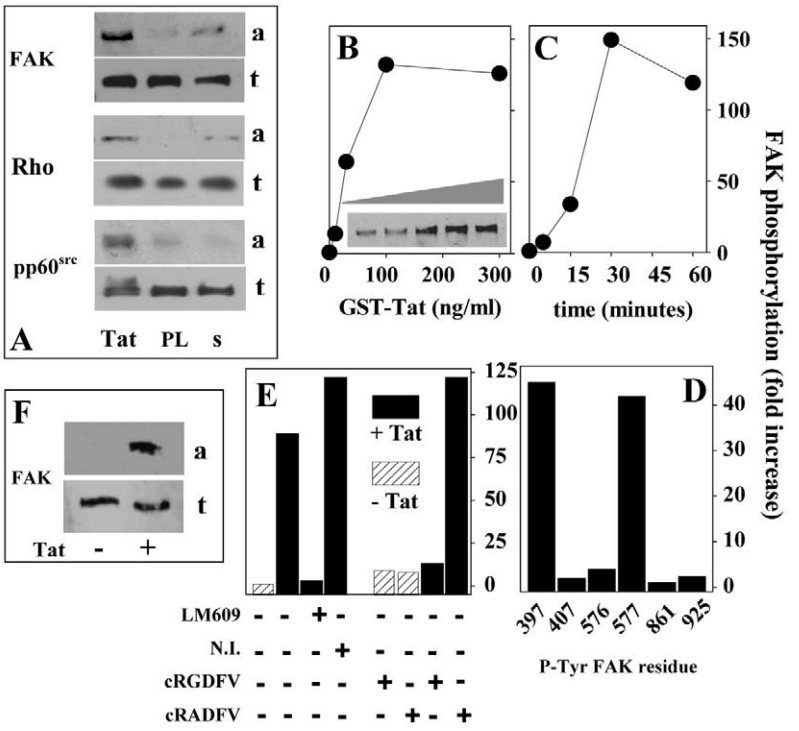
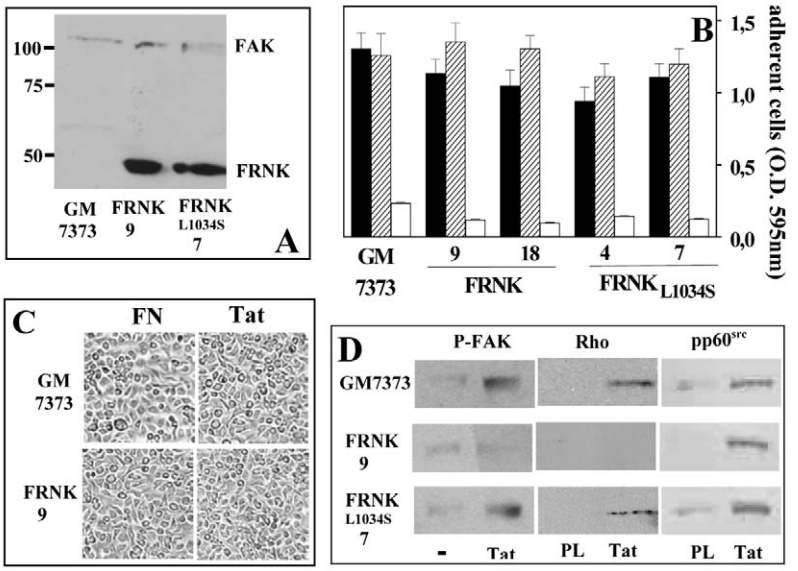


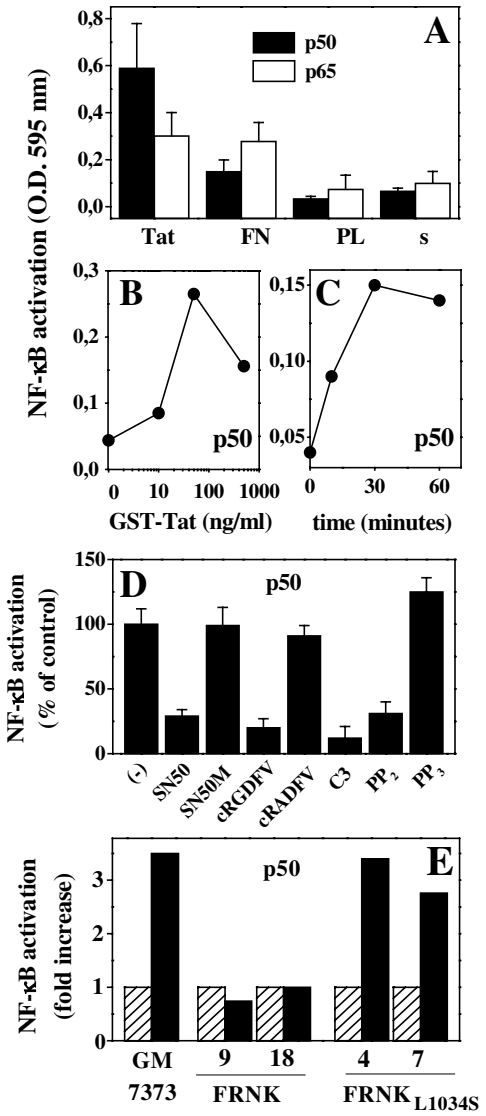
Fig. 3. FAK, RhoA and pp60^{src} activation by Tat in ECs. (A) GM7373 cells were allowed to adhere to plastic coated with 20 μg/ml of GST-Tat or PL, or were maintained in suspension (s) for 30 minutes. Then, cells were lysed, and analysed for FAK, RhoA or pp60^{src} activation (a, activated/phosphorylated protein; t, total protein). (B,C) GM7373 cells adherent to cell culture plates were incubated for 30 minutes at 37°C with the indicated concentrations of free GST-Tat (B) or with 100 ng/ml of GST-Tat for the indicated periods of time (C). Then, cells were lysed, immunoprecipitated with anti-FAK antibodies and immunoblotted with anti-phosphotyrosine antibody. (D) GM7373 cells adherent to cell culture plates were incubated for 30 minutes at 37°C with 100 ng/ml of GST-Tat, lysed, and immunoblotted with antibodies specific for the indicated FAK phosphotyrosine residues. (E) GM7373 cells adherent to cell culture plates were incubated for 30 minutes at 37°C with or without GST-Tat (100 ng/ml) in the absence or in the presence of anti-$\alpha_v\beta_3$ (LM 609) or irrelevant (N.I.) antibodies (both at 75 μg/ml) or of cRGDfV and cRADfV peptides (both at 3 μM). Then, cells were lysed and immunoblotted with antibodies specific for FAK phospho-Tyr$_{397}$ residue. In B, C, E and F, the extent of FAK phosphorylation was quantified by image analysis and expressed as fold increase with respect to basal FAK phosphorylation observed in Tat-untreated cells. The data are representative of two to four independent experiments that gave similar results. (F) MAE cells adherent to cell culture plates were incubated for 30 minutes at 37°C in the absence or in the presence of 100 ng/ml of GST-Tat. Then, cells were lysed, immunoprecipitated with anti-FAK antibodies, and immunoblotted with anti-FAK or anti-phosphotyrosine antibodies. a, phosphorylated protein; t, total protein.

To assess whether FAK connects $\alpha_v\beta_3$/Tat interaction to RhoA/pp60^{src} activation in ECs, we stably transfected GM7373 cells with the cDNA encoding for the FAK C-terminal domain (FRNK). FRNK exerts a dominant negative effect on FAK activation promoting its dephosphorylation at Tyr$_{397}$. This dominant-negative effect is abolished by the L1034S point mutation that prevents FRNK localization to focal contact sites (Sieg et al., 1999). After transfection with expression vectors harbouring the cDNA encoding for FRNK or FRNK$_{L1034S}$ both tagged with a triple-HA, GM7373 transfectants were firstly screened for transgene expression by western blotting using specific anti-HA antibodies (data not shown). Only clones characterized by a similar expression of HA were utilized further. Among them, two clones expressing FRNK (named FRNK-9 and FRNK-18) and two clones expressing FRNK$_{L1034S}$ (named FRNK$_{L1034S}$-4 and FRNK$_{L1034S}$-7) were used for further experiments. Parental GM7373 cells, FRNK-9 and FRNK$_{L1034S}$-7 clones (Fig. 4A), as well as FRNK-18 and FRNK$_{L1034S}$-4 (data not shown), are characterized by similar levels of expression of total FAK protein. Also, the different transfectants, but not parental GM7373 cells, express similar levels of FRNK or FRNK$_{L1034S}$ proteins.

On these bases, EC adhesion and spreading and consequent

Fig. 4. Effect of FRNK overexpression in GM7373 endothelial cells. (A) Parental GM7373 cells or the indicated transfectant clones were grown on tissue culture plates, lysed and immunoblotted with an antibody directed against the C terminus of FAK. (B) The different clones were allowed to adhere to plastic coated with 20 μg/ml of GST-Tat (black bars), FN (hatched bars) or BSA (white bars). At the end of incubation, cell adhesion was quantified. Each point is the mean ± s.e.m. of three determinations in duplicate. (C) Representative images showing parental and FRNK-transfected GM7373 cells adherent to GST-Tat or FN (magnification 100×). (D) The different clones were seeded on cell culture plates, incubated for 30 minutes with or without free GST-Tat (100 ng/ml), and immunoblotted with antibodies specific for FAK phospho-Tyr$_{397}$ residue. Alternatively, the different clones were allowed to adhere to plates coated with GST-Tat or PL and analysed for RhoA or pp60^{src} activation. No significant differences were found in the total/starting levels of FAK, pp60^{src} or RhoA (data not shown). The data are representative of two to three independent experiments that gave similar results.





activation of FAK, RhoA and pp60^{src} were evaluated in the different cell lines. As shown in Fig. 4B,C, FRNK or FRNK$_{L1034S}$ overexpression does not hamper cell adhesion and spreading of ECs to immobilized Tat or FN. Nevertheless, Tat-adherent FRNK cells show a remarkable decrease in FAK phosphorylation when compared to parental or FRNK$_{L1034S}$ transfected cells (Fig. 4D). Accordingly, FRNK cells adherent to immobilized Tat failed to activate RhoA but they retained the capacity to phosphorylate pp60^{src} (Fig. 4D).

Taken together, the data indicate that $\alpha_v\beta_3$-mediated adhesion of ECs to immobilized Tat triggers FAK phosphorylation, which, in turn, is required for the activation of RhoA, but not of pp60^{src}.

Tat/$\alpha_v\beta_3$ interaction promotes NF-κB activation

FAK phosphorylation triggers NF-κB activation (Funakoshi-Tago et al., 2003; Scatena et al., 1998) and NF-κB is activated by Tat in different cell types (Demarchi et al., 1996; Nath et

Fig. 5. NF-κB activation by Tat in ECs. (A) GM7373 were allowed to adhere to plastic coated with the indicated proteins or maintained in suspension (s) for 30 minutes. (B,C) GM7373 adherent to cell culture plates were incubated for 1 hour at 37°C with the indicated concentrations of free GST-Tat (B), or with 100 ng/ml of GST-Tat for the indicated periods of time (C). (D) GM7373 cells adherent to cell culture plates were incubated for 1 hour at 37°C with 100 ng/ml of GST-Tat in the absence (–) or in the presence of SN50, SN50M (both at 25 μg/ml), cRGDfV, cRADfV (both at 3 μM), exoenzyme C3 (5 μg/ml), PP$_2$ or PP$_3$ (both at 100 μM). (E) The different clones adherent to cell culture plates were incubated for 1 hour with (black bars) or without (hatched bars) GST-Tat (100 ng/ml). At the end of incubation, cells were lysed and the activation of the indicated NF-κB subunit was evaluated. In D, data are expressed as percentage of NF-κB activation measured in the absence of any inhibitors. In E, data are expressed as fold increase in respect to basal p50 NF-κB activation observed in Tat-untreated cells. In A and D each point is the mean ± s.e.m. of three determinations in duplicate; the data in B, C and E are representative of three independent experiments that gave similar results.

al., 1999). On these bases, we assessed whether $\alpha_v\beta_3$-dependent EC adhesion to Tat and consequent FAK, RhoA and/or pp60^{src} activation triggers NF-κB activation. As shown in Fig. 5A, the p50 and p65 subunits of NF-κB are activated in GM7373 cells adherent to immobilized GST-Tat or FN [here used as a positive control (Scatena et al., 1998)] but not to PL nor in cells maintained in suspension.

GST-Tat also induces NF-κB activation in a time- and dose-dependent manner when administered as a free molecule to GM7373 cells (Fig. 5B,C). Tat-dependent NF-κB activation is inhibited by the NF-κB inhibitor SN50 but not by its inactive analogue SN50M (Fig. 5D). The capacity of cRGDfV, but not of cRADfV, to hamper NF-κB activation by Tat indicates the role of $\alpha_v\beta_3$ engagement in this biological response (Fig. 5D). Furthermore, Tat fails to activate NF-κB in FRNK but not in FRNK$_{L1034S}$ transfectants (Fig. 5E). Finally, NF-κB activation is inhibited by the RhoA inhibitor exoenzyme C3 and by the pp60^{src} inhibitor PP_2 but not by its inactive control PP_3 (Fig. 5D). Taken together, the data indicate that NF-κB activation by Tat requires $\alpha_v\beta_3$/Tat interaction and FAK, RhoA and pp60^{src} activation.

Activation of FAK, RhoA, pp60^{src}, and NF-κB are required for Tat-induced motogenic activity in ECs

In a final series of experiments we assessed the involvement of the second messengers activated by Tat in the induction of motogenic activity in ECs. As shown in Fig. 6A, FRNK overexpression hampers the capacity of immobilized Tat to induce motogenic activity in adherent ECs. No inhibition was instead exerted in this assay by overexpression of the inactive FRNK$_{L1034S}$ mutant.

Also, the RhoA inhibitor exoenzyme C3 and the pp60^{src} inhibitor PP_2, but not its inactive control PP_3, prevented the migration of the wounded EC monolayer on immobilized Tat. A similar effect is also exerted by the NF-κB inhibitor SN50, but not by its inactive analogue SN50M (Fig. 6B). Thus, FAK, RhoA, pp60^{src} and NF-κB activation are all implicated in the motogenic activity triggered by immobilized Tat in ECs.

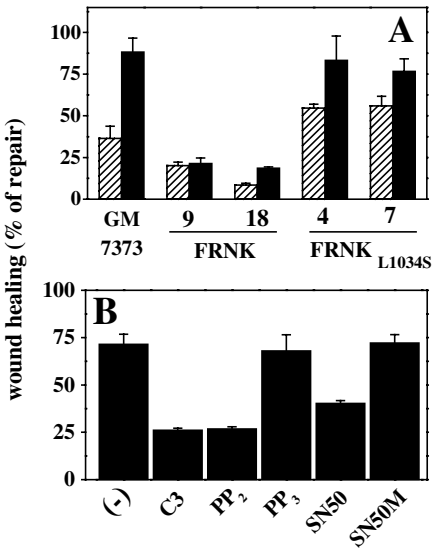


Fig. 6. Characterization of the motogenic activity induced by immobilized-Tat in adherent ECs. (A) Monolayers of the indicated GM7373 clones adherent to immobilized GST-Tat were wounded and incubated for 24 hours (hatched bars) or 48 hours (black bars) in medium containing 0.4% FCS. (B) Monolayer of parental GM7373 cells adherent to immobilized GST-Tat were incubated for 48 hours in the presence of the indicated inhibitors (same doses as in Fig. 5D). At the end of the incubation period the area of the wound was quantified by image analysis. Each point is the mean ± s.e.m. of four to eight fields measured in one experiment out of three that gave similar results. The results are expressed as percentage repair with respect to untreated monolayers adherent to immobilized Tat.

Discussion

HIV-1 Tat interacts with $\alpha_v\beta_3$ integrin of ECs inducing their adhesion to the substratum, but little is known about the molecular bases and biological consequences of this process.

By exploiting real-time surface plasmon resonance, here we shown the high affinity (K_d=32-36 nM) and rapid kinetics (k_{on} and k_{off}=1.16×10^7 M^{-1} s^{-1} and 3.78×10^{-1} s^{-1}, respectively) of Tat/$\alpha_v\beta_3$ interaction. Interestingly, the binding of $\alpha_v\beta_3$ to classical adhesive proteins such as fibrinogen or vitronectin (VN) is characterized by similar affinity (27 nM and 64 nM for fibrinogen and VN, respectively) but slower dissociation rate (k_{off}=9.8×10^{-4} s^{-1} and 2.1×10^{-4} s^{-1} for fibrinogen and VN, respectively) (Takagi et al., 2002), demonstrating that these latter interactions are more stable than that of Tat. In vivo, the high stability of $\alpha_v\beta_3$/adhesive proteins interaction is required to provide a firm anchorage to adherent ECs. At variance with this, the rapid kinetics of Tat/$\alpha_v\beta_3$ interaction suggest a more 'dynamic' adhesion, functional to migration of ECs adherent on substratum-immobilized Tat. Accordingly, here we reported that immobilized Tat stimulates a motogenic activity in adherent ECs.

To date, the process of angiogenesis has been viewed as the result of two distinct sets of inputs conveyed to ECs by free angiogenic growth factors interacting with their specific receptors of the luminal aspect of ECs, and by ECM components mainly interacting with integrins at the basal aspect of ECs. Here we observed that Tat acts simultaneously as an adhesive protein and as a free molecule, inducing ECs adhesion, signal transduction and motogenic activity. Accordingly, Tat triggers activation of intracellular second messengers both when presented to ECs as a substratum-immobilized or a free protein. However, we were not able to induce repair of a wounded monolayer of ECs adherent to tissue culture plastic by administering free Tat (Fig. 2E) at doses sufficient to induce second messengers activation in the same experimental conditions (see Figs 3 and 5) and comparable to the amount of Tat that remains coating the plastic (Rusnati et al., 1998). Accordingly, no significant differences were seen in wound repair when free Tat was administered to ECs adherent to Tat itself, FN or PL. These observations suggest that, in its substratum-immobilized form, Tat may be presented to ECs in a more appropriate way or may be more persistent in its stimulation. Alternatively, substratum-immobilized Tat, but not free Tat may alter cell-matrix interaction and cell traction, favouring EC motility.

Tat can be found immobilized in the ECM, where it associates with heparin-like HSPGs, and is protected from proteolitic degradation (Chang et al., 1997). Relevant to this point, a single heparin chain is able to bind up to six molecules

of Tat (Rusnati et al., 1999) and Tat interaction with substratum-immobilized heparin is far more stable (k_{off}=2.7×10^{-3} s^{-1}) (Rusnati et al., 2001) than that with $\alpha_v\beta_3$ (see above). These findings suggest the possibility that Tat accumulates in the ECM and increases its concentration in the microenvironment providing a firm substratum for EC adhesion and migration during angiogenesis in vivo. This possibility is further supported by the observation that the binding of Tat to HSPGs or to $\alpha_v\beta_3$ are not mutually exclusive, since free heparin does not inhibit Tat/$\alpha_v\beta_3$ interaction or EC adhesion to Tat (Urbinati et al., 2004). The capacity of substratum-immobilized Tat to induce signal transduction and motogenic activity in adherent ECs can find a physiological counterpart in vivo, where Tat released by HIV-positive cells in the lymph nodes may remain immobilized in the HSPGs-rich basal lamina of the capillaries during diffusion to the blood stream.

$\alpha_v\beta_3$ engagement by immobilized Tat induces FAK phosphorylation that, in turn, triggers the activation of different intracellular second messengers. In neurons, the residue Leu_{1034} of FAK acts as a docking site for p190RhoGEF, a Rho-specific GDP/GTP exchange factor (Zhai et al., 2003). Accordingly, we found that abrogation of FAK activity hampers the capacity of ECs to activate RhoA in response to their adhesion to immobilized Tat. Also, phosphorylation of FAK Tyr_{397} creates a docking site for SH_2-containing src kinases, including pp60^{src} (Hsia et al., 2003). Here we found that, even though adhesion of ECs to plastic-immobilized Tat induces pp60^{src} phosphorylation, abrogation of FAK activity by FRNK overexpression does not affect the capacity of ECs to phosphorylate pp60^{src} in response to Tat. This lack of inhibition can be explained by the possible activation of pp60^{src} via a direct interaction with the cytoplasmic domain of β integrin subunit (Arias-Salgado et al., 2003). Alternatively, Tat can bind and activate tyrosine kinase VEGF receptor KDR, whose engagement can lead to a direct activation of src kinases (Chou et al., 2002). Further experiments are required to elucidate the complex interplay among integrin, KDR, FAK, RhoA and pp60^{src} in Tat-stimulated ECs.

Whatever their mutual interactions, $\alpha_v\beta_3$, FAK, RhoA and pp60^{src} activation are essential for motogenesis of Tat-adherent ECs. Interestingly, FAK abrogation inhibits the motogenic activity of Tat without affecting EC adhesion and spreading. However, we observed that FRNK overexpression inhibits Tat-induced proliferation of ECs (C.U., unpublished data), suggesting that, beside adhesion and spreading, the activation of $\alpha_v\beta_3$, FAK, RhoA and pp60^{src} are also implicated in the modulation of the mitogenic activity of Tat. Interestingly, Tat-induced FAK activation has been associated with migration and permeability of brain microvascular ECs (Avraham et al., 2004).

Our data demonstrate that NF-κB connects $\alpha_v\beta_3$, FAK, RhoA and pp60^{src} activation to the motogenic activity triggered by Tat in ECs. Tat has been shown to activate NF-κB in different cell types including astrocytes (Conant et al., 1998), macrophages (Kumar et al., 1999), T-cells (Li-Weber et al., 2000) and ECs (Cooper et al., 1996; Cota-Gomez et al., 2002; Pieper et al., 2002; Toborek et al., 2003), but the mechanism(s) of activation are not fully elucidated. Indeed, Tat can induce NF-κB activation both when administered extracellularly (Cota-Gomez et al., 2002) and when produced endogenously (Pieper et al., 2002). Here, we show that NF-κB is activated in ECs following extracellular interaction of Tat with $\alpha_v\beta_3$ and the consequent activation of FAK, RhoA and pp60^{src}. In ECs, NF-κB activation by Tat has, to date, been connected to the overexpression of surface adhesive molecules and/or activation of inflammatory pathways (Cooper et al., 1996; Cota-Gomez et al., 2002; Pieper et al., 2002; Toborek et al., 2003). The data here reported implicate NF-κB in the motogenic activity exerted by Tat in vitro, suggesting its involvement also in the neovascularization induced by Tat in vivo. Accordingly, adhesion of ECs to FN activates a NF-κB-dependent program of gene expression related to angiogenesis (Klein et al., 2002). These findings point to a possible general involvement of NF-κB in neovascularization triggered by 'more classical' angiogenic growth factors. To date, the role of NF-κB in FGF2 biology is still being debated (Klein et al., 2002; Kroon et al., 2001; Messmer et al., 2000; Mohan et al., 2000; Pollet et al., 2003; Stoltz et al., 1996) and little data are available about NF-κB in VEGF-dependent angiogenesis. However, the possibility that NF-κB is involved in the biology of these two growth factors is also inferred by their capacity to accumulate in the ECM, thus inducing integrin-dependent EC adhesion and pro-angiogenic programs (Hutchings et al., 2003; Rusnati et al., 1997; Tanghetti et al., 2002).

Beside ECs, Tat induces substratum adhesion of Kaposi's sarcoma-derived spindle cells (Barillari et al., 1993), neurons (Cornaglia-Ferraris et al., 1995), myoblasts (Vogel et al., 1993), and epithelial cells (C.U. and C. Ravelli, unpublished), FAK phosphorylation in neurons (Milani et al., 1998) and NF-κB activation in various cell types (see above). Taken together, these observations suggest that Tat/$\alpha_v\beta_3$-dependent signal transduction pathway(s) are implicated in a broad array of biological effects connected with AIDS-associated pathologies, making them a suitable target for the design of anti-Tat strategies.

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